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Angiotensinogen Gene-Inducible Enhancer-Binding Protein 1, a Member of a New Family of Large Nuclear Proteins That Recognize Nuclear Factor κB-Binding Sites through a Zinc Finger Motif

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Transcriptional activation of the rat angiotensinogen gene during the acute-phase response is dependent on a previously characterized acute-phase response element (APRE) that binds at least two types of nuclear proteins: a cytokine-inducible activity indistinguishable from nuclear factor κ-B (NFκB) and a family of C/EBP-like proteins. We screened a rat liver cDNA expression library with a labeled APRE DNA probe and isolated a single clone that encodes a sequence-specific APRE-binding protein. This new protein, the angiotensinogen gene-inducible enhancer-binding protein 1 (AGIE-BP1), is encoded by a large continuous open reading frame and contains a zinc finger motif virtually identical to the DNA-binding domain of a recently described human protein, MBP-1/PRDII-BF1, and a homologous mouse protein, αA-CRYBP1. Outside the binding domain, the sequences diverged considerably. Southern blot analysis indicated that AGIE-BP1 and αA-CRYBP1 are encoded by separate genes, thus defining a new family of DNA-binding proteins. Electrophoretic mobility shift assays, methylation interference, and DNase I footprint protection assays with the bacterially expressed DNA-binding domain of AGIE-BP1 demonstrated a binding specificity indistinguishable from that of purified NFκB. Antiserum raised against the bacterially expressed DNA-binding domain of AGIE-BP1 detected on immunoblots of cellular proteins a large (>250-kDa) nuclear protein. Northern (RNA) blot analysis of RNAs from different rat tissues and cell lines indicated different levels of expression of the large (>10-kb) AGIE-BP1 transcript in different tissues. The potential role of AGIE-BP1 in the regulation of gene expression is discussed.

Activation of rat angiotensinogen gene transcription during the acute-phase response is mediated by a *cis*-acting DNA element located 552 bases upstream of the transcription start site (21). This acute-phase response element (APRE) binds at least two distinct proteins identifiable in rat liver nuclear extracts: a protein indistinguishable from nuclear factor κB (NFκB) that is inducible by either interleukin-1 or tumor necrosis factor, referred to previously as BPi (binding protein inducible), and a family of C/EBP-like constitutive proteins referred to as BPc (binding proteins constitutive). These two classes of proteins have distinct yet overlapping contact points within the APRE, and distinct point mutations of nucleotides within the APRE selectively effect BPi and BPc binding (5, 21). Analysis of the regulation of the transcription of reporter gene constructs containing such selective mutations of the APRE have led to the conclusion that both BPi and BPc are transcriptional activators of the APRE. Differences in their relative potency and abundance, as well as in their interaction with the adjacent glucocorticoid-responsive elements of the rat angiotensinogen gene, account for the acute-phase activation of the gene in hepatocytes (5, 20).

To further our understanding of the regulation of rat angiotensinogen gene transcription during the acute-phase response, we sought to identify recombinant cDNA clones present in a rat liver library which encode proteins capable of binding specifically to the APRE. Data derived from previous studies on the binding specificity of the cellular proteins that interact with the APRE would allow us to assign a potential role for such proteins. We isolated a cDNA clone that encodes a previously undescribed protein that binds specifically to the APRE: angiotensinogen gene-inducible enhancer-binding protein 1 (AGIE-BP1). In this report, we present the available sequence of the transcript encoding this large protein as well as a detailed analysis of its binding characteristics, tissue distribution, and regulation during development. We discuss the relationship of this protein to other, previously identified proteins capable of binding to the APRE.

Isolation of a cDNA clone encoding an APRE-binding protein. To identify recombinant clones that encode proteins capable of binding to the rat angiotensinogen APRE, a lambda gt11 rat liver cDNA library was screened by probing nitrocellulose filter blots of the plated library (following isopropyl-β-D-thiogalactopyranoside [IPTG] induction of synthesis of fusion protein) with a body-labeled fragment of double-stranded DNA containing four copies of the APRE sequence (26, 29; the sequence of the APRE probe used was 5′-GATCCACAGTTGGGATTTCCCAACCTGACCAGA-3′). Several recombinant clones that reproducibly bound the multimeric APRE probe were isolated after screening of 5×10^5 recombinant clones of an amplified library. Initial characterization of the binding specificity of the encoded fusion proteins upon blotting onto nitrocellulose demonstrated that two clones bound the APRE but not an APRE mutant sequence. The two clones, C1.1 and C1.2, contained an identical 0.8-kb insert. DNA sequence analysis of the insert disclosed that it encoded an uninterrupted open reading frame continuous with the β-galactosidase gene of the cloning vector. The predicted protein sequence of this open reading frame was noted to contain conserved residues

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found in the zinc finger class of DNA-binding proteins (7). A striking similarity was noted to the putative DNA-binding domain of a recently described human protein, MBP-1/PRDII-BF1 (2, 8), and a homologous mouse protein, αA-CRYBP1 (19) (Fig. 1). The sequence identity is greater than 90% over a stretch of 87 amino acids, with conservation of all but one cysteine and histidine postulated to be of importance in coordinating the zinc ion in the binding domain at the base of the two postulated zinc fingers.

To obtain further sequence data on the transcript encoding this DNA-binding protein, we rescreened the original cDNA library as well as another rat liver cDNA library, using the insert from clone C1.1 as a nucleic acid hybridization probe. We isolated three partially overlapping cDNA clones encoding a continuous open reading frame of 916 residues extending from the 5′ end of the upstream clone to a stop codon, followed by a long (1-kb) 3′ untranslated tract in the downstream-most clone (Fig. 2). Comparison of the composite sequence of all available clones with that of the aforementioned homologous DNA-binding proteins demonstrated that whereas the sequence similarity between the two previously identified human and mouse clones extended throughout the available sequence (based on a stringent protein matrix comparison [10] that detects only blocks of 18 amino acids with >80% identity), the newly identified clone matched these two proteins only in the zinc finger motif-containing DNA-binding domain (based on a matrix comparison at relatively low stringency that detects blocks of 12 residues with as little as 60% sequence identity). This analysis suggested to us that we had isolated a member of a new family of proteins rather than simply the rat homolog of MBP-1/PRDII-BF1/αA-CRYBP1.

Examination of the available sequence of the isolated clones revealed that the predicted encoded protein contains, in addition to the two zinc finger motifs, a highly charged adjacent acidic region (also present in MBP-1/PRDII-BF1/αA-CRYBP1) as well as a series of 10 direct repeats of a motif, characterized by a hydrophobic residue (usually leucine) followed by S-P-X-R/K (where X is a nonacidic, nonhydrophobic residue, usually lysine or arginine). We termed this new predicted protein, the partial sequence of which we had succeeded in cloning, AGIE-BP1.

To determine whether AGIE-BP1 represents an alternatively processed transcript emanating from a single genomic locus that would encode AGIE-BP1 as well as the homologous protein, or whether AGIE-BP1 is encoded by a different, unique genomic locus, identical blots of restriction endonuclease-digested rat genomic DNA were probed with either an AGIE-BP1-binding domain probe or a homologous probe from the mouse αA-CRYBP1 cDNA (kindly provided by Keiko Ozato). Bands of similar size were not detected in the differentially probed but otherwise identical blots, indicating that AGIE-BP1 and the rat homolog of αA-CRYBP1 (which is easily detected by the murine probe) are encoded by separate genomic loci (Fig. 3).

Sequence-specific DNA binding by AGIE-BP1 in vitro and in vivo. To evaluate the possible relationship of AGIE-BP1 to the functionally characterized nuclear proteins that have been shown to bind overlapping yet distinct sites within the rat angiotensinogen gene APRE (5, 21), we sought to determine the precise binding specificity and contact points which AGIE-BP1 makes with the APRE. To this end, we expressed in bacteria (using the pET3b expression plasmid and the plysS strain of *Escherichia coli* BL21DE3; gifts of F. W. Studier [27]) a 105-amino-acid-long peptide corresponding to the putative binding domain of AGIE-BP1 (binding fragment

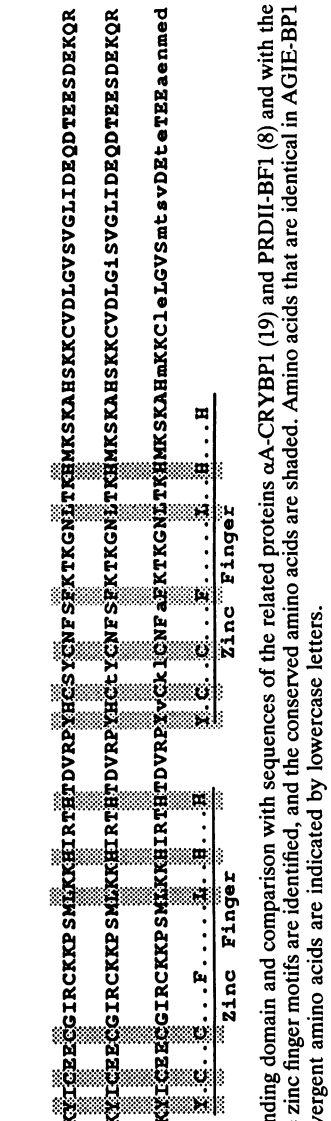

FIG. 1. Predicted amino acid sequence of the AGIE-BP1-binding domain and comparison with sequences of the related proteins αA-CRYBP1 (19) and PRDII-BF1 (8) and with the consensus sequence for a zinc finger motif (7). The two putative zinc finger motifs are identified, and the conserved amino acids are shaded. Amino acids that are identical in AGIE-BP1 and the related proteins are indicated in capital letters, and divergent amino acids are indicated by lowercase letters.

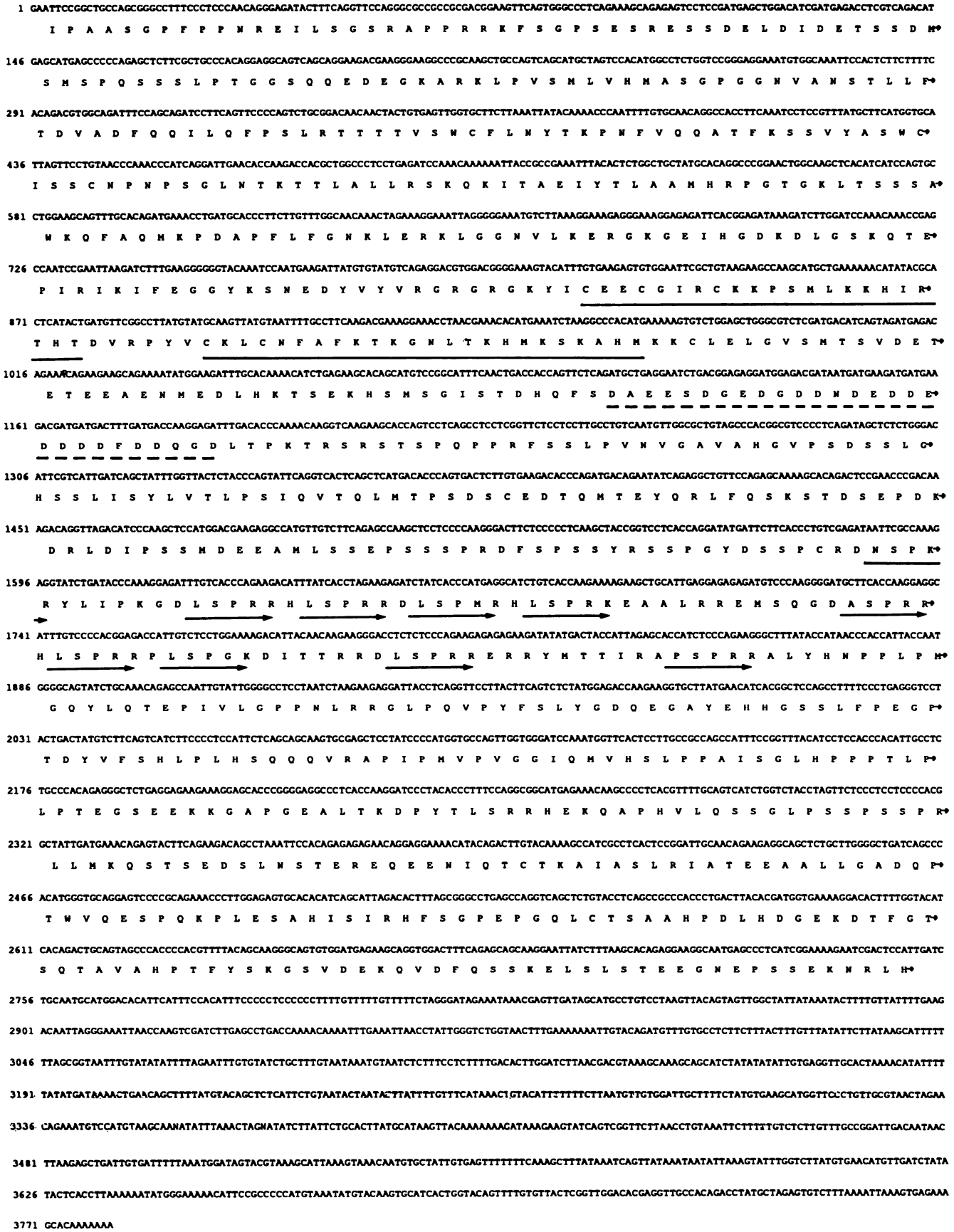

FIG. 2. Nucleic acid sequence (top line) and predicted amino acid sequence (bottom line) of the carboxy terminus of AGIE-BP1. The two zinc finger motifs are underlined, the highly charged acidic domain is underlined by the broken line, and the direct repeats of the motif, X-L-S-P-X-K/R, are underlined by arrows.

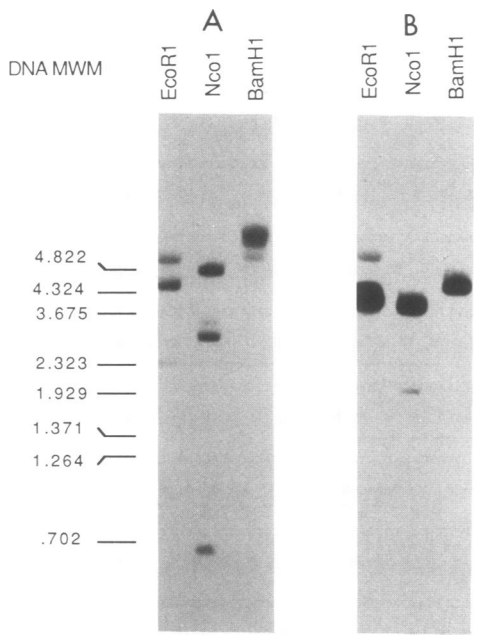

FIG. 3. Evidence that nucleic acid probes corresponding to the binding domain of AGIE-BP1 and the homologous binding domain of αA-CRYBP1 hybridize to different-size DNA fragments in a blot of restriction endonuclease-digested rat genomic DNA. Rat genomic DNA (30 μg) was extensively digested with the indicated restriction endonucleases. Identical blots of the DNA, fractionated on a 1% agarose gel, were hybridized (in 50% formamide at 42°C) with a body-labeled AGIE-BP1 probe encoding the DNA-binding domain (insert of clone C1.1; A) or a probe to the homologous region of αA-CRYBP1 (the 1.2-kb fragment of *Hin*dIII-digested plasmid pYTN8.1 [19]; B).

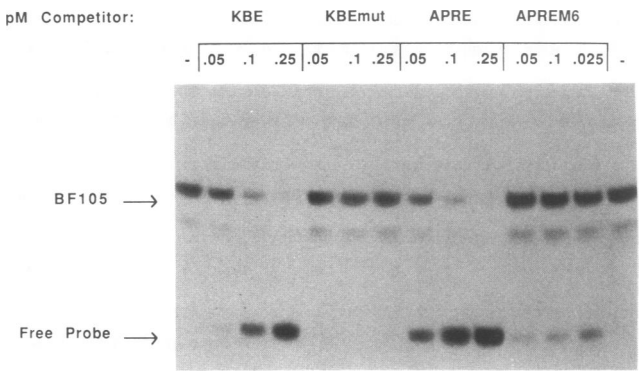

FIG. 4. EMSA of a labeled APRE oligonucleotide by the bacterially expressed binding domain of AGIE-BP1. Lysates of bacteria transformed with an expression vector encoding a 105-amino-acid peptide spanning the binding domain of AGIE-BP1 (BF105) were used to shift 0.02 pmol of labeled APRE probe in nondenaturing 7% polyacrylamide gel electrophoresis. Various quantities (in picomoles) of the indicated unlabeled oligonucleotides were included in the binding assay as competitors (for sequences of APRE, KBE, and KBEmut, see reference 21; for the sequence of APREM6, see reference 5). Positions of migration of the free probe and bound complex are indicated by arrows.

105 [BF105]), spanning nucleotides 693 to 1005 of the available sequence presented in Fig. 2. Lysates from bacteria transformed with the BF105 expression vector (pET3b BF105) contained a protein that bound the APRE in an electrophoretic mobility shift assay (EMSA) with high affinity and specificity (Fig. 4). Preparations of BF105-expressing lysates variably gave between one and three specific bands on EMSA; lysates from control bacteria, transformed with the parent prokaryotic expression vector (pET3b), variably gave only a nonspecific band. The specific bands of different mobility may represent artifactual degradation of the protein-binding species or different conformations assumed by an undegraded protein-DNA complex under the nondenaturing gel electrophoresis conditions. Competition of binding, with an excess of unlabeled oligonucleotide probes, suggested that AGIE-BP1 shares the binding specificity of the inducible, NFκB-like BPi because the binding of BF105 to the APRE was abolished by an excess of the murine kappa light-chain gene NFκB-binding site oligonucleotide KBE but not by a KBE mutant or by the APREM6 oligonucleotide, which has been shown to bind only the constitutive BPc (not BPi [5]). In addition to binding the KBE sequence derived from the murine kappa light-chain enhancer, AGIE-BP1 also binds the related NFκB-binding site of the major histocompatibility complex alpha-chain gene promoter (data not shown).

Methylation interference analysis of the contact points made by BF105 on the APRE disclosed that AGIE-BP1 contacted the same guanosine residues as did BPi (−549, −548, and −547 on the coding strand and −542, −541, and −540 on the noncoding strand; Fig. 5). Though less obvious on the autoradiographic exposure presented here, methylation of the central guanosine in each triplet also interferes with BPi binding, and prolonged exposure of the gel reveals that BF105 is subject to an identical hierarchy of methylation interference; with the outermost residues being more important contact points than the inner residue. BF105 failed to contact guanosine −537 on the noncoding strand, an essential contact point for BPc (Fig. 5B). DNase I footprint analysis demonstrated that BF105 binding protected the APRE from digestion and confirmed that while the pattern of protection was different from that seen for BPc, the two proteins overlap in their binding sites on the angiotensinogen gene promoter (Fig. 5). We are unable to obtain a consistent protection from DNase I digestion by the available purified cellular BPi. However, the region protected by BF105 overlaps the contact points made by BPi, as determined by the methylation interference analysis. On the basis of this, we concluded that AGIE-BP1 binds the APRE in vitro in a manner indistinguishable from that of cellular BPi but clearly distinct from that of BPc.

To confirm that zinc coordination by the putative zinc fingers of AGIE-BP1 is involved in structuring the DNA-binding domain of this protein, we denatured the bacterially expressed BF105 peptide with 6 M guanidine-HCl in the presence of large quantities of EDTA to chelate the zinc present in the bacterial lysate. Following removal by dialysis of the guanidine-HCl and EDTA, the bacterially expressed peptide failed to bind the APRE probe unless zinc was added to the EMSA binding reaction (data not shown). Similar treatment of cellular proteins BPi and BPc failed to demonstrate such zinc-dependent binding, suggesting that their binding domain is different from that of AGIE-BP1.

To address the question of whether AGIE-BP1 is capable of binding DNA in vivo, we constructed a eukaryotic expression vector encoding a 674-amino-acid protein (pECE674) spanning the binding domain of AGIE-BP1 (from nucleotides 144 to 2166, with an in-frame initiator methionine present

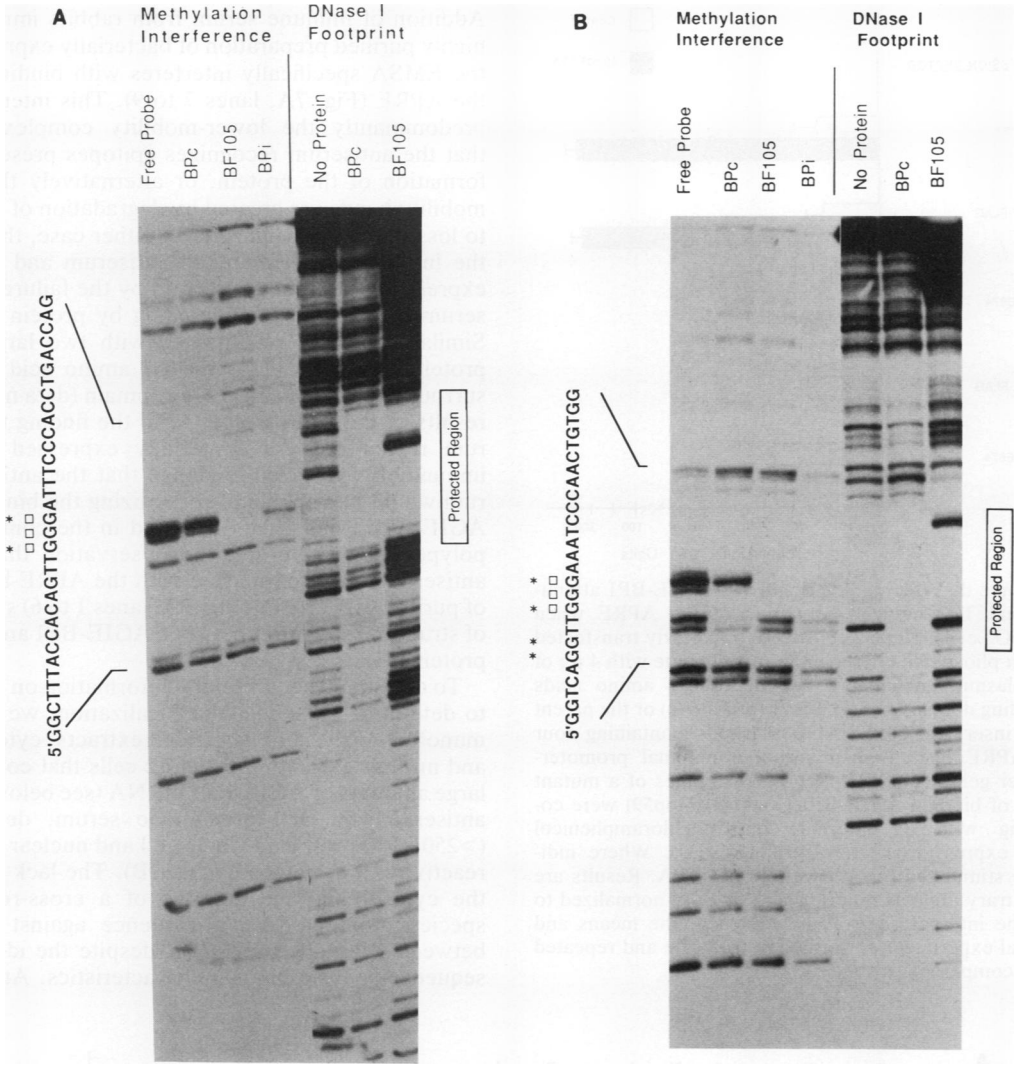

FIG. 5. Methylation interference analysis of AGIE-BP1 binding to the rat angiotensinogen gene promoter and DNase I footprint protection by AGIE-BP1. Bacterially expressed BF105 or cellular BPi and BPc (purified from rat liver) were used in methylation interference analysis and a DNase I footprint protection assay of a fragment of the rat angiotensinogen gene promoter from nucleotides 615 to −526. The probe was labeled on the coding (A) or noncoding (B) strand. Residues whose methylation interferes with DNA binding by BPi and AGIE-BP1 are indicated by the boxes to the left of each panel; residues that interfere with BPc binding are indicated by asterisks. The region protected in the footprinting assay, corresponding to the functionally defined APRE, is indicated by the rectangle to the right of each panel.

within a translation initiation consensus sequence [16] in the expression vector pECE [22]). When cotransfected into HepG2 cells along with a luciferase reporter construct consisting of four multimerized copies of the APRE upstream of a minimal promoter (X4APREp59 [21]), pECE674 gave a 7-fold inhibition of basal reporter gene activity and an 18-fold inhibition of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced, BPi-mediated activity (Fig. 6). The control parent expression vector, lacking the AGIE-BP1-encoding insert (pECE-FLAG), led to no such inhibition of reporter gene activity. As a further control for the specificity of inhibition of reporter gene activity by pECE674, we found no effect on the transcriptional activity of a reporter construct (X4APREM6p59) that contains a mutant APRE to which AGIE-BP1 does not bind in vitro (Fig. 4). Because this APRE mutant construct has been shown to be unresponsive to TPA (consistent with its inability to bind BPi [5]), only the basal activity of this BPc-binding enhancer was tested. Because pECE674 directs the expression of only a small fragment of AGIE-BP1, these studies do not address the functional role of the intact protein; they do, however, suggest that the binding domain of AGIE-BP1, when expressed in HepG2 cells, is capable of displacing the endogenous, APRE-binding, transactivating proteins from the APRE, implying the ability to bind DNA in a sequence-specific manner in vivo as well as in vitro. Consistent with these observations, the bacterially expressed binding domain of AGIE-BP1 competed with BPi and BPc for binding to the APRE in EMSA (data not shown).

Anti-AGIE-BP1 antiserum detects a large nuclear protein distinct from previously identified APRE-binding proteins. The relationship between AGIE-BP1 and the cellular proteins that bind the APRE was characterized further with an antiserum raised against the binding domain of AGIE-BP1.

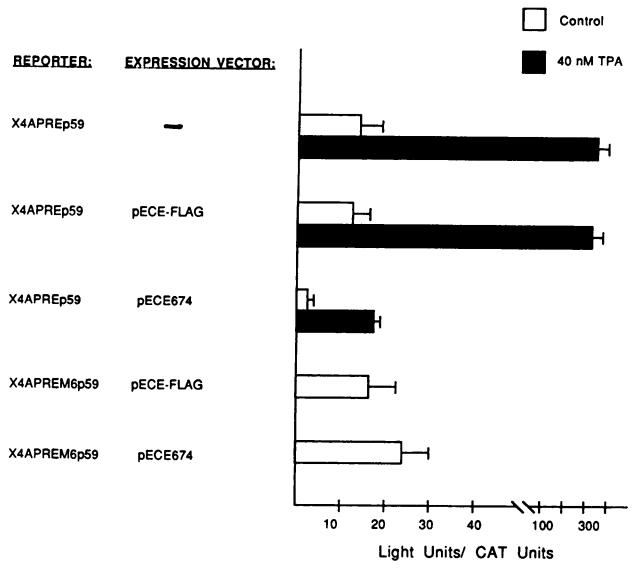

FIG. 6. Evidence that the binding domain of AGIE-BP1 attenuates the basal and TPA-induced activation of the APRE when expressed in HepG2 cells. HepG2 cells were transiently transfected (6) by the calcium phosphate coprecipitation technique with 4 μg of an expression plasmid encoding a peptide of 674 amino acids spanning the binding domain of AGIE-BP1 (pECE674) or the parent vector with no insert (pECE-FLAG). Plasmids containing four copies of the APRE ligated upstream of a minimal promoter-luciferase reporter gene (X4APREp59) or four copies of a mutant APRE incapable of binding AGIE-BP1 (X4APREM6p59) were co-transfected along with an internal control chloramphenicol acetytransferase expression vector (pRSVcat [11]). Where indicated, cells were stimulated for 4 h with 40 nM TPA. Results are expressed in arbitrary units as reporter gene activity normalized to the activity of the internal control and represent the means and ranges of a typical experiment performed in triplicate and repeated three times with comparable results.

Addition of immune serum from rabbits immunized with a highly purified preparation of bacterially expressed BF105 to the EMSA specifically interferes with binding of BF105 to the APRE (Fig. 7A, lanes 7 to 9). This interference affects predominantly the lower-mobility complexes, suggesting that the antiserum recognizes epitopes present in this conformation of the protein, or alternatively that the higher-mobility bands are created by degradation of BF105, leading to loss of the antigenic site. In either case, the specificity of the interaction between the antiserum and the bacterially expressed protein is attested to by the failure of preimmune serum to alter the DNA binding by protein in the EMSA. Similar results were obtained with two larger AGIE-BP1 protein fragments (224 and 603 amino acid residues long) surrounding the DNA-binding domain (data not shown). The results of the EMSA along with the finding that the antiserum recognized the bacterially expressed proteins in a immunoblot provided evidence that the anti-BF105 antiserum would be capable of recognizing the binding domain of AGIE-BP1 even when presented in the context of a larger polypeptide. Therefore, the observation that the immune antiserum failed to interfere with the APRE-binding activity of purified BPi or BPc (Fig. 7A, lanes 1 to 6) suggested a lack of structural similarity between AGIE-BP1 and these cellular proteins.

To obtain further structural information on AGIE-BP1 and to determine its subcellular localization, we performed immunoblot analysis of whole cell extracts, cytosolic extracts, and nuclear extracts from PC12 cells that contain relatively large amounts of AGIE-BP1 mRNA (see below). Anti-BF105 antiserum, but not preimmune serum, detected a large (>250-kDa) protein in whole cell and nuclear extracts but no reactivity in the cytosol (Fig. 7B). The lack of reactivity in the cytosol and the absence of a cross-reacting 50-kDa species provided further evidence against a relationship between BPi and AGIE-BP1, despite the identical in vitro sequence-specific binding characteristics. Attempts to con-

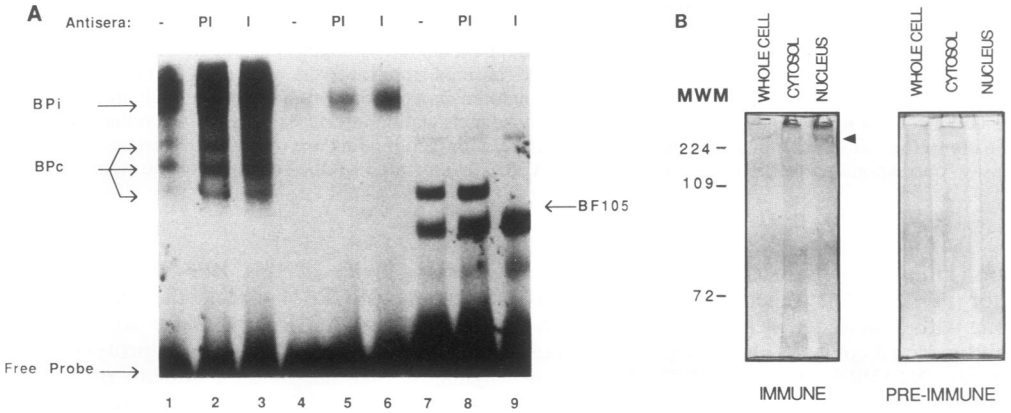

FIG. 7. Evidence that an antiserum raised against the binding domain of AGIE-BP1 interferes with DNA binding and recognizes a large nuclear protein on Western immunoblots. (A) The anti-AGIE-BP1 antiserum interferes with DNA binding by bacterially expressed BF105 but not by the cellular proteins BPi and BPc. Undiluted serum (0.75 μl) from a rabbit immunized with a highly purified preparation of BF105 (I) or equal amounts of preimmune serum from the same animal (PI) or phosphate-buffered saline (−) were added to a 20-μl binding reaction mixture containing crude nuclear extract from acute-phase response rat liver (lanes 1 to 3), purified BPi (lanes 4 to 6), or bacterially expressed BF105 (lanes 7 to 9). Following a 2-h incubation on ice, labeled APRE oligonucleotide was added and EMSA was performed. The individual DNA-protein complexes are identified by the arrows. (B) Anti-AGIE-BP1 antiserum recognizes a large nuclear protein in immunoblots of PC12 cell extracts. PC12 cell lysates were fractionated into nuclear and cytosolic extracts; the proteins were precipitated in acetone and separated on a 4.5% polyacrylamide-sodium dodecyl sulfate gel. Nitrocellulose blots of two identical protein gels were reacted with a 1:300 dilution of BF105 peptide affinity-purified anti-AGIE-BP1 antiserum or identically treated preimmune serum from the same animal. A specific reacting protein migrating above the 224-kDa marker (indicated by the arrowhead) was identified in whole cell extract and nuclear fractions. Other, smaller nonspecific bands are seen with both immune and preimmune sera. MWM, Molecular weight markers, indicated in thousands.

firm the nuclear localization of AGIE-BP1 by immunocytochemistry were frustrated by a high background perinuclear reactivity in the PC12 cells even when the cells were reacted with BF105 affinity-purified preimmune serum. We were similarly unable to detect specific antiserum binding to proteins on an immunoblot using rat tissues. We speculate that our failure to detect proteins in rat tissues that are reactive with the anti-BF105 antiserum may be due to a low abundance of AGIE-BP1 in tissues and a greater difficulty in preventing in vitro artifactual degradation of large proteins isolated from tissue than from cultured cells.

Variable levels of AGIE-BP1 mRNA in tissues and cell lines. To obtain clues as to a possible physiological role for AGIE-BP1 in modulating angiotensinogen gene expression, we determined the distribution of AGIE-BP1 mRNA in various rat tissues and correlated these findings with expression of the angiotensinogen mRNA in the same tissues. Northern (RNA) blot analysis of poly(A)$^+$ RNA from various rat tissues demonstrated a single large (>10-kb) transcript which varied considerably in abundance, being easily detectable in brain, heart, spleen, lung, and skeletal muscle but very scarce in liver, kidney, testes, and prostate. The transcript was similarly abundant in pheochromocytoma-derived PC12 cells (regardless of their state of differentiation) yet scarce in liver-derived H35 cells (Fig. 8A). When the same blot was hybridized with a rat angiotensinogen cDNA probe, we noted no obvious relationship between expression of angiotensinogen and expression of AGIE-BP1 among the different tissues. In the adult brain, however, angiotensinogen and AGIE-BP1 are both expressed to high levels. We therefore sought to determine whether a temporal correlation could be made between the developmental regulation of angiotensinogen in brain and the expression of AGIE-BP1. Interestingly, when poly(A)$^+$ RNA from brains of developing rat embryos was analyzed by Northern blot analysis, a transient peak of expression of both transcripts was noted on embryonic day 17 (Fig. 8B), suggesting the possibility of a role for AGIE-BP1 in modulating the expression of the angiotensinogen gene in the developing rat brain.

The isolation of a cDNA clone encoding a previously undescribed APRE-binding protein, AGIE-BP1, adds a second member to the family of cloned sequence-specific DNA-binding proteins capable of binding to this acute-phase-inducible enhancer sequence of the rat angiotensinogen gene. *cis*-acting DNA elements similar to the APRE, of which the NFκB-binding site in the enhancer of the kappa light-chain gene is prototypical (24), are present in many other inducible genes, such as the human alpha interferon, the human interleukin-2 receptor, the murine serum amyloid A protein, and the human immunodeficiency virus type 1 enhancer genes (reviewed in reference 17). In the rat angiotensinogen gene, AGIE-BP1 binds to the APRE, making contacts with DNA that are indistinguishable from those made by the cytokine inducible, NFκB-like BPi.

The potent transcriptional activator NFκB has been shown to be composed of a tetrameric complex between two 50-kDa subunits that are responsible for DNA binding and two 65-kDa polypeptide chains that subserve a regulatory function (1). BPi, as identified in interleukin-1-induced HepG2 cells, has an apparent molecular size of 50 kDa (21). Despite the apparently indistinguishable binding characteristics, multiple lines of evidence suggest that AGIE-BP1 is distinct from the NFκB-like BPi. NFκB, in latent form, is found in the cytoplasm of all cells tested (25), whereas the AGIE-BP1 transcript varies considerably in its abundance between different cells, being, for example, abundant in

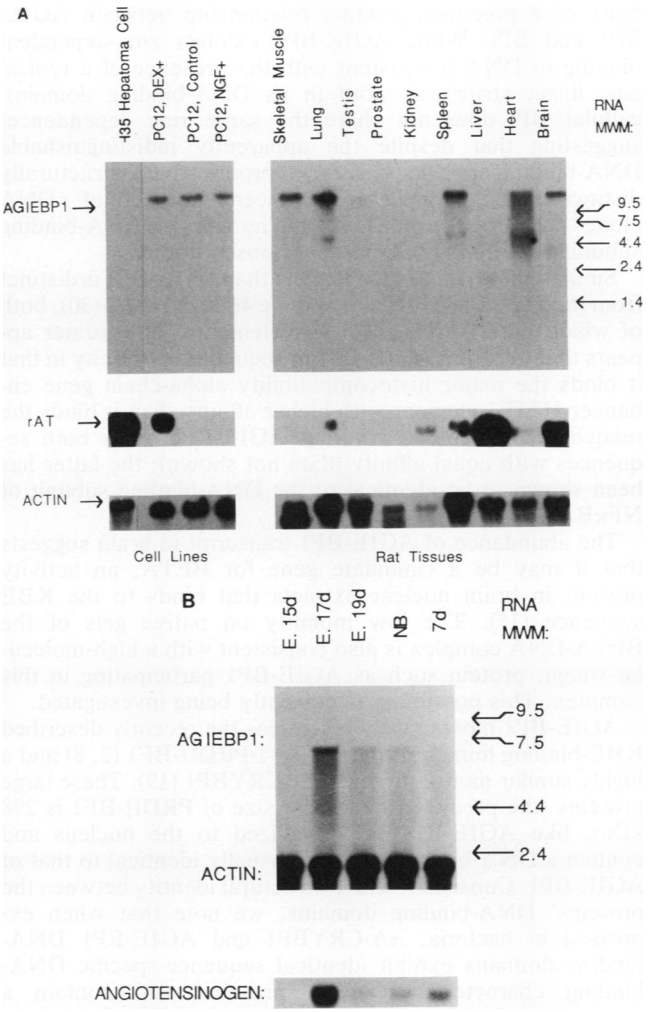

FIG. 8. Different levels of expression of AGIE-BP1 in rat tissues and cell lines. (A) Poly(A)$^+$ RNAs (10 μg) from the indicated cell lines and rat tissues were fractionated on a 1% agarose-formaldehyde gel, blotted onto a nylon membrane, and hybridized with a body-labeled AGIE-BP1 cDNA probe (the insert from clone S10.1; top panel). The same blot was then hybridized with a rat angiotensinogen (rAT) cDNA probe (bottom panel). PC12 cells were treated with 5×10^{-7} dexamethasone (DEX+) or 40 ng of nerve growth factor per ml (NGF+) for 72 h prior to harvest. (B) AGIE-BP1 is expressed during development of rat brain. Poly(A)$^+$ RNA (10 μg) from heads of rat embryos at the indicated embryonic days (E.) and brains of newborn (NB) and 7-day-old pups (7d) were fractionated on a 1% agarose-formaldehyde gel, blotted onto a nylon membrane, and hybridized with a body-labeled AGIE-BP1 cDNA probe and an oligonucleotide probe complementary to rat actin (top panel); the blot was then stripped and hybridized to a rat angiotensinogen cDNA probe (bottom panel).

PC12 cells and scarce in H35 cells, though both cells contain roughly equal amounts of TPA-inducible NFκB DNA-binding activity (data not shown). Furthermore, immunoblots of cellular proteins, when probed with anti-AGIE-BP1 antiserum, reveal a large (>250-kDa) protein which is nuclear in its subcellular distribution. A 50-kDa protein is not detected by anti-AGIE-BP1 antiserum in any subcellular fraction or in immunoblots of whole cell extracts, and this antiserum, capable of interfering with DNA binding by AGIE-BP1, does not effect DNA binding by BPi, speaking against the possi-

bility of a precursor-product relationship between AGIE-BP1 and BPi. While AGIE-BP1 exhibits zinc-dependent binding to DNA (consistent with the presence of a typical zinc finger structural motif in its DNA-binding domain), cellular BPi does not share the same zinc dependence, suggesting that despite the apparently indistinguishable DNA-binding specificity, the two proteins have structurally distinct binding domains. The recent isolation of cDNA clones encoding the mouse (9) and human (14) DNA-binding subunits of NFκB confirms these observations.

Similar lines of evidence suggest that AGIE-BP1 is distinct from the 110-kDa H2TF1 (3) and the 48-kDa KBF1 (30), both of which bind APRE-like DNA elements. The former appears to differ from AGIE-BP1 in sequence specificity in that it binds the major histocompatibility alpha-chain gene enhancer H2TF1 element with higher affinity than it binds the related KBE element, whereas AGIE-BP1 binds both sequences with equal affinity (data not shown); the latter has been shown to be identical to the DNA-binding subunit of NFκB (14).

The abundance of AGIE-BP1 transcript in brain suggests that it may be a candidate gene for BETA, an activity present in brain nuclear extracts that binds to the KBE sequence (15). The low mobility on native gels of the BETA-DNA complex is also consistent with a high-molecular-weight protein such as AGIE-BP1 participating in this complex. This possibility is currently being investigated.

AGIE-BP1 most closely resembles the recently described KBE-binding human protein MBP-1/PRDII-BF1 (2, 8) and a highly similar mouse protein, αA-CRYBP1 (19). These large proteins (the predicted molecular size of PRDII-BF1 is 298 kDa), like AGIE-BP1, are localized to the nucleus and contain a DNA-binding domain virtually identical to that of AGIE-BP1. Consistent with a structural identity between the proteins' DNA-binding domains, we note that when expressed in bacteria, αA-CRYBP1 and AGIE-BP1 DNA-binding domains exhibit identical sequence-specific DNA-binding characteristics. Both proteins also contain a negatively charged region immediately carboxy terminal to the DNA-binding domain. The functional role of this conserved region is unknown, but despite the strong net negative charge, this region in and of itself is insufficient to impart transactivation potential to the DNA-binding protein fragment of AGIE-BP1. When expressed in HepG2 cells, such a protein represses the activity of an APRE-containing promoter (Fig. 6). When analyzed together with data indicating mutually exclusive binding of AGIE-BP1 and the endogenous cellular APRE-binding proteins, this result suggests that whatever transactivation potential the acidic region may have, it is considerably less than that of the endogenous APRE-binding BPi and BPc that AGIE-BP1 presumably displaces.

PRDII-BF1 has two functionally identical DNA-binding domains (8). The amino-terminal set of zinc fingers that comprise this second DNA-binding domain are separated from the carboxy-terminal set by 1,630 amino acids. In view of the fact that we have available to us sequence data encompassing only the 916 carboxy-terminal residues of what is predicted to be, on the basis of protein size in the immunoblot, a polypeptide of greater than 2,000 residues, it is conceivable that AGIE-BP1 also has a second DNA-binding domain.

On the basis of nucleic acid sequence, AGIE-BP1 is predicted to have, downstream of the acidic domain, a series of direct repeats of the amino acid sequence X-S-P-X-K/R (single-letter code; X is any amino acid). A similar series of repeats is not found in MBP-1/PRDII-BF1 or αA-CRYBP1. Multiple repeats of a similar motif are present in the H1 and H2B histones of sea urchin spermatozoa and appear to be sites for phosphorylation by a kinase that is active in a developmental-stage-specific manner in spermatids and upon fertilization in the zygote. This observation has suggested a role for these sites in regulating packaging of DNA through phosphorylation-dependent DNA-protein interactions (13). Similar peptide sequences have also been suggested to play a role in DNA binding by many other nuclear proteins through an interaction of this structural motif with the minor groove of the DNA double helix (28). We addressed the possibility of a role for the X-S-P-X-K/R repeats in DNA-binding by AGIE-BP1 by expressing in bacteria protein fragments of AGIE-BP1 that contain the DNA-binding domain alone or in a construct that includes these repeats. We then compared the DNase I footprint protection patterns over the APRE obtained with these different proteins, reasoning that, to the extent that DNase I attacks DNA through the minor groove, interactions between the aforementioned repeat motifs and the DNA should result in qualitatively different protection patterns by peptides that do or do not contain the repeats. No differences in the footprinted region were noted (data not shown). We also compared the activity in transfected HepG2 cells of a mammalian expression vector that encoded a fragment of AGIE-BP1 spanning the binding domain (but excluding the repeat motifs) with that of pECE674 (which encodes both the DNA-binding domain and the repeats). Both constructs repressed basal and TPA-induced activity of the cotransfected APRE-containing promoter reporter construct, suggesting that in vivo as well as in vitro, the repeat motifs are not necessary for DNA binding (data not shown).

Analysis of the tissue distribution of AGIE-BP1 transcripts reveals a wide variation in its abundance in different rat tissues. Interestingly, with respect to the regulation of the rat angiotensinogen gene during the acute-phase response, AGIE-BP1 transcripts are relatively less abundant in tissues such as liver and kidney, in which the rat angiotensinogen gene is induced during the acute-phase response, than in brain and heart, in which the gene is not induced (4, 21). Analysis of mRNA from various cell lines demonstrated that rat hepatoma-derived H35 cells, in which the endogenous rat angiotensinogen gene is induced by cytokines and glucocorticoids (20, 21), have very little AGIE-BP1 mRNA, whereas pheochromocytoma-derived PC12 cells, in which the endogenous angiotensinogen gene is induced only by glucocorticoids but not by cytokines, have relatively large quantities of AGIE-BP1 mRNA and protein. This finding suggested a model whereby AGIE-BP1 could function as a repressor of NFκB-mediated activation of the angiotensinogen gene. However, promoters containing APRE or KBE elements, which bind NFκB as well as AGIE-BP1, are no less inducible (by stimuli that activate NFκB) in the AGIE-BP1-rich PC12 cells than in AGIE-BP1-poor hepatoma cells (data not shown).

The data implicating AGIE-BP1 in binding to the angiotensinogen APRE in vivo as well as in vitro, together with our observation of a temporal relationship between AGIE-BP1 expression and angiotensinogen expression in the developing rat brain, suggest the possibility of a role for AGIE-BP1 in the regulation of angiotensinogen gene transcription. An understanding of this role, if any, awaits isolation of a full-length AGIE-BP1 cDNA and its expression in cells naturally deficient in this protein.

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