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An Inducible 50-Kilodalton NFκB-Like Protein and a Constitutive Protein Both Bind the Acute-Phase Response Element of the Angiotensinogen Gene

DAVID RON,* ALLAN R. BRASIER, KATHY A. WRIGHT, JAMES E. TATE, AND JOEL F. HABENER

Laboratory of Molecular Endocrinology, Massachusetts General Hospital, and Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts 02114

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The rat angiotensinogen gene is induced in the course of the hepatic acute-phase response. We demonstrate that monocyte conditioned medium can stimulate transcription of a stably introduced reporter construct driven by 615 base pairs of the angiotensinogen 5′-flanking sequence, as well as the endogenous gene, in Reuber H35 cells. Point mutations of a *cis*-acting element, located 545 base pairs from the transcription start site and sharing sequence identity with known nuclear factor kappa B (NFκB)-binding sites, led to loss of cytokine inducibility. When cloned upstream of a minimal promoter, this *cis*-acting element imparted transcriptional inducibility by monocyte conditioned medium, interleukin-1, and tumor necrosis factor on a luciferase reporter gene in HepG2 cells. Two distinct proteins bound this element in vitro: a heat-stable, constitutively present, hepatic nuclear protein that gave rise to a DNase I-protected footprint covering the functionally defined element; and a binding protein of different mobility, induced by monocyte conditioned medium, which also recognized the NFκB-binding site of the murine kappa light-chain enhancer. UV cross-linking showed this inducible protein to have an apparent molecular mass of 50 kilodaltons, similar to that described for NFκB and distinct from the constitutively present protein that was shown by Southwestern (DNA-protein) blot to have a molecular mass of 32 kilodaltons. Methylation interference analysis showed that the induced species made contact points with guanine residues in the NFκB consensus sequence typical of NFκB. Induction of this binding activity did not require new protein synthesis, and 12-O-tetradecanoylphorbol-13-acetate could mimic the induction by cytokines. We thus provide direct evidence for involvement of NFκB or a similar factor in the hepatic acute-phase response and discuss the potential role of the presence of a constitutive nuclear factor binding the same *cis* element.

The hepatic acute-phase response represents a coordinate change in the profile of genes expressed by the liver in response to inflammation or tissue injury. Underlying it, as a predominant mechanism, is the inducible alteration of transcription rates of the acute-phase response genes in response to various cytokines acting at the liver cell membrane and steroid hormones acting at the nucleus (reviewed in reference 5).

Recently, *cis*-acting DNA elements have been identified in the 5′-flanking regions of various liver acute-phase response genes that confer responsiveness to cytokines on inert reporters when introduced into responsive cell lines. Such elements have been identified for the interleukin-6 (IL-6) response genes coding for human haptoglobin (33), rat alpha-1 acid glycoprotein (36), human C-reactive protein (1), and rat alpha-2 macroglobulin (21). Similarly, a phorbol ester response element with sequence identity to well-characterized transcriptional activator protein nuclear factor kappa B (NFκB)-binding sites has been identified in the 5′-flanking region of the human serum amyloid A (SAA) gene, a known liver acute-phase response gene (19). The responsive sequence in the SAA gene bears little resemblance to the IL-6 response elements identified in the other genes. This suggests that NFκB may be involved in the acute-phase response mediated by cytokines other than IL-6, such as IL-1, a known stimulator of the homologous SAA gene in the mouse (37), or the functionally related tumor necrosis factor (TNF), which, along with IL-1, induces transcription of the human immunodeficiency virus (HIV) in lymphoid cells via NFκB-binding sites present in its long terminal repeat (31, 34).

The rat angiotensinogen (rAT) gene which codes for the protein precursor of the potent pressor hormone angiotensin II, is known to be rapidly induced in rat liver in the course of the acute-phase response (8, 24). The published sequence of the 5′-flanking region of the rAT gene (9, 32) contains a sequence similar to that found in NFκB-binding sites. In this study, we demonstrate the critical role of this element in the hepatic acute-phase transcriptional responsiveness of the rAT gene and, by analyzing the nuclear proteins binding to this element, implicate an inducible factor, indistinguishable from NFκB, directly in the hepatic acute-phase response.

MATERIALS AND METHODS

Animal studies and cell culture. Adult male CD rats were injected intraperitoneally with 3 mg of lipopolysaccharide (LPS) from *Escherichia coli* φ127:88 (Sigma, St. Louis, Mo.) per kg of body weight, animals were killed by CO_2 asphyxiation 4, 12, and 24 h later, and total liver RNA was prepared by the guanidine thiocyanate method as previously described (12). Raw 264.7, a mouse monocytic cell line, was obtained from the American Type Culture Collection (Rockville, Md.) and maintained in Cellgro RPMI (Mediatech, Washington, D.C.) supplemented with 10% fetal calf serum. LPS stimulation and preparation of conditioned medium (Raw CM) was essentially as described by Beutler et al. (6), with the modification that the medium was concentrated 20-fold and underwent two cycles of volume exchange with

* Corresponding author.

10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4) by diafiltration in a model 8200 ultrafiltration device through a YM-5 membrane (Amicon Corp., Danvers, Mass.). Reuber H35 cells, a rat hepatoma cell line (a gift of John Koontz) were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum in the presence of 5×10^{-7} M dexamethasone. Raw CM was added to the culture medium to a final concentration of 0.4%, and total cellular RNA was isolated 4, 8, and 24 h later. Total cellular RNA (20 μg per lane) was fractionated on 1.5% agarose-formaldehyde gels and analyzed by Northern (RNA) blotting. The blots were probed with multiple-primer-labeled rAT cDNA, exposed, stripped of probe, and rehybridized to a rat actin complementary probe (11).

Plasmids and transfections. Mutation of the six nucleotides (nt), nt −547 to −541, in the rAT gene 5'-flanking sequence to an *Nco*I site was accomplished by amplifying, in the polymerase chain reaction, the sequences upstream (nt −615 to −548) and downstream (nt −540 to −443) of the desired mutation site, using oligonucleotide primers that had, in their 5' ends, sequences coding for an *Nco*I site (at nt −547 and −541 for the upstream and downstream primers, respectively), a *Bam*HI site (at nt −615), or a *Bgl*II site (at nt −442). The products of these polymerase chain reactions, when digested with *Nco*I and ligated one to the other, resulted in the creation of a fragment of the rAT gene containing the sequence between nt −615 and −442, except that the six nucleotides between nt −547 and −541 had been mutated to an *Nco*I site. The *Bam*HI and *Bgl*II sites flanking this fragment allowed, upon their digestion, its cloning into the *Bam*HI site at nt −442 of a fusion construct between the rAT 5'-flanking sequence and a luciferase reporter gene as described previously in detail (9). The resulting mutant plasmid was named Mp615RLG. An identical wild-type plasmid (p615RLG) was created by amplification of the rAT sequence from nt −615 to −442, using identical primers, and ligating the fragment, after digestion with *Bam*HI and *Bgl*II, into the same *Bam*HI site at nt −442 of the fusion construct described above. These two constructs were used to stably transfect H35 cells.

Reporter constructs used to test the enhancer properties of the putative rAT acute-phase response element (APRE) and to compare it with the previously described (38) NFκB-binding site present in the murine immunoglobulin kappa light-chain enhancer (κBE) were prepared by annealing complementary single-stranded oligonucleotides containing these sequences, ligating them through four nucleotide complementary overhangs to create multiple copies of these double-stranded oligonucleotide cassettes, and cloning them into the *Bam*HI site immediately upstream of nt −59 of a minimal rAT promoter-luciferase reporter construct (p59RLG). The resulting constructs were named X1-, X2-, and X4APREp59RLG and X2- and X4KBEp59RLG. The rAT APRE oligonucleotide sequence is that from nt −557 to −528, with the addition of GAT at the 5' end (to allow cloning into *Bam*HI sites), and reads on the coding strand as follows: 5'-GATCCACAGTTGGGATTTCCCAACCTGAC CAGA-3'. The κBE oligonucleotide was prepared as described previously (27) except that the complementary overhangs were modified to be *Bam*HI-*Bgl*II compatible; it reads on the coding strand as follows: 5'-GATCCAGAGGG GACTTTCCGAGAGGA-3'. A mutant APRE oligonucleotide was also constructed with the sequence 5'-GATCCACA TGTTGGATTTCCGATACTGACCAGA-3' (underlined nucleotides represent the mutations introduced), and a four-copy concatemerized multimer of the double-stranded oligonucleotide cassette was cloned into the *Bam*HI site of p59RLG to create X4MAPREp59RLG. The sequences of all constructs described were confirmed by sequencing with the dideoxynucleotide termination method.

H35 cells stably transfected with p615RLG and Mp615RLG were obtained by transfecting 5×10^6 cells with 9 μg of test DNA and 1 μg of pSV_2 Neo (41) by the calcium phosphate precipitation technique, and resistant clones were isolated by selecting for aminoglycoside resistance in the presence of Geneticin (0.3 mg/ml; Sigma). The presence of multiple tandem arrays of integrated test plasmid was confirmed by Southern blot analysis of genomic DNA from cloned transformants that had been passaged several times in the absence of Geneticin. Stably transfected cells were stimulated for 4 h with 0.4% Raw CM or 250 U of recombinant human TNF-α (hereafter referred to as TNF; Bachem, Torrance, Calif.) per ml and assayed for luciferase activity (9). Results were normalized to protein content of the cell lysate as determined by the Bio-Rad assay (Bio-Rad, Laboratories, Richmond, Calif.).

Transient transfections of HepG2 cells and luciferase assays were performed as previously described (9) except that placental alkaline phosphatase activity from cotransfected pSV_2PAP (22; a gift of Tom Kadesch) was used as the internal recovery marker. The medium was changed 48 h after transfection, and cells were stimulated for 4 h with 30 or 100 U of recombinant human IL-1α (hereafter referred to as IL-1; a gift of Steven Gillis, Immunex Corp., Seattle, Wash.) per ml or 40 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma) and harvested for assay. Stimulation of transiently transfected HepG2 cells in the presence of cycloheximide was performed by a modification of a reported technique (42). Transfected cells were stimulated with IL-1 in the presence of cycloheximide (10 μg/ml) for 2 h and then washed three times with phosphate-buffered saline to remove both IL-1 and cycloheximide. Protein synthesis was allowed to resume for 2 h in the absence of IL-1, and cells were harvested for assay of luciferase activity. All transfections were carried out in duplicate, and experiments were repeated at least three times with consistent results.

Mapping of the initiation site of transcription from the X4APREp59RLG construct was performed by primer extension analysis (10) of poly(A)$^+$ RNA prepared from IL-1-stimulated (100 U/ml) and unstimulated HepG2 cells transfected with the construct. The primer used was complementary to nt +86 to +55 of the luciferase cDNA (17), and the expected extension product was 152 bases long.

Nuclear extracts and binding assays. Rat liver nuclear extracts after LPS injection were prepared by the method of Costa et al. (15). Nuclear extracts from HepG2 cells were prepared essentially as described by Dignam et al. (18), with the modification that after the chromatin extraction step an ammonium sulfate precipitation was included to concentrate proteins before dialysis. Electrophoretic mobility shift assays (EMSA) were performed with a double-stranded oligonucleotide (APRE or κBE) labeled by filling in the four-base overhangs with Klenow fragment of DNA polymerase incorporating [α-^{32}P]dATP. Binding reactions were performed as described by Sen and Baltimore (38) except that the binding buffer contained 1 mM dithiothreitol instead of β-mercaptoethanol. Complexes were resolved on a 4% nondenaturing polyacrylamide gel. DNase I footprinting reactions were carried out as previously described (9). The probe was labeled on the coding strand at nt −615, and fragments were resolved on a 7% polyacrylamide–8 M urea gel. A methyla-

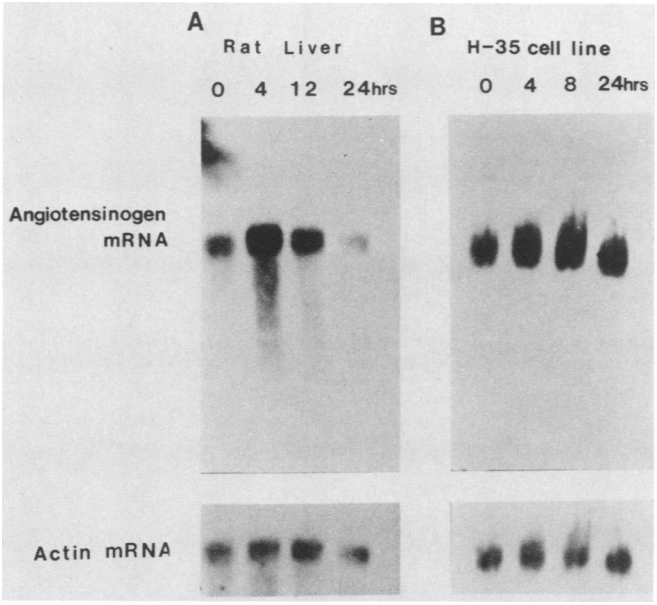

FIG. 1. Induction of rAT mRNA levels by acute-phase reactants in vivo and in a cell culture model. (A) Effect of LPS injection on levels of rAT mRNA in rat liver. Shown is a Northern blot analysis of 20 μg of total RNA harvested at the indicated times after LPS injection and hybridized with a rAT cDNA probe or, as a control, an actin probe. (B) Effect of LPS-stimulated Raw CM on rAT mRNA levels in H35 cells. Total RNA (20 μg) from cells stimulated with Raw CM for the indicated periods of time was analyzed as for panel A.

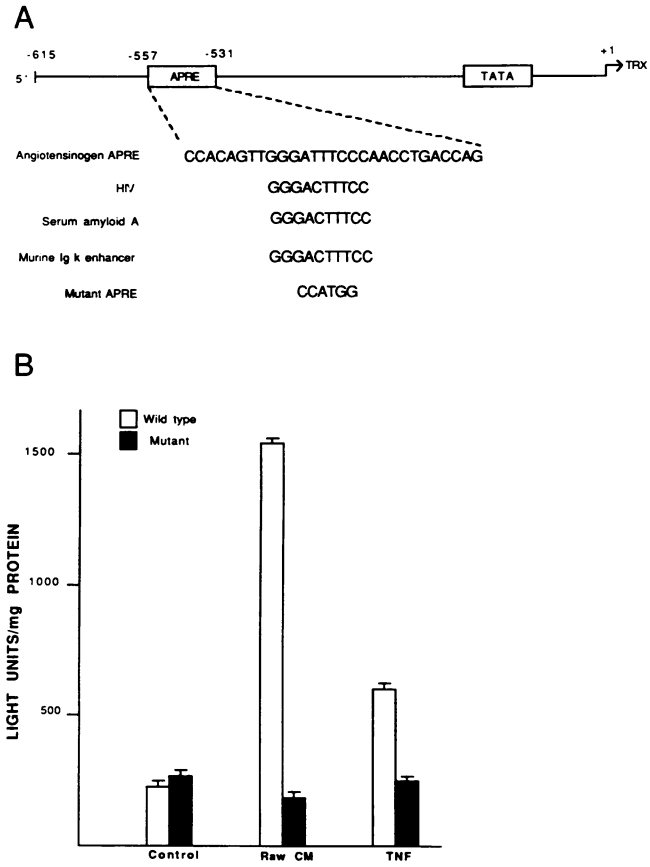

FIG. 2. Attenuation of transcriptional activation by a mutation in the APRE. (A) Location and sequence of the rAT gene APRE in the 5'-flanking region of the gene and comparison with similar sequences in other genes. Negative numbers indicate numbers of bases from the transcription start site at +1. (B) Activation of wild-type (p615RLG) and mutant (Mp615RLG) angiotensinogen-luciferase reporter constructs stably transfected into H35 cells by 0.4% Raw CM and 250 U of TNF per ml. Cells were stimulated for 4 h before harvest. Luciferase activities were normalized to protein content of the cell lysate. Data represent means and ranges of transfections performed in duplicate and are representative of experiments repeated at least three times.

tion interference assay was performed by using a probe labeled on the coding strand at nt −615 (38).

UV cross-link labeling of nuclear proteins (13) was performed by using a body-labeled probe synthesized with incorporated bromodeoxyuridine and [α-^{32}P]dATP by Klenow fragment extension from an SP6 promoter primer, using as a template four copies of the APRE oligonucleotide cloned into the *Bam*HI site of pGEM3 (Promega Biotec, Madison, Wis.) After binding for 10 min of 6 μg of HepG2 nuclear extract to 10 fM labeled derivatized probe, UV irradiation for 15 min, and digestion of the free probe, the labeled proteins were denatured and resolved on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel.

Southwestern (DNA-protein) ligand blot was performed as described by Miskiminis et al. (30) as follows: rat liver nuclear extract (100 μg per lane) was denatured and fractionated on a 10% SDS-polyacrylamide gel and electroblotted onto nitrocellulose paper, and without further renaturation steps the filter was blocked for 3 h at 4°C with 5% nonfat dry milk in TNE-50 (50 mM NaCl, 10 mM Tris [pH 7.5], 1 mM EDTA, 1 mM dithiothreitol). Binding reactions were performed for 12 h at 4°C in TNE-50 with labeled APRE double-stranded oligonucleotide as a probe. Specific nonlabeled competitor was used where indicated, and poly(dI-dC) (10 μg/ml) was included as a nonspecific competitor in all reactions. Before autoradiography, filters were washed in TNE-50 three times for 20 min at room temperature.

RESULTS

Cell culture model for the hepatic rAT acute-phase response. We confirmed the acute-phase inducibility of rAT mRNA by injecting rats with LPS (3 mg/kg of body weight) and harvesting liver RNA at 4, 12, and 24 h later. The Northern blot shown in Fig. 1A demonstrates a rapid increase in rAT mRNA (normalized to actin mRNA) to fourfold over base line by 4 h persisting for at least 12 h, and falling to control levels by 24 h.

Because it is difficult to control for the various contributions of rising levels of glucocorticoids and cytokines, both induced by LPS injection, to the induction of the rAT mRNA in the animal model, we used a cell culture model. H35 rat hepatoma cells were stimulated with Raw CM as a source for cytokines (Fig. 1B). rAT mRNA rose rapidly to 3.5 × base line and began to fall by 24 h, demonstrating that the H35 cell line is valid for studying the cytokine-responsive elements in the angiotensinogen gene.

Direct-site mutagenesis of six nucleotides centered around nt −545 abolished the cytokine responsiveness of rAT 5'-flanking sequence. On the basis of the similarity between known NFκB-binding sites and the sequence centered around nt −545 in the rAT gene (Fig. 2A), we introduced constructs containing wild-type and mutant 5'-flanking sequence of the rAT gene fused to a luciferase reporter gene into the cytokine-responsive H35 cells. Stably transfected cells were

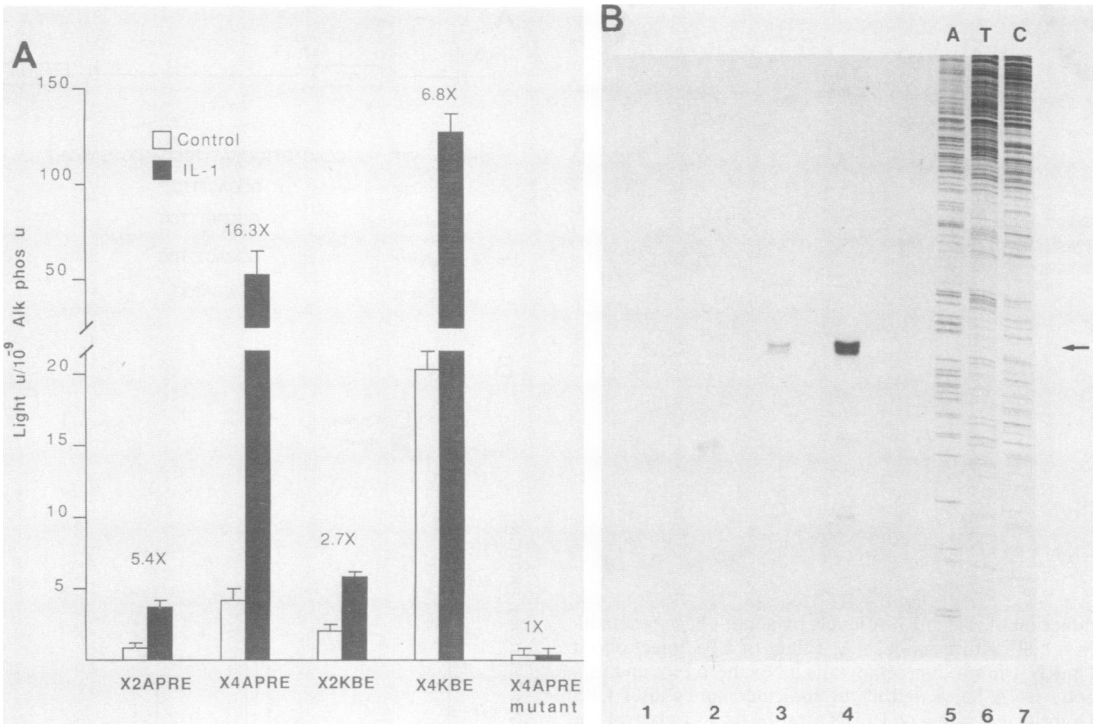

FIG. 3. Demonstration that the APRE linked to a minimal promoter activates transcription in response to IL-1. (A) IL-1 activation of angiotensinogen promoter-luciferase reporter constructs containing two or four copies of the APRE or κBE site transfected into HepG2 cells. Stimulation was carried out for 4 h with 100 U of human recombinant IL-1 per ml. Luciferase activity was normalized to placental alkaline phosphatase activity expressed from a cotransfected pSV_2PAP plasmid. Results are presented as means and ranges of transfections performed in duplicate; the experimental points were repeated three to five times with consistent results. The mutant APRE had no response to IL-1. (B) Primer extension analysis of poly(A)$^+$ RNA from HepG2 cells transfected with X4APREp59RLG and stimulated with IL-1 (100 U/ml) for 4 h (lane 4) or from unstimulated controls (lane 3). RNA from nontransfected HepG2 cells (lane 2) and yeast transfer RNA (lane 1) served as negative controls. ←——, Site of correctly initiated transcript, as determined by the dideoxy termination sequencing reactions (lanes 5 to 7).

selected for by cotransfecting a neomycin resistance plasmid and treating cells with Geneticin. Three antibiotic-resistant clones transfected with wild-type sequence from nt −615 to nt +39 (p615RLG) and two clones bearing the mutation depicted in Fig. 2A (Mp615RLG) were isolated. The wild-type clones all responded to Raw CM with a 5- to 10-fold increase in reporter gene activity and with a 3- to 4-fold increase in response to TNF. The response was rapid, occurred within 4 h of stimulation, and persisted for up to 24 h. The magnitude of the response of the reporter gene activity was comparable to that of the endogenous rAT mRNA, suggesting that sequences within the reporter construct used were sufficient for full cytokine responsiveness. The mutant clones, on the other hand, were unresponsive even though a comparable basal reporter activity was attained (Fig. 2B).

The sequence spanning nt −557 to −528 of the rAT gene confers cytokine responsiveness on an inert promoter. The direct-site mutagenesis data suggesting that a cytokine-responsive *cis*-acting DNA element is centered around nt −545, in conjunction with the DNase I footprinting data presented below, enabled us to postulate the boundaries of such an element, the APRE. To test whether the identified element is capable of conferring cytokine inducibility on a nonresponsive reporter construct, we designed an oligonucleotide cassette with linkers based on the sequences in the rAT gene between nt −557 and −528 and ligated multiple copies upstream of a rAT minimal promoter luciferase reporter construct (p59RLG). These plasmids, X2APREp59RLG and X4APREp59RLG, when transfected into HepG2 cells (a human hepatoblastoma cell line known to be easily transfectable), responded with 15- to 30-fold activation of luciferase activity to IL-1 (Fig. 3A), Raw CM, or TNF (not shown). The response was dependent both on the dose of the stimulating cytokine and on the copy number of APRE cassettes in the reporter construct. A construct bearing four copies of a mutant APRE, X4MAPREp59RLG, was unresponsive, as was the promoter alone. Similar responses were seen in another hepatoma cell line, Hep3b (not shown).

The cytokine-induced increase in reporter activity was correlated with an increase in correctly initiated transcripts, as assayed by primer extension (Fig. 3B), and thus in all likelihood represented enhanced transcription of reporter mRNA. We further demonstrated the independence of the cytokine inducibility of the APRE from any particular promoter or reporter interactions by showing that four copies of the APRE conferred cytokine responsiveness on the thymidine kinase promoter linked to a chloramphenicol acetyltransferase reporter (results not shown).

In view of the similarity between NFκB-binding sites and the identified rAT APRE, we tested the ability of an oligonucleotide cassette designed after the sequence of the NFκB site present in the mouse κ light-chain gene enhancer to confer cytokine responsiveness on the same minimal rAT promoter. Constructs containing κBE sequences were in-

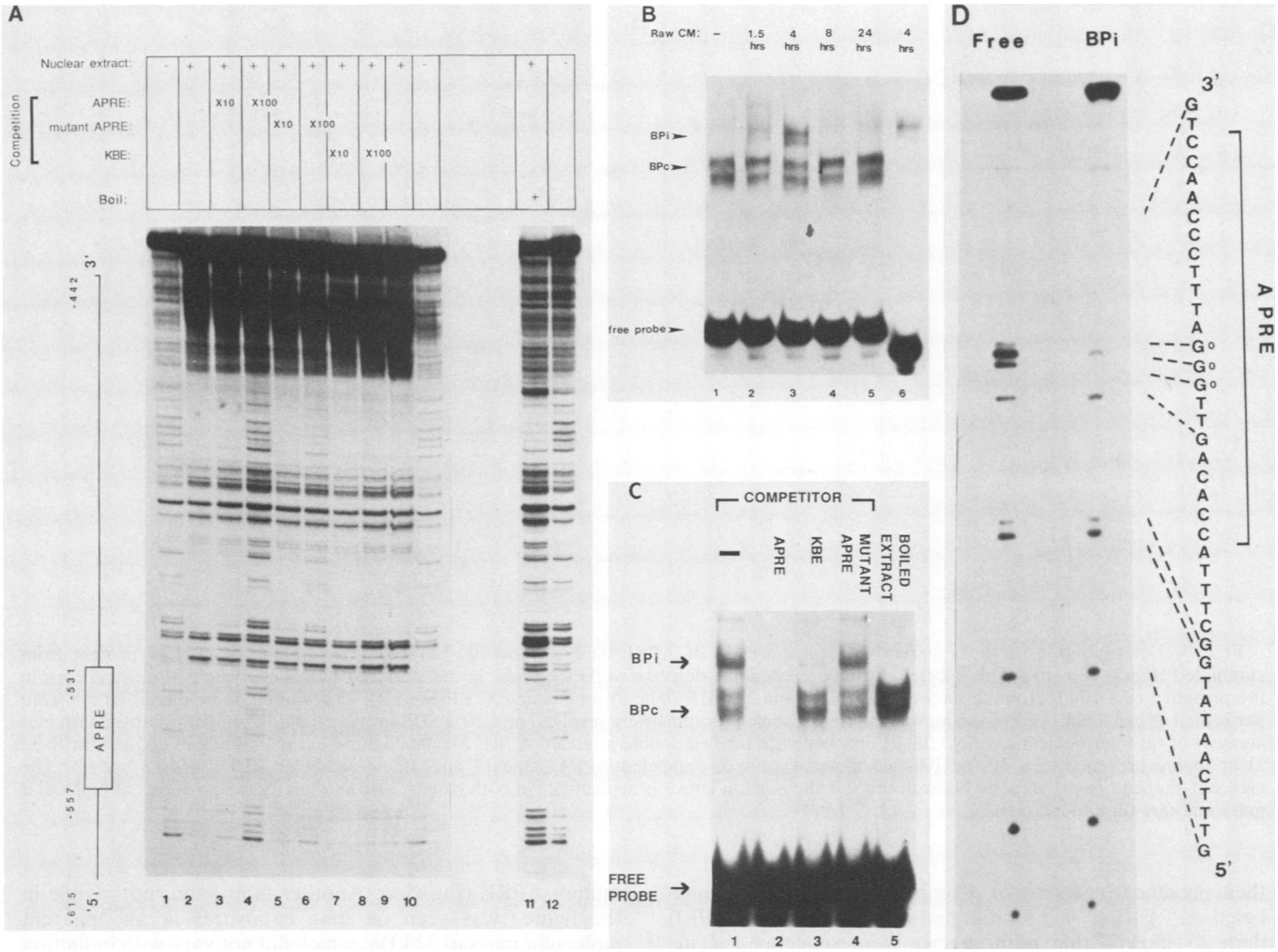

FIG. 4. Binding of the APRE by both a constitutive and an inducible protein. (A) DNase I footprint analysis of a rAT gene fragment (nt −615 to −442), using liver nuclear extract from LPS-injected rats (50 μg per reaction). Shown are control digests with no nuclear extracts (lanes 1, 10, and 12), no competition (lanes 2 and 9), and competition with the indicated molar excess of double-stranded oligonucleotides: APRE (lanes 3 and 4), mutant APRE (lanes 5 and 6), and κBE (lanes 7 and 8). The extract in lane 11 was heated to 100°C for 2 min before the binding reaction. (B) EMSA of labeled double-stranded APRE oligonucleotide (lanes 1 to 5) or κBE oligonucleotide (lane 6) by nuclear extract (6 μg) from Raw CM-stimulated HepG2 cells. Arrowheads identify the constitutive (BPc) and inducible (BPi) binding proteins. (C) EMSA of APRE probe with 4 h Raw CM-stimulated HepG2 nuclear extract (6 μg) (lane 1), in the presence of a 100-fold molar excess of unlabeled competitor APRE (lane 2), κBE (lane 3), and mutant APRE (lane 4). Lane 5 contained 24 μg of the same extract after heat treatment. (D) Methylation interference of binding by BPi to a rAT gene probe 5′ labeled on the coding strand at nt −615. Guanine residues whose methylation interfered with binding are indicated (○).

duced by IL-1 in HepG2 cells; furthermore, the basal and ultimate activities of these constructs were greater than those of the APRE constructs containing the same number of cassettes (Fig. 3A), suggesting a functional difference between the two elements beyond the obvious similarities in nucleotide sequences.

Identification of two distinct proteins binding the rAT APRE. We performed DNase I footprinting assays on a probe spanning the rAT APRE, using untreated and LPS-treated rat liver nuclear extract and unstimulated and Raw CM-stimulated HepG2 nuclear extracts. An identical area of protection from nt −557 to −531 was seen with all extracts, which was specifically competed for by an APRE oligonucleotide cassette but not by a mutant APRE or the κBE sequence. This result suggested the presence of a constitutive nuclear factor binding to this enhancer sequence (Fig. 4A). Two other protected regions were detected downstream of the APRE, but they did not appear to represent binding by the same factor since they were competed for by a mutant APRE (Fig. 4A, lane 6) and abolished by heating the nuclear extract, whereas the footprint over the APRE persisted (lane 11).

By EMSA using the same extracts and a labeled double-stranded APRE oligonucleotide probe, two distinct complexes were identified: a constitutively present binding factor (BPc) and an inducible one of different mobility (BPi), which could be seen as early as 1.5 h after Raw CM stimulation of HepG2 cells (Fig. 4B). A similar species was present in LPS-treated rat liver nuclear extract, although the high abundance of BPc all but overshadowed its presence (not shown). The proteinaceous nature of these binding species is attested to by the ability of proteinase K to abolish

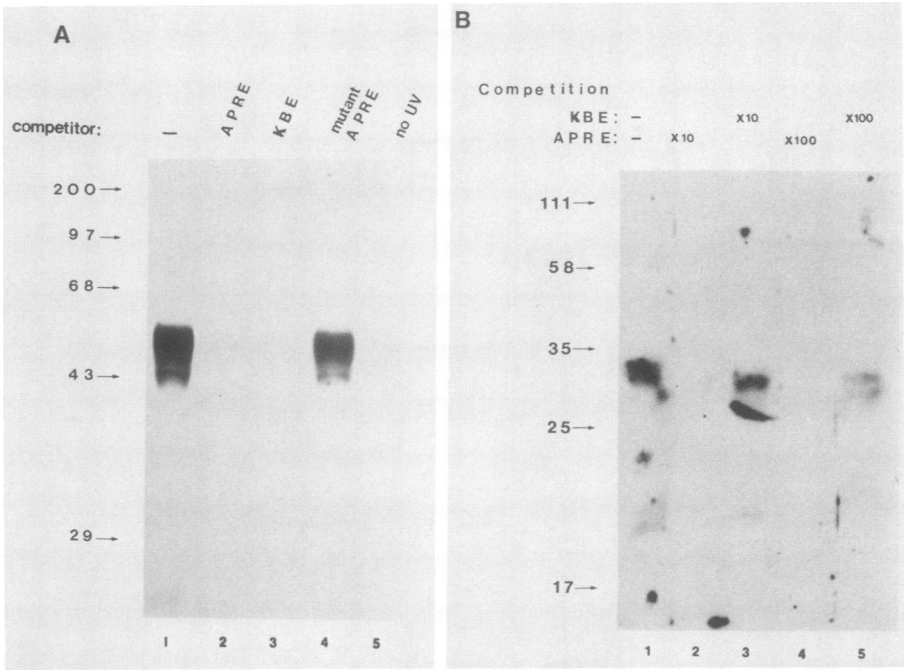

FIG. 5. Determination of the apparent molecular weight of APRE-binding proteins. (A) UV cross-linking of nuclear protein from stimulated HepG2 cells to a body-labeled, bromodeoxyuridine-derivatized APRE probe in the absence of unlabeled competitor (lane 1) or in the presence of a 100-fold molar excess of APRE (lane 2), κBE (lane 3), or mutant APRE (lane 4). The binding reactions in lane 5 were performed as usual but UV irradiation was omitted. Labeled proteins were resolved on a 10% SDS-polyacrylamide gel. (B) Southwestern blot analysis of rat liver nuclear extract (100 μg) probed with labeled double-stranded APRE oligonucleotide in the absence of competitor (lane 1) or in the presence of a 10- or 100-fold molar excess of unlabeled APRE (lanes 2 and 4), or unlabeled κBE (lanes 3 and 5). The crescent-shaped opacity directly beneath the specific band in lane 3 is artifactual. In both panels, arrows identify the positions of molecular mass markers (in kilodaltons).

their presence (not shown). Competition with excess unlabeled APRE (Fig. 4C, lane 2) and unlabeled mutant APRE (lane 4) showed that both species were sequence-specific DNA-binding proteins. They did, however, differ in specificity; the κBE sequence could bind the inducible protein directly (Fig. 4B, lane 6) and compete for its binding to the labeled APRE (Fig. 4C, lane 3) whereas the constitutive protein failed to bind κBE. The two binding proteins were further separable by the relative resistance of BPc to boiling (Fig. 4C, lane 5). This difference in the heat stability of the two proteins was further exploited to examine their role in giving rise to the DNase I-resistant footprint over the APRE. The persistence of the footprint after boiling of the nuclear extract (Fig. 4A, lane 11) and failure of the κBE sequence to alter the appearance of the protected region in both induced and uninduced extracts implies that BPc alone was responsible for this in vitro DNase I protection.

Since BPi failed to consistently alter the DNase I digestion pattern of our probe, we resorted to a methylation interference binding assay to establish the specific contact points made. Methylation on the coding strand of three guanine residues within the APRE interfered with BPi binding (Fig. 4D). These residues correspond to the expected contact points that NFκB makes with the similar sequence in the κBE (38). To further characterize the two binding proteins, we cross-linked a labeled derivatized APRE probe with UV light to uninduced and induced HepG2 nuclear extracts. Raw CM induced a binding protein with an apparent molecular mass of 50 kilodaltons (kDa), the binding characteristics of which were identical to those of BPi: it was competed for by an excess of both unlabeled APRE and κBE but not by a mutant APRE (Fig. 5A). Another faint band, not visible in the figure, was seen on long exposures at an apparent molecular mass of 32 kDa, which did not vary with induction and may have corresponded to BPc. However, to obtain a more reliable estimate of the molecular mass of BPc, we performed Southwestern ligand blot analysis of rat liver nuclear extract, which by EMSA appeared greatly enriched in BPc compared with the HepG2 extract. Rat liver nuclear extract was denatured, fractionated by SDS-polyacrylamide gel electrophoresis, and blotted onto a nitrocellulose filter, which was then probed with a labeled APRE oligonucleotide. A doublet migrating at an apparent molecular mass of 32 kDa was seen with the binding characteristics of BPc: unlabeled APRE competed effectively for binding, whereas κBE did not (Fig. 5B). The faint bands migrating with an apparent molecular mass of 20 kDa had the same binding specificity as did the 32-kDa species; the fact that they were not seen in all preparations suggests to us that they may represent in vitro degradation products.

Phorbol esters induce APRE-binding activity indistinguishable from that of BPi and activate transcription from an APRE-containing template. In view of the evidence that BPi is an NFκB-like factor, we tested whether TPA, a phorbol ester known to induce nuclear translocation of NFκB (2) could induce BPi activity in nuclear extracts of HepG2 cells and whether reporter activity, driven by APRE-containing constructs, could be likewise induced. Figure 6B shows the induction, by 4 h of TPA stimulation, of a binding species identical in mobility to BPi that was competed for by both unlabeled κBE and APRE (not shown).

TPA induced a 20- to 50-fold increase in luciferase activity

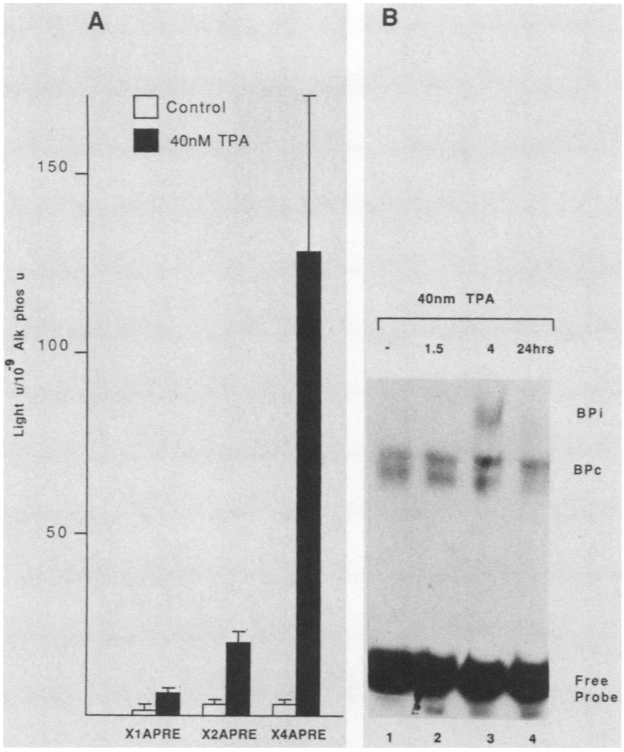

FIG. 6. Demonstration that phorbol ester mimics cytokine activation of transcription via the APRE. (A) Phorbol ester activation of angiotensinogen promoter-luciferase reporter constructs containing one, two, and four copies of APRE oligonucleotide cassettes. Transfected HepG2 cells were stimulated for 4 h in the presence of 40 nM TPA. Luciferase activities was normalized to placental alkaline phosphatase activity from a cotransfected pSV_2PAP plasmid. (B) EMSA of APRE probe with nuclear extract prepared from TPA (40 nM)-stimulated HepG2 cells.

driven by X4APREp59RLG and a lesser induction of constructs containing fewer copies of APRE (Fig. 6A). The promoter alone was not induced by TPA. Of note, the internal control used in all of our transfection assays, pSV_2PAP, which contains the complete simian virus 40 enhancer, was unresponsive to TPA stimulation; this result conflicts with the reported responsiveness of this enhancer to TPA in HepG2 cells (23). Since the reported TPA responsiveness was not seen in all hepatoma cell lines (being absent in Hep3B cells [23]), we speculate that our HepG2 cells, originally obtained from the American Type Culture Collection but since serially passaged in our laboratory for over 50 passages, may have acquired a phenotype different from that of cells used by Imbra and Karin (23).

Cytokine induction of BPi does not depend on new protein synthesis. The appearance of nuclear NFκB-binding activity in response to inducers proceeds in the absence of new protein synthesis (39). We therefore tested whether cytokines could induce the appearance of BPi activity in HepG2 nuclei in the presence of concentrations of cycloheximide inhibitory to new protein synthesis. IL-1 induced an APRE-binding factor with a mobility expected of BPi in the presence of cycloheximide (Fig. 7B). Competition experiments demonstrated that the induced complex could be competed for by both unlabeled κBE and APRE; the bands visible above and below BPi corresponded to BPc, since they were competed for by unlabeled APRE but not unlabeled κBE.

By treating HepG2 cells transfected with X4APREp59RLG with IL-1 in the presence of 10 μg of cycloheximide per ml for 2 h and then washing the cells free of inhibitor and stimulator and allowing reporter expression to proceed for another 2 h, we could assay for reporter activation by an event occurring in the absence of new protein synthesis. Luciferase activity, after IL-1 treatment in the presence of cycloheximide, was superinduced to levels higher than in cells similarly treated in the absence of cycloheximide (Fig. 7A), thus demonstrating that the activation event did not depend on early new protein synthesis. As a control for the adequacy of protein synthesis inhibition, we demonstrated that failure to wash the cells free of cycloheximide led to a total lack of activation. Similar results were obtained in H35 cells stably transformed with a cytokine-responsive reporter construct, p615RLG (data not shown).

To confirm that the increase in reporter activity induced by IL-1 in the presence of cycloheximide resulted from an increase in abundance of specific transcripts, we performed primer extension analysis of luciferase mRNA, using poly(A)$^+$ RNA obtained from HepG2 cells transfected with X4APREp59RLG. A 4-h treatment with IL-1 (100 U/ml) in the presence of cycloheximide (10 μg/ml) led to a marked increase in correctly initiated transcripts (Fig. 7C, lane 3) compared with results for cells treated with cycloheximide alone (lane 2) or untreated cells (lane 1). Transcripts initiating from the simian virus 40 early early gene promoter of cotransfected pSV_2PAP did not change with treatment, serving as an internal control for transfection efficiency and RNA recovery.

DISCUSSION

We have identified, by directed mutagenesis, a *cis*-acting DNA element essential for cytokine-induced transcription of the rAT gene in liver cells. This element, termed the rAT APRE, when fused in *cis* to a nonreactive promoter, is capable of conferring cytokine responsiveness to a reporter gene. LPS-stimulated monocyte conditioned medium, a source of IL-1 and TNF, as well as highly purified preparations of the recombinant bacterially expressed cytokines elicited similar responses, suggesting that signal transduction pathways responsive to both cytokines can converge on the same *cis* element. An identical situation has been described for these two cytokines in inducing transcription via a common element in the HIV long terminal repeat (34). Comparison of the nucleotide sequence of the HIV response element with that of the rAT APRE shows a high degree of sequence similarity. Furthermore, these IL-1 and TNF response elements are extensively similar in sequence to the NFκB-binding site present in the mouse κBE (38) and to other inducible sites in the beta interferon gene (27), the IL-2 receptor alpha gene (29), and to a phorbol ester responsive element in the human SAA gene (19), a known hepatic acute-phase response gene. Our positive assignment of a role for such a *cis* element in a cell culture model of the rAT hepatic acute-phase response supports a similar role for the SAA gene NFκB-binding site as suggested by Edbrooke et al. (19) and is in keeping with the hypothesized pleiotropic nature of NFκB as a mediator of intracellular signal transduction (27).

Binding studies identified two distinct liver nuclear proteins that can bind to the APRE: one constitutively present (BPc) and one inducible by the same stimuli that activate transcription through the rAT APRE (BPi). Competition experiments reveal that the inducible factor also recognizes the murine kappa light-chain gene NFκB-binding site with

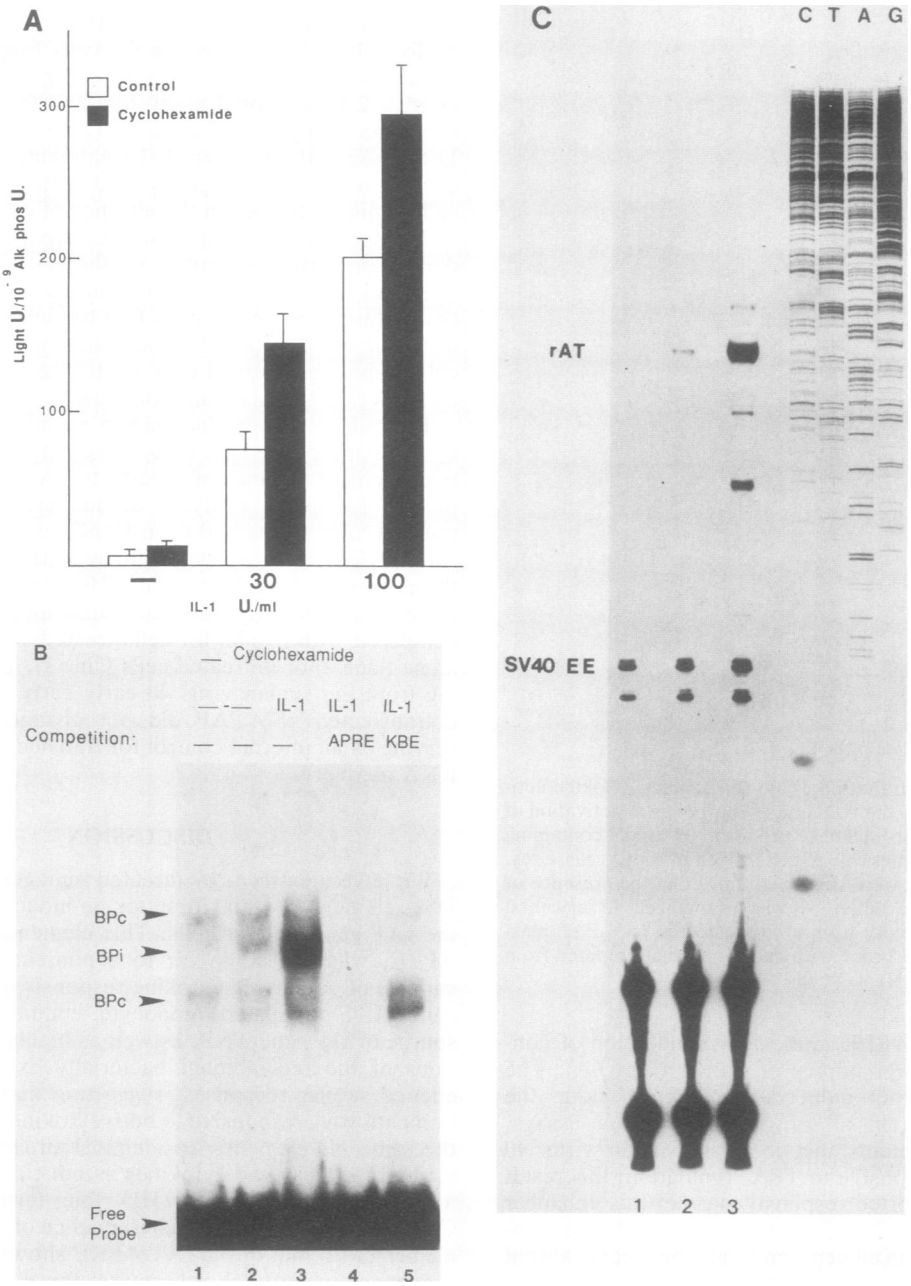

FIG. 7. Demonstration that activation of transcription mediated via the APRE occurs by a posttranslational mechanism. (A) Activation of X4APREp59RLG by IL-1 in the presence of cycloheximide. Transfected cells were stimulated with IL-1 (100 U/ml) in the presence of cycloheximide (10 μg/ml) for 2 h, washed free of cycloheximide, and allowed to resume protein synthesis for 2 h in the absence of IL-1 before harvest. (B) EMSA of an APRE probe with HepG2 nuclear extracts from control cells (lane 1) and cells incubated with cycloheximide (10 μg/ml) for 4 h in the absence (lane 2) or presence (lanes 3 to 5) of IL-1 (100 U/ml). In lanes 4 and 5, 100-fold molar excesses of unlabeled APRE and κBE, respectively, were included in the binding assay. (C) Primer extension analysis of poly(A)$^+$ RNA from HepG2 cells transfected with X4APREp59RLG and cultured for 4 h in the presence of both cycloheximide and IL-1 (lane 3), cycloheximide alone (lane 2), or neither (lane 1). Indicated on the left are positions of the correctly initiated reporter construct transcript (rAT) and transcripts initiated from the cotransfected pSV_2PAP plasmid (SV40 EE).

similar affinity. Moreover, methylation interference analysis of the contact points made by BPi shows that methylation of guanine residues in the rAT gene homologous to those whose methylation is known to interfere with NFκB binding to its cognate site in the κBE (38) interferes with BPi binding, lending further support to the notion that cytokines can induce, in liver cells, a transcriptional activator protein similar to NFκB. UV cross-linking identifies a 50-kDa inducible protein in HepG2 cells that binds to both the APRE and murine NFκB-binding sites. This protein is therefore similar in size to purified NFκB from human Namalwa cells as reported by Kawakami et al., 51 kDa (25), and within the range of that purified from bovine spleen by Lenardo et al., 44 kDa (28), and the inducible proteins shown by a similar technique to bind to the IL-2 receptor alpha gene NFκB-binding site (16), suggesting a structural similarity with these

proteins. We find no evidence for a larger 86 kDa protein reported to bind to the HIV NFκB site by Bohnlein et al. (7). The induction of NFκB-like binding activity in hepatoma cells and the activation of transcription mediated by the rAT APRE share two other features with the induction of NFκB: phorbol esters, known to lead to the nuclear translocation of NFκB in many cell types (2), can mimic the effects of cytokines in our liver cell system, and the cytokine response can proceed in the absence of new protein synthesis, as has been shown for the LPS stimulation of NFκB-binding activity in pre-B lymphocytes (39). Furthermore, we provide evidence that reporter gene activity activated via the APRE is superinduced in the presence of protein synthesis inhibition, as has been shown for NFκB (39).

The functional role of the constitutive binding protein, BPc, is less clear. Its high-affinity binding to the wild-type APRE, as attested to by the ability of a mere 10-fold molar excess of unlabeled competitor to compete significantly in the various binding assays as well as failure of a transcriptionally inactive mutant APRE sequence to compete, suggests sequence-specific binding. BPc is detectable only in nuclear extracts, being totally absent from the cytosolic fraction by EMSA (data not shown), suggesting that it plays a role in a nuclear event. A constitutive factor capable of binding the APRE appears to contribute to multiple DNA-protein interactions on the rAT gene 5′-flanking sequence, as demonstrated by the ability of unlabeled APRE cold competitor oligonucleotide to compete for several DNase I-protected regions in the footprint assay (Fig. 4A). However, these interactions either are of lower affinity than the binding to the APRE site or require the presence of ancillary, heat-labile factors, since boiling the nuclear extract for 2 min, a procedure to which BPc activity is relatively resistant, leads to retention of the DNase I-protected area predominantly over the APRE (Fig. 4A, lane 11).

A role for a functional interaction between BPc and the NFκB-like BPi is suggested by the complete overlapping of their binding sites in the rAT 5′-flanking sequence. Comparing the shifting pattern of a κBE probe with that of an APRE probe by extracts that contains both BPi and BPc, we fail to detect a complex of intermediate or lower mobility, consistent with one being composed of both proteins binding to the APRE simultaneously, thus suggesting that their binding is mutually exclusive. Other studies have suggested that the functional enhancer properties of those NFκB-binding sites which also bind other constitutively present nuclear proteins is consistent with a model of competition between factors of varying transactivating potency for the same binding site. This is reflected in the basal enhancer activity of such sites and in their attenuated inducibility by stimuli that activate NFκB (27). Our findings concerning the rAT APRE fit this model. The apparent inability of BPc to bind the κBE sequence, coupled with the evidence from a side-by-side comparison of equal-dose κBE-site-containing promoters and APRE-containing ones which reveals the former to be more active, suggests a role for BPc in attenuating the BPi-mediated activation of angiotensinogen transcription. Such a model could account for the decreased basal enhancer activity of APRE versus κBE in HepG2 cells by postulating that the basal activity of κBE is dependent on basal levels of BPi being present in the nucleus.

Inducibility of NFκB-binding sites varies considerably between different genes. This may be due to differences either in the core sequence or in the surrounding bases. The IL-2 receptor alpha gene, as an example, contains an NFκB-binding site that cross-competes with κBE for a phorbol ester-inducible nuclear protein yet is not active as a phorbol ester-inducible enhancer by itself (29). When present in the context of the surrounding bases of the IL-2 receptor alpha gene, however, the same element is inducible (16); this implies an interaction between NFκB and other adjacent regulatory factors. Other proteins may bind the same site as does NFκB, as in the case of the heavy-chain gene of the mouse class I major histocompatibility complex antigen system (*H-2K^b*) (4) or the viral inducible element in the beta interferon gene (26, 43). In both cases, the NFκB binding site colocalizes to an element that appears to be important for basal expression of the gene and that binds a constitutively present protein (3, 20). The two identified constitutive proteins appear to be similar in that cDNA clones obtained by screening direct-expression libraries with recognition site probes from both genes are reported to bear considerable sequence identity (27). That these proteins are distinct from the APRE BPc is suggested by the difference between the apparent molecular mass of BPc as identified on the Southwestern blot, 32 kDa, and that predicated by the open reading frame of the fusion protein coded for by the aforementioned clones (40). BPc also appears distinct from the constitutive *H-2K^b*-binding protein, KBF1, purified from mouse thymoma cells and reported to have an apparent molecular mass of 48 kDa (44). The distinction between the two proteins is further supported by the fact that BPc binding to the APRE is not competed for by an oligonucleotide based on the *H-2K^b* protein sequence (data not shown), suggesting different binding specificities of the two proteins.

The rAT single-copy APRE is distinct from other known APREs in its distant location relative to the promoter, situated some 545 base pairs upstream from the cap site. A similar sequence is identically located in the mouse angiotensinogen gene promoter (14), implying evolutionary conservation of the spacing. In gene transfer experiments, single-copy NFκB-binding sites are relatively inactive over such distances (20, 35; our unpublished observations), suggesting that there may be some pressure to control the degree of activation of the angiotensinogen gene. The possibility that BPc plays a role in such an attenuation and the even more intriguing possibility that an interaction between the APRE and the rAT glucocorticoid response elements leads to a coordinate modulation of the rAT acute-phase response by glucocorticoids are now under investigation.

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LITERATURE CITED

1. **Arcone, R., G. Gualandi, and G. Ciliberto.** 1988. Identification of sequences responsible for acute-phase induction of human C-reactive protein. Nucleic Acids Res. **16**:3195–3207.
2. **Baeuerle, P., and D. Baltimore.** 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell **53**:211–217.
3. **Baldwin, A. S., Jr., and P. A. Sharp.** 1987. Binding of a nuclear factor to a regulatory sequence in the promoter of the mouse *H-2K^b* class I major histocompatibility gene. Mol. Cell. Biol. **7**:305–313.
4. **Baldwin, A. S., Jr., and P. A. Sharp.** 1988. Two transcription factors, NF-κB and H2TF1, interact with a single regulatory sequence in the class I major histocompatibility promoter. Proc. Natl. Acad. Sci. USA **85**:723–727.
5. **Bauman, H.** 1989. Hepatic acute phase reaction in vivo and in

vitro. In Vitro Cell. Dev. Biol. 25:115–126.
6. **Beutler, B., J. Mahoney, N. Le Trang, P. Pekela, and A. Cerami.** 1985. Purification of cachectin, a lipoprotein lipase-suppressing hormone secreted by endotoxin-induced RAW264.7 cells. J. Exp. Med. **161**:984–995.
7. **Bohnlein, E., J. W. Lowenthal, M. Siekevitz, D. W. Ballard, B. R. Franza, and W. C. Green.** 1988. The same inducible nuclear protein regulates mitogen activation of both interleukin-2 receptor alpha gene and type 1 HIV. Cell **53**:827–836.
8. **Bouhnik, J., F. Savoie, and P. Corvol.** 1988. Differential effects of inflammation models on rat T-kininogen and rat angiotensinogen. Biochem. Pharmacol. **37**:1099–1102.
9. **Brasier, A. R., J. E. Tate, D. Ron, and J. F. Habener.** 1989. Multiple cis-acting DNA regulatory elements mediate hepatic angiotensinogen gene expression. Mol. Endocrinol. **3**:1022–1034.
10. **Calzone, F. J., R. J. Britten, and E. H. Davidson.** 1987. Mapping of gene transcripts by nuclease protection assay and cDNA primer extension. Methods Enzymol. **152**:611–632.
11. **Campbell, D. J., and J. F. Habener.** 1986. Angiotensinogen gene is expressed and differentially regulated in multiple tissues of the rat. J. Clin. Invest. **78**:31–39.
12. **Chirgwin, J. M., A. E. Przybyla, R. J. Macdonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources rich in ribonuclease. Biochemistry **18**:5294–5299.
13. **Chodosh, L. A., R. W. Carthew, and P. A. Sharp.** 1986. A single polypeptide possesses the binding and transcription activities of the adenovirus major late transcription factor. Mol. Cell. Biol. **6**:4723–4733.
14. **Clouston, W. M., B. A. Evans, J. Haralambidis, and R. I. Richards.** 1988. Molecular cloning of the mouse angiotensinogen gene. Genomics **2**:240–248.
15. **Costa, R. H., D. R. Grayson, K. G. Xanthopoulous, and J. E. Darnell, Jr.** 1988. A liver-specific DNA binding protein recognizes multiple nucleotide sites in regulatory regions of transthyretin, alpha-1 antitrypsin, albumin, and simian virus 40 genes. Proc. Natl. Acad. Sci. USA **85**:3840–3844.
16. **Cross, S. L., N. R. Halden, M. J. Lenardo, and W. J. Leonard.** 1989. Functionally distinct NFκB binding sites in the immunoglobulin κ and IL-2 receptor alpha chain genes. Science **244**: 466–469.
17. **De Wet, J. R., K. V. Wood, M. DeLuca, D. R. Helsinki, and S. Subramani.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7**:725–737.
18. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
19. **Edbrooke, M. R., D. W. Burt, J. K. Cheshire, and P. Woo.** 1989. Identification of cis-acting sequences responsible for phorbol ester induction of human serum amyloid A gene expression via a nuclear factor kappa B like transcription factor. Mol. Cell. Biol. **9**:1908–1916.
20. **Fan, C. M., and T. Maniatis.** 1989. Two different virus-inducible elements are required for human β-interferon gene regulation. EMBO J. **8**:101–110.
21. **Fey, G. H., M. Hattori, W. Northemann, L. J. Abraham, M. Baumann, T. A. Braciak, R. G. Fletcher, J. Gauldie, F. Lee, and M. F. Reymond.** 1989. Regulation of rat liver acute phase genes by interleukin-6 and production of hepatocyte stimulating factors by rat hepatoma cells. Annl. N.Y. Acad. Sci. **557**:317–333.
22. **Henthorn, P., P. Zervos, M. Raducha, H. Harris, and T. Kadesch.** 1988. Expression of a human placental alkaline phosphatase gene in transfected cells: use as a reporter for studies of gene expression. Proc. Natl. Acad. Sci. USA **85**:6342–6346.
23. **Imbra, R. J., and M. Karin.** 1986. Phorbol ester induces the transcriptional stimulatory activity of the SV40 enhancer. Science **323**:555–558.
24. **Kageyama, R., H. Ohkubu, and S. Nakanishi.** 1985. Induction of rat liver angiotensinogen mRNA following acute inflammation. Biochem. Biophys. Res. Commun. **129**:826–832.
25. **Kawakami, K., C. Scheidereit, and R. G. Roeder.** 1988. Identification and purification of a human immunoglobulin-enhancer binding protein (NF-κB) that activates transcription from a human immunodeficiency virus type 1 promoter in vitro. Proc. Natl. Acad. Sci. USA **85**:4700–4704.
26. **Lenardo, M. J., and D. Baltimore.** 1989. NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. Cell **58**:227–229.
27. **Lenardo, M. J., C. M. Fan, T. Maniatis, and D. Baltimore.** 1989. The involvement of NF-κB in β-interferon gene regulation reveals its role as widely inducible mediator of signal transduction. Cell **57**:287–294.
28. **Lenardo, M. J., A. Kuang, A. Gifford, and D. Baltimore.** 1988. NF-κB protein purification from bovine spleen: nucleotide stimulation and binding site specificity. Proc. Natl. Acad. Sci. USA **85**:8825–8829.
29. **Leung, K., and G. J. Nabel.** 1988. HTLV-1 transactivator induces interleukin-2 receptor expression through an NF-κB-like factor. Nature (London) **333**:776–778.
30. **Miskiminis, W. K., M. P. Roberts, A. McClelland, and F. H. Ruddle.** 1985. Use of a protein-blotting procedure and a specific DNA probe to identify nuclear proteins that recognize the promoter region of the transferrin receptor gene. Proc. Natl. Acad. Sci. USA **82**:6741–6744.
31. **Nabel, G., and D. Baltimore.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature (London) **326**:711–713.
32. **Ohkubo, H., K. Nakayama, T. Tanaka, and S. Nakanishi.** 1986. Tissue distribution of rat angiotensinogen mRNA and structural analysis of its heterogeneity. J. Biol. Chem. **261**:319–323.
33. **Oliviero, S., G. Morrone, and R. Cortese.** 1987. The human haptoglobin gene: transcriptional regulation during development and acute phase response. EMBO J. **6**:1905–1912.
34. **Osbourne, L., S. Kunkel, and G. J. Nabel.** 1989. Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa-B. Proc. Natl. Acad. Sci. USA **86**:2336–2340.
35. **Pierce, J. W., M. Lenardo, and D. Baltimore.** 1988. Oligonucleotide that binds nuclear factor NF-κB acts as a lymphoid-specific and inducible enhancer element. Proc. Natl. Acad. Sci. USA **85**:1482–1486.
36. **Prowse, K. R., and H. Baumann.** 1988. Hepatocyte-stimulating factor, β2 interferon, and interleukin-1 enhance expression of the rat alpha-1-acid glycoprotein gene via a distal upstream regulatory region. Mol. Cell. Biol. **8**:42–51.
37. **Ramadori, G., J. D. Sipe, C. A. Dinarello, S. B. Mizel, and H. R. Colton.** 1985. Pretranslational modulation of acute phase hepatic protein synthesis by murine recombinant interleukin 1 (IL-1) and purified human IL-1. J. Exp. Med. **162**:930–942.
38. **Sen, R., and D. Baltimore.** 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell **46**:705–716.
39. **Sen, R., and D. Baltimore.** 1986. Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. Cell **47**:921–928.
40. **Singh, H., J. H. LeBowitz, A. S. Baldwin, Jr., and P. A. Sharp.** 1988. Molecular cloning of an enhancer binding protein: isolation by screening of an expression library with a recognition site DNA. Cell **52**:415–423.
41. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
42. **Subramaniam, M., L. J. Schmidt, C. E. Crutchfield, and M. J. Getz.** 1989. Negative regulation of serum-responsive enhancer elements. Nature (London) **340**:64–68.
43. **Visvanthan, K. V., and S. Goodbourn.** 1989. Double-stranded RNA activates binding of NF-κB to an inducible element in the human β-interferon promoter. EMBO J. **8**:1129–1138.
44. **Yano, O., J. Kanellopoulous, M. Kieran, O. Le Bail, A. Israel, and P. Kourilsky.** 1987. Purification of KBF1, a common factor binding to both H-2 and β2-microglobulin enhancers. EMBO J. **6**:3317–3324.