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A family of constitutive C/EBP-like DNA binding proteins attenuate the IL-1α induced, NFκB mediated *trans*-activation of the angiotensinogen gene acute-phase response element

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The gene encoding angiotensinogen, the glycoprotein precursor of the potent vasopressor peptide angiotensin II, is transcriptionally activated in hepatocytes during the acute-phase response through interactions of mutually cooperative glucocorticoid receptors and proteins that bind to an acute-phase response element (APRE) 5'-AGTTGGGATTTCCCAACC-3'. The APRE binds a family of constitutive proteins (BPcs) and a cytokine inducible protein (BPi) that is indistinguishable from nuclear factor κB (NFκB). The interactions of purified proteins with the APRE were studied by *in vitro* binding and *in vivo* transcriptional *trans*-activation assays. BPc is a family of heat-stable DNA binding proteins, the different sized members of which are capable of forming heterodimers. BPcs are recognized by anti-C/EBP antiserum and produce a footprint similar to bacterially expressed C/EBP on the APRE. BPi has a 4- to 5-fold greater affinity for the APRE than the BPcs, and contacts guanosine residues distinct from those contacted by the BPcs, demonstrating that these two classes of proteins contain functionally distinct DNA binding domains. Assays of APRE-luciferase reporter plasmids transfected into HepG2 cells show that a mutated APRE that binds only BPi functions as an IL-1α inducible enhancer, whereas a mutated APRE that binds only BPc does not. The APRE mutant that binds the C/EBP-like BPcs to the exclusion of BPi functions as an uninducible basal enhancer both in the native context of the angiotensinogen gene and when multimerized and placed upstream of a minimal angiotensinogen promoter. The wild-type APRE that binds both BPi and BPc is less inducible by IL-1α than the mutated APRE that binds only BPi. Gel shift competition assays demonstrate *in vitro* that the mechanism of transcriptional regulation by the APRE involves a competition between BPc and the inducible BPi for binding to the APRE. IL-1α stimulation of hepatocytes leads to nuclear translocation of the NFκB-like BPi which competes with the constitutive C/EBP-like BPcs for overlapping binding sites on the APRE and thereby replaces weak transcriptional activators with a stronger one.

Key words: acute-phase response/angiotensinogen gene/C/EBP/DNA binding proteins/nuclear factor κB

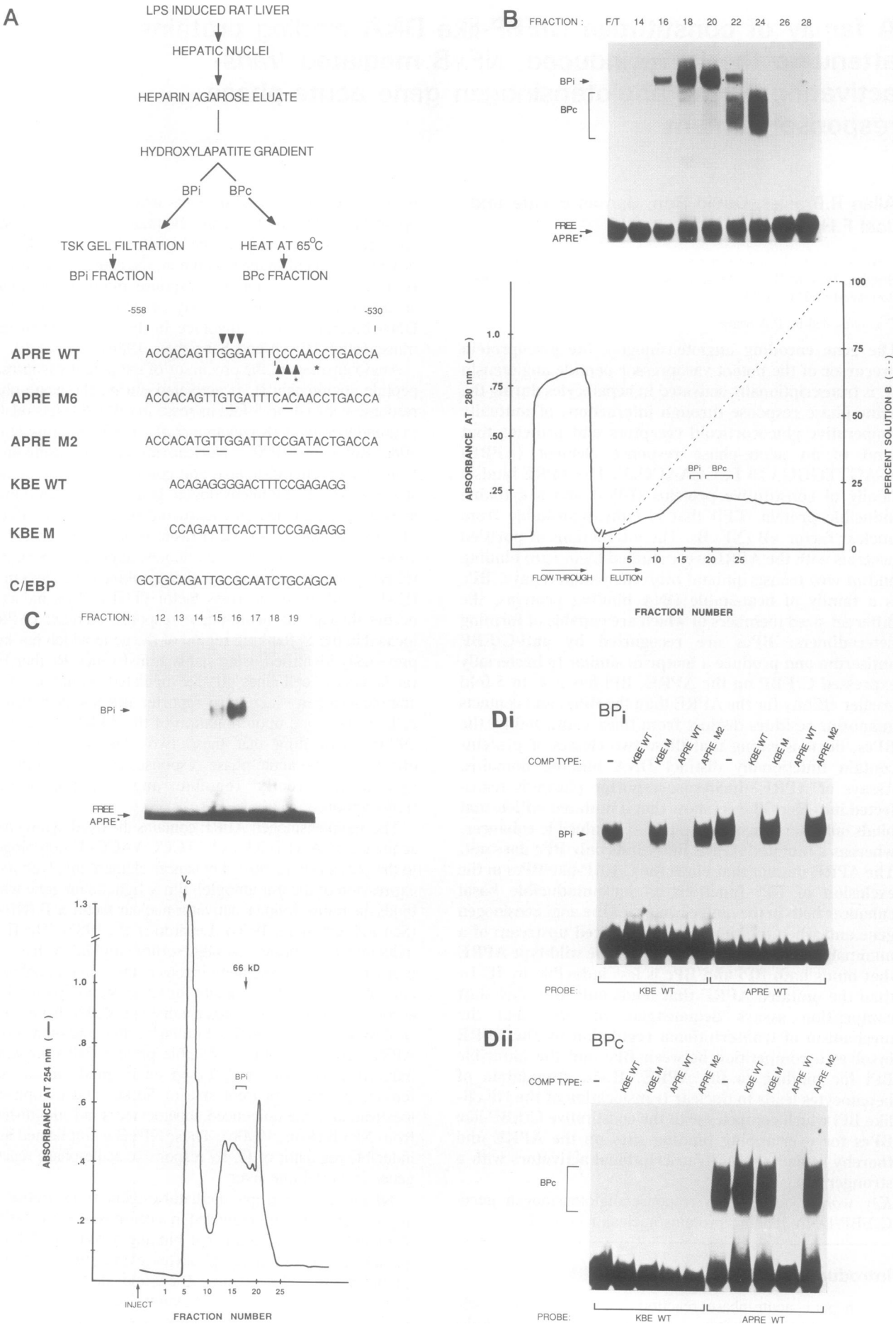
Introduction

The hepatic acute-phase reaction is a response to tissue inflammation that switches the synthetic capacity of the liver to the secretion of proteins involved in macrophage opsonization and wound repair (Baumann, 1989). Consistent with the limited capacity of the liver to store presynthesized proteins and regulate their secretion, the acute-phase reaction is primarily a transcriptional response and therefore makes an informative model for study of inducible *trans*-acting DNA binding proteins involved in the regulation of gene transcription (Birch and Schrieber, 1986).

Angiotensinogen, the precursor of the potent vasopressor peptide angiotensin II, is activated during the acute-phase response with a 4- to 5-fold increase in mRNA levels relative to uninduced liver (Kageyama *et al.*, 1985; Bouhnik *et al.*, 1988; Ron *et al.*, 1990b). Nuclear run-on assays using nuclei from glucocorticoid-treated and control cells have demonstrated that rat angiotensinogen gene is transcriptionally activated by liganded glucocorticoid receptor, one effector of the acute-phase response (Brasier *et al.*, 1986; Ron *et al.*, 1990a). In addition to glucocorticoids, angiotensinogen gene transcription is induced by the cytokines interleukin-1α (IL-1α) and tumor necrosis factor (TNF). This induction occurs through an acute-phase response element (APRE) located in the 5' flanking region of the gene which has been previously identified using stably transformed Reuber H35 rat hepatoma cell lines. IL-1α mediated induction of the angiotensinogen-luciferase reporter mRNA in hepatoma cells is abolished upon mutation of the APRE (Ron *et al.*, 1990b), indicating that these two classes of hormonal effectors of the acute-phase response, glucocorticoids and cytokines, directly regulate angiotensinogen gene transcription.

The angiotensinogen APRE contains the dyad-symmetrical sequence 5'-AGTTGGGATTTCCCAACC-3' homologous to the previously identified enhancer element involved in the expression of the immunoglobulin κ light chain gene which binds the transcriptional activator nuclear factor κ B (NFκB) (Sen and Baltimore, 1986a; Lenardo *et al.*, 1987). The IL-1α inducible acute-phase reactant, serum amyloid A, has in its promoter a *cis*-element homologous to the κ immunoglobulin enhancer that is a binding site for tetradecanoylphorbol-13-acetate (TPA) inducible factor indistinguishable from NFκB (Edbrooke *et al.*, 1989). Similarly, the angiotensinogen APRE binds a cytokine inducible protein (BPi) present in induced hepatocytes that, based on its methylation interference pattern, apparent size of 50 kd, and cytoplasmic localization in the uninduced hepatocyte, is indistinguishable from NFκB (Ron, 1990b). Thus, NFκB is implicated as an inducible regulator of IL-1α responsive acute-phase reactant genes in vertebrate liver.

NFκB is a pleiotropic mediator of gene transcription that is post-translationally regulated in a cell-type specific fashion. Although constitutive nuclear binding activity of NFκB is present in mature B lymphocytes expressing κ immunoglobulin light chain genes (Sen and Baltimore, 1986a; Lenardo *et al.*, 1987), NFκB binding activity is sequestered in the cytoplasm of non-lymphocyte cell types by virtue of


A
LPS INDUCED RAT LIVER
HEPATIC NUCLEI
HEPARIN AGAROSE ELUATE
HYDROXYLAPATITE GRADIENT
BPi
BPc
TSK GEL FILTRATION
HEAT AT 65°C
BPi FRACTION
BPc FRACTION
-558
-530
APRE WT ACCACAGTTGGGATTTCCCAACCTGACCA
APRE M6 ACCACAGTTGTGATTTCACAACCTGACCA
APRE M2 ACCACATGTTGGATTTCCGATACTGACCA
KBE WT ACAGAGGGGACTTTCCGAGAGG
KBE M CCAGAATTCACTTTCCGAGAGG
C/EBP GCTGCAGATTGCGCAATCTGCAGCA
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being bound by an inhibitor protein IκB (Baeuerle and Baltimore, 1988a,b). The sequestered NFκB is translocated into the nucleus upon treatment with the phorbol ester TPA (Sen and Baltimore, 1986b) or the cytokines IL-1α/TNF (Osborn *et al.*, 1989; Duh *et al.*, 1989). This occurs rapidly, even in the absence of new protein synthesis (Sen and Baltimore, 1986b; Ron *et al.*, 1990a). *Cis*-elements that bind NFκB include the immunoglobulin κ light chain enhancer (Sen and Baltimore, 1986a,b; Lenardo *et al.*, 1987), simian virus (SV40) enhancer (Sen and Baltimore, 1986b; Macchi *et al.*, 1989; Espel *et al.*, 1990), class I major histocompatibility antigen promoter (Baldwin and Sharp, 1988; Israel *et al.*, 1989), interleukin 2 receptor α-chain promoter (Bohnlein *et al.*, 1988; Ballard *et al.*, 1989), tumor necrosis factor alpha promoter (Collart *et al.*, 1990), human β-interferon promoter (Goodbourn *et al.*, 1985; Lenardo *et al.*, 1989; Visvanathan and Goodbourn, 1989) and the HIV enhancer (Nabel and Baltimore, 1987), implicating this ubiquitous transcriptional regulator as a participant in the cytokine mediated network of immune regulation. In addition to binding the NFκB-like cytokine inducible BPi, the angiotensinogen APRE also binds a class of heat-stable constitutive nuclear proteins (BPcs). The relationship of these two proteins that bind to the APRE and the mechanism by which they modulate the transcription of the angiotensinogen promoter during the acute-phase response is the subject of this study.

We have isolated the inducible BPi and the constitutive BPc DNA binding proteins from lipopolysaccharide-treated rat liver and characterized the binding sites and interaction of these two proteins *in vitro*. Reporter gene constructs containing specific DNA templates that bind each protein selectively were used to assay independently for the transcriptional activities of these factors and to demonstrate the consequences of their interactions *in vivo*. We demonstrate that these two factors bind the angiotensinogen gene APRE in a mutually exclusive manner and that competition between the inducible, strong transcriptional *trans*-activator BPi (NFκB-like) and the constitutive, weaker *trans*-activator BPc (C/EBP-like) for binding to the APRE represents a mechanism for modulation of angiotensinogen gene transcription.

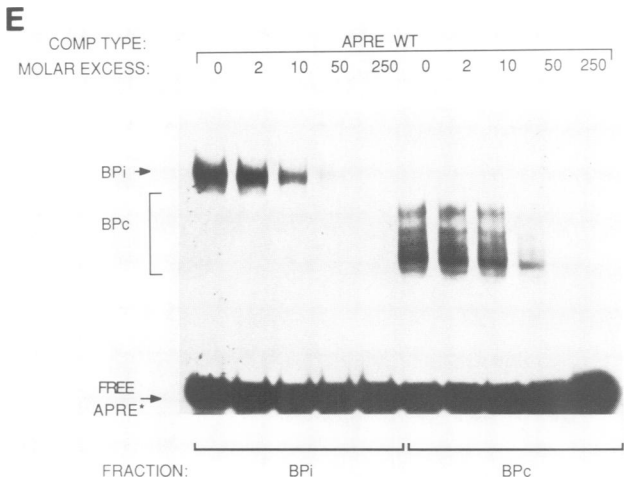
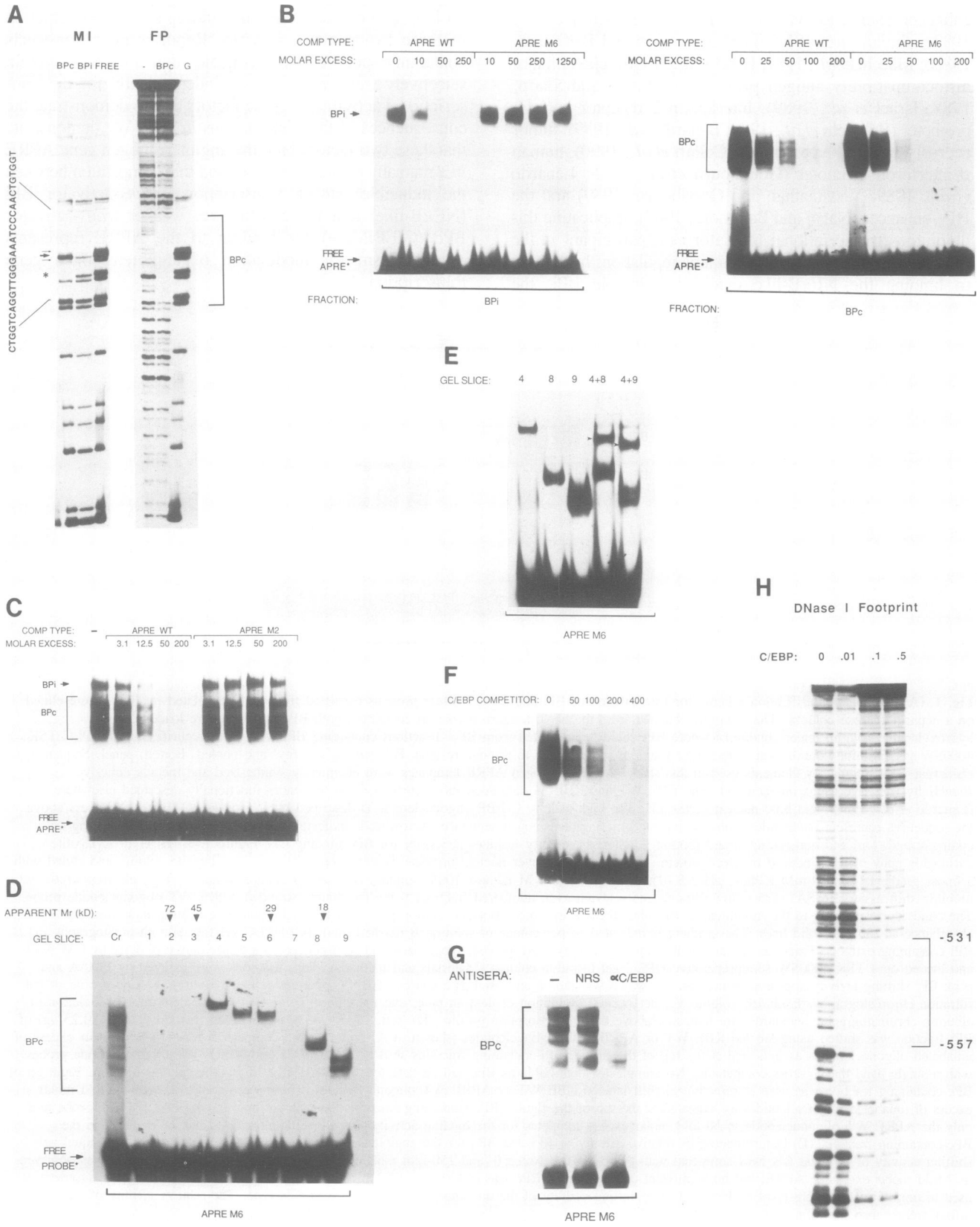


Fig. 1. (**A**) Top panel. Purification scheme for fractionation of BPi and BPc. Nuclear proteins extracted from LPS-stimulated rat livers were eluted on a heparin agarose column. The fractions obtained from the heparin agarose column containing both BPc and BPi were loaded onto a hydroxylapatite column under conditions where BPi could be separated from BPc. Fractions containing BPi were further purified on a HPLC TSK 4000SW gel filtration column and stored. BPc fractions were heated to remove residual BPi binding activity, and pooled. Bottom panel. Sequences of *cis*-acting DNA regulatory elements used in this study. Angiotensinogen APRE templates were chemically synthesized and include cohesive *Bam*HI/*Bgl*II 5′ overhangs on each end. The KBE WT and KBE M oligonucleotides correspond to sequences functionally described elsewhere (Lenardo *et al.*, 1989), and bind nuclear factor κB. The high affinity C/EBP oligonucleotide is described by Vinson *et al.* (1989). Numbers above the sequences represent nucleotides from the reported rat angiotensinogen sequence. Arrowheads indicate residues necessary for BPi binding on coding strand (top) and non-coding strand (bottom). Asterisks identify residues necessary for BPc binding (see Figure 2A). (**B**) Hydroxylapatite chromatography of LPS induced rat liver protein. LPS induced rat liver nuclear proteins were run on a 2 ml hydroxylapatite column and eluted with a linear gradient from 50 mM KPhos, pH 7.5 (0% solution B) to 1 M KPhos (100% solution B) over 20 column volumes. Top, electrophoretic gel mobility shift assay (EMSA) across flow-through and relevant even numbered fractions using the double stranded APRE WT oligonucleotide probe. The bands corresponding to the mobilities of BPi and BPc are indicated. Bottom, elution profile of the hydroxylapatite column monitored by absorbance at 280 nm (solid line). The gradient is indicated as percentage of solution B (dashed line). (**C**) HPLC gel filtration chromatography of BPi containing extracts. Fractions containing BPi shifting activity were pooled, lyophilized and resuspended to a final volume of 1 ml in 1 M Nacl and loaded on a TSK 4000SW semipreparative HPLC gel filtration column (Materials and methods). Top, fractions were assayed by EMSA and peak BPi shifting activity appeared in fraction 16, which was stored and used as a source of BPi-containing extracts. Bottom, elution profile of gel filtration chromatography. Excluded volume V_o, determined by elution of dextran blue, and the elution volume of 66 kd, determined by elution of albumin chromatographic standards, are indicated above the UV absorption profile. (**D**) Left, EMSA of BPi-containing fraction. Ten μl (0.25 μg) of BPi fraction was shifted using labeled KBE WT or APRE WT oligonucleotides (shown in A, bottom), and competed with a 50-fold molar excess of unlabeled oligonucleotides as indicated at the top of the figure. BPi-containing fractions shift both KBE WT and APRE WT oligonucleotide probes with a single band that is cross competable. No complexes identifiable as BPc can be detected. Right, EMSA of BPc-containing fraction. Eight μg of BPc-containing fraction was used to directly gel shift labeled KBE WT or APRE WT oligonucleotides. These were competed with 50-fold molar excess of unlabeled oligonucleotides as indicated at the top of the figure. BPc-containing fractions have no shifting activity of KBE WT probe and only the APRE WT oligonucleotide in 50-fold molar excess competes for the binding activity. No BPi binding activity can be detected in the BPc-containing fraction. (**E**) Determination of relative affinity of BPi and BPc for the angiotensinogen APRE. EMSA of approximately equal gel shifting activity of BPi and BPc was competed with APRE WT between 0- and 250-fold molar excess. Significant competition of BPi was observed in 2-fold molar excess of APRE, but no significant competition of BPc was observed until competitor was 50-fold excess. Scanning densitometry was used to normalize the intensity of each band to the shifting activity of the uncompeted lane for each protein type. The relative affinity of BPi is 5-fold greater than the affinity of BPc for the APRE.

Results

Isolation of angiotensinogen APRE binding proteins

In hepatoma cells cultured *in vitro* and LPS induced rat liver nuclei preparations *in vivo*, the two angiotensinogen APRE binding proteins are distinguished by their unique electrophoretic mobilities under nondenaturing conditions and by their distinct affinities for mutant templates (Ron *et al.*, 1990b). The APRE binding proteins from these two tissues therefore appear to be indistinguishable, but because of the greater abundance of protein in liver as compared with cultured cells, we used rat liver for purification of the two proteins designated as BPc and BPi (Figure 1A). Crude nuclear protein from LPS-stimulated rats was concentrated



on a heparin agarose column and the two APRE binding proteins were resolved on a hydroxylapatite column using a shallow linear elution of potassium phosphate. Separation of the two binding activities was monitored by electrophoretic gel mobility shift assay (EMSA). The inducible BPi species eluted at a lower ionic strength (~350 mM KPhos) than BPc (400 mM KPhos) (Figure 1B). Fractions containing BPi were pooled and BPi was further purified by size exclusion chromatography on an HPLC TSK 4000SW column after concentration by lyophilization (Figure 1C). Fractions containing peak EMSA activity were pooled and referred to as BPi. Further purification of the BPc species was accomplished by heating the hydroxylapatite peak BPc fractions at 65°C for 10 min, a treatment which inactivates contaminating BPi.

To demonstrate that each fraction with specific APRE binding activity is pure with respect to the other, EMSA was performed on the BPi and BPb containing extracts using both the wild-type (BPi only binding) KBE sequence (KBE WT) and the wild-type (BPi and BPc binding) APRE sequence (APRE WT) as probes. Extracts containing BPi produced a single gel-shifted band on the KBE WT probe which was competed completely by a 50-fold molar excess of either unlabeled KBE WT or APRE WT, but not by the mutant sequences KBE M or APRE M2 (for sequences of probes, see Figure 1A). Conversely, a single band that closely comigrated with the retarded band on the KBE WT gel shift was detected using the APRE as a probe. This band was also competed by an excess of unlabeled wild-type KBE or APRE sequence but not by the mutants. Importantly, in the BPi fraction, no BPc shifting activity was demonstrated. In the BPc fraction, no direct binding to the KBE WT probe was seen, demonstrating that this fraction was free of contaminating BPi (Figure 1D). Consistent with previous observations using unfractionated extracts (Ron *et al.*, 1990b), the BPc proteins are not competed by excess KBE WT, KBE M or APRE M2 sequences (see also Figure 2E). Thus, the gel shifting, and hence DNA binding activities, of BPi and BPc were completely separated by this protein purification scheme. In EMSA, the BPc fractions gave rise to many shifted bands indicating the presence of multiple BPc proteins.

To quantitate the relative binding affinities of each protein for the APRE WT template, equivalent amounts of gel shifting activities of purified BPi and BPc fractions were competed with increasing amounts of unlabeled APRE WT oligonucleotide competitor (Figure 1E). Under these conditions, 50% of BPi gel shifting activity was competed by 0.8 nM concentration of APRE WT, whereas 4.2 nM was required to compete 50% of BPc gel shifting activity. Thus, BPi has an ~5-fold greater affinity for the APRE as compared with BPc.

BPc and BPi recognize distinct sites on the angiotensinogen APRE

Methylation interference was performed using the individual purified BPi- and BPc-containing protein fractions and a probe consisting of a methylated fragment of DNA spanning nt −526 to nt −615 of the rat angiotensinogen gene (Figure 2). Methylation of guanosine residues on non-coding strands at positions nt −537, nt −540 and nt −542 prevented BPc species from binding to the APRE template. Methylation of the three guanosines between nt −540 and nt −542 abolished BPi binding, consistent with earlier methylation interference analysis of BPi binding on the angiotensinogen APRE using crude cellular extracts (Ron *et al.*, 1990b). Both BPc and BPi have contact points within the functionally defined APRE element. Overlapping, yet distinct DNA contact sites for these two proteins demonstrate that they have functionally distinct binding domains. Furthermore, all the BPi DNA contact points defined by methylation interference lie within the DNase footprint produced by purified BPc (Figure 2A, right panel).

Identification of nt −541 as essential for binding by BPi,

Fig. 2. (**A**) Left, methylation interference of purified proteins on the angiotensinogen APRE. The non-coding strand of the angiotensinogen APRE was labeled at nt −526 and digested at nt −615, methylated and used as a probe for EMSA. The shifted bands produced by purified proteins were electroeluted, cleaved with piperidine and electrophoresed in a denaturing 8% polyacrylamide−8 M urea gel. Free lane corresponds to methylation pattern of non-shifted probe after piperidine cleavage. The site whose methylation prevents BPc from binding is indicated by an asterisk (*), sites that interfere with BPi, but not BPc, are indicated by large arrows and sites important for both factors are indicated by thin arrows. Angiotensinogen non-coding sequences are displayed on the left. Right, DNase I footprint of purified BPc. The identical probe used for methylation interference was footprinted by BPc. 0, no nuclear extract; BPc, 16 μg of purified BPc fraction; G, Maxam−Gilbert 'G' sequencing ladder. Sequences protected from DNase digestion are indicated by a bracket. These sequences correspond to the footprinted region previously described using LPS induced or non-induced nuclear extracts. (**B**) Left, APRE M6 is not bound by BPi. EMSA using BPi-containing fraction to shift APRE WT probe competed with APRE WT (left) and APRE M6 (right). APRE M6 did not detectably compete for gel shifting activity of BPi even at 1250-fold molar excess. Right, APRE M6 is bound by BPc with wild-type affinity. EMSA using BPc-containing fraction to shift APRE WT probe competed with APRE WT (left) and APRE M6 (right) oligonucleotides over a range of 0−200 molar excess of unlabeled competitor. The affinity of BPc for APRE M6 was indistinguishable from that for APRE WT. (**C**) APRE M2 is not bound by either factor. EMSA using either BPi- or BPc-containing fractions competed with unlabeled APRE WT on left and APRE M2 on the right panel. The relative mobilities of BPi and BPc are indicated on the left of the autoradiogram. APRE M2 did not significantly compete for either factor up to 200-fold molar excess. (**D**) Renaturation of BPc binding activity after SDS−PAGE. Rat liver nuclear extract (150 μg) was electrophoresed on a 10% reducing SDS−polyacrylamide gel in parallel with prestained mol. wt markers. The gel was cut perpendicular to the direction of electrophoresis into 1 cm thick slices, the proteins were eluted and renatured with guanidine−HCl. The renatured proteins were then used for EMSA using the BPc binding probe APRE M6. Crude liver nuclear extract is used in the first lane. The migration of mol. wt standards (in kd) is indicated above the relevant gel slice. All five bands compete with APRE M6 but not KBE or APRE M2 oligonucleotides (not shown). (**E**) Subunit interaction between members of the BPc family. Renatured proteins eluted from SDS−polyacrylamide gels corresponding to fraction numbers 4, 8 and 9 of (D) were assayed for gel shifting activity alone and in combination. Mixing equal amounts of fractions 4 and 8 produce a new gel shifted band of intermediate mobility (arrows) and produce a corresponding diminution of the intensity of the original gel shifted bands as compared with either fraction in isolation. Similarly subunit interaction could be demonstrated on APRE M6 template for mixtures of fractions 4 and 9. (**F**) C/EBP oligonucleotide competes for BPc binding. EMSA using BPc-containing fractions and APRE M6 probe were competed with the indicated fold molar excess of unlabeled C/EBP oligonucleotide (sequence displayed in Figure 1A and described by Vinson *et al.*, 1989). C/EBP competes for BPc binding activity. (**G**) C/EBP polyclonal antiserum blocks BPc binding. EMSA using BPc-containing fractions preincubated for 3 h at 4°C with PBS, normal rabbit serum or polyclonal antiserum raised to C-terminal 15 amino acids of C/EBP. Anti-C/EBP blocks binding of the specific BPc binding species. A non-specific band seen in this preparation is unaffected by the anti-C/EBP antiserum. (**H**) DNase I footprint of angiotensinogen sequences using bacterially expressed C/EBP. Bacterially expressed C/EBP was incubated with uniquely end-labeled angiotensinogen fragment spanning nt −615 to nt −526 and subjected to DNase I footprint. C/EBP produces a footprint over the APRE identical to that of BPc (compare A, right), although an adjacent sequence is protected at high levels of recombinant C/EBP.

but not BPc, allowed the synthesis of an oligonucleotide DNA probe that is recognized by BPc only. An oligonucleotide containing a purine to non-complementary pyridine (G to T mutation of nt −541 and its mirror image on the palindromic APRE at nt −548, see Figure 1A) generates a BPc specific template (APRE M6). APRE M6 was used to compete the purified BPc and BPi gel shifting activity on the APRE WT probe (Figure 2B). APRE M6 fails to compete for the gel shifting activity of the BPi species on the APRE WT template, even at a 1250-fold molar excess. Conversely, the affinities of BPc for APRE WT and APRE M6 were indistinguishable; a reduction of gel shifting activity of 50% was observed at competition with a molar excess of between 25- and 50-fold of either oligonucleotide (Figure 2B, right). Thus, BPc proteins bind to the APRE M6 with high affinity. Mutation of contact points common to BPi and to BPc generates APRE M2, a DNA template that, by competition in EMSA (Figure 2C), fails to compete for binding of either BPi or BPc.

BPc species are a family of C/EBP-like proteins

The BPc proteins give rise to several distinct gel shifting bands under non-denaturing conditions (for example, see Figures 1E and 2B and C). We were unable to separate individual BPc species further by heating, non-denaturing gel filtration, ion exchange chromatography or lectin affinity chromatography. Therefore, to determine whether these variably sized BPc−DNA complexes are the result of a single binding domain loosely associating with other proteins or whether the multiple species represent distinct proteins with a common DNA binding domain, the BPc binding proteins were analyzed by denaturing SDS gel electrophoresis. Proteins in slices prepared from the gels were eluted, renatured and assayed for gel shifting activity on the APRE M6 template (Figure 2D). Five distinct gel shifting activities were detected that migrate at apparent molecular masses between 18 and 45 kd. The distinct species identified by this technique all have identical affinities for APRE M6 and are not completed by APRE M2 or KBE WT oligonucleotides (Figures 1E and 2B and data not shown). Furthermore, when mixed together in varying combinations these BPc proteins with distinct electrophoretic mobilities on a SDS gel are capable of forming heterodimers that bind the APRE (Figure 2E).

Because the BPcs have the properties of heat stability, the ability to form heterodimers, and their presence in rat liver, we asked whether purified BPc was similar to the CCAAT/enhancer binding protein, C/EBP (Landschulz *et al.*, 1988b). BPc gel shifting activity of the APRE WT probe can be competed with an excess of unlabeled consensus C/EBP oligonucleotides (sequences in Figure 1A) and identical gel shifting patterns using BPc fractions are produced with either APRE M6 or C/EBP-labeled probes (Figure 2F and data not shown). Antiserum raised to the C-terminal 15 amino acids of C/EBP (Birkenmeier *et al.*, 1989) blocks binding to the APRE WT probe of all the BPc binding species (Figure 2G); however the α14 polyclonal antiserum raised to a peptide N-terminal to the C/EBP DNA binding domain (Landschulz *et al.*, 1988b) only crossreacts with the BPc proteins in gel slice #4 (not shown). Thus the DNA binding protein represented in gel slice #4 is highly homologous to bona fide C/EBP, whereas the smaller BPc species share C-terminal epitopes with C/EBP. Furthermore, bacterially expressed C/EBP footprints sequences corresponding to the angiotensinogen APRE in a fashion identical to purified BPc (compare Figure 2H with Figure 2A, right panel). Although high concentrations of recombinant C/EBP give rise to another footprint in an adjacent region on the angiotensinogen promoter, the functional significance of this protein binding site remains to be determined.

The precise nature of the variably sized BPcs, their relationships to C/EBP, as well as the possibility that they may represent artifactual proteolysis of C/EBP requires further analysis.

BPc attenuates IL-1α inducible transcriptional activity mediated by BPi

The identification of DNA probes that are selective targets for binding by the BPi or BPc proteins *in vitro* allowed us to examine separately the transcriptional activities of these proteins in HepG2 cells. Basal and IL-1α induced activities of reporter genes containing four copies of APRE templates binding specifically to either protein were ligated upstream of the angiotensinogen core promoter and transfected into HepG2 cells (Figure 3A). This angiotensinogen core promoter was previously demonstrated to initiate faithfully transcripts at the nt +1 cap site (Ron *et al.*, 1990a,b). Under basal conditions, luciferase activity driven by APRE WT sequences was 5-fold greater than the activity of the null mutation APRE M2. The APRE WT constructs were further 40-fold inducible by IL-1α. The basal activity of the KBE WT driven reporter was 4-fold greater than the basal activity of the APRE WT construct, and the magnitude of IL-1α induced luciferase activity was 5-fold greater than that of the APRE WT sequence. These differences in transcriptional activity were reproduced in five independent experiments internally controlled for transfection efficiency. Concatenated APRE M6 functions as an uninducible basal enhancer with a 20-fold greater luciferase activity compared with the null binding mutation APRE M2. Although our observation that the KBE WT binding site functions as an enhancer in unstimulated cell lines is in apparent contradiction to initial reports that the location of NFκB is restricted to the cytoplasm in non-lymphoid cell lines (Baeuerle and Baltimore, 1988a,b), our findings are consistent with more recent observations demonstrating basal transcription activity of NFκB binding sites (Collart *et al.*, 1990) and the presence of NFκB protein in the nucleus (Macchi *et al.*, 1990) in a variety of unstimulated non-lymphocyte cell lines.

To examine the enhancer activity of these binding sites within the context of the angiotensinogen promoter sequence, we tested the IL-1α induced transcriptional activity of the enhancer sequence between nt −615 and nt −526 transfected into HepG2 cells (Figure 3B). This enhancer, which contains the APRE and has previously been demonstrated to be sufficient for IL-1α induction of reporter gene activity (Ron *et al.*, 1990a,b), was cloned as a single copy upstream of the angiotensinogen minimal promoter. A similar construct was generated containing site-directed mutations within the APRE to generate a KBE sequence (that binds only BPi, designated KBE). In response to IL-1α, APRE WT invoked a dose dependent increase in luciferase reporter activity, plateauing at 5.6-fold when using 100 U/ml IL-1α. As observed with the multimerized binding site (Figure 3A), the BPi binding sequence KBE has a markedly higher basal luciferase activity (10-fold) and a higher IL-1α

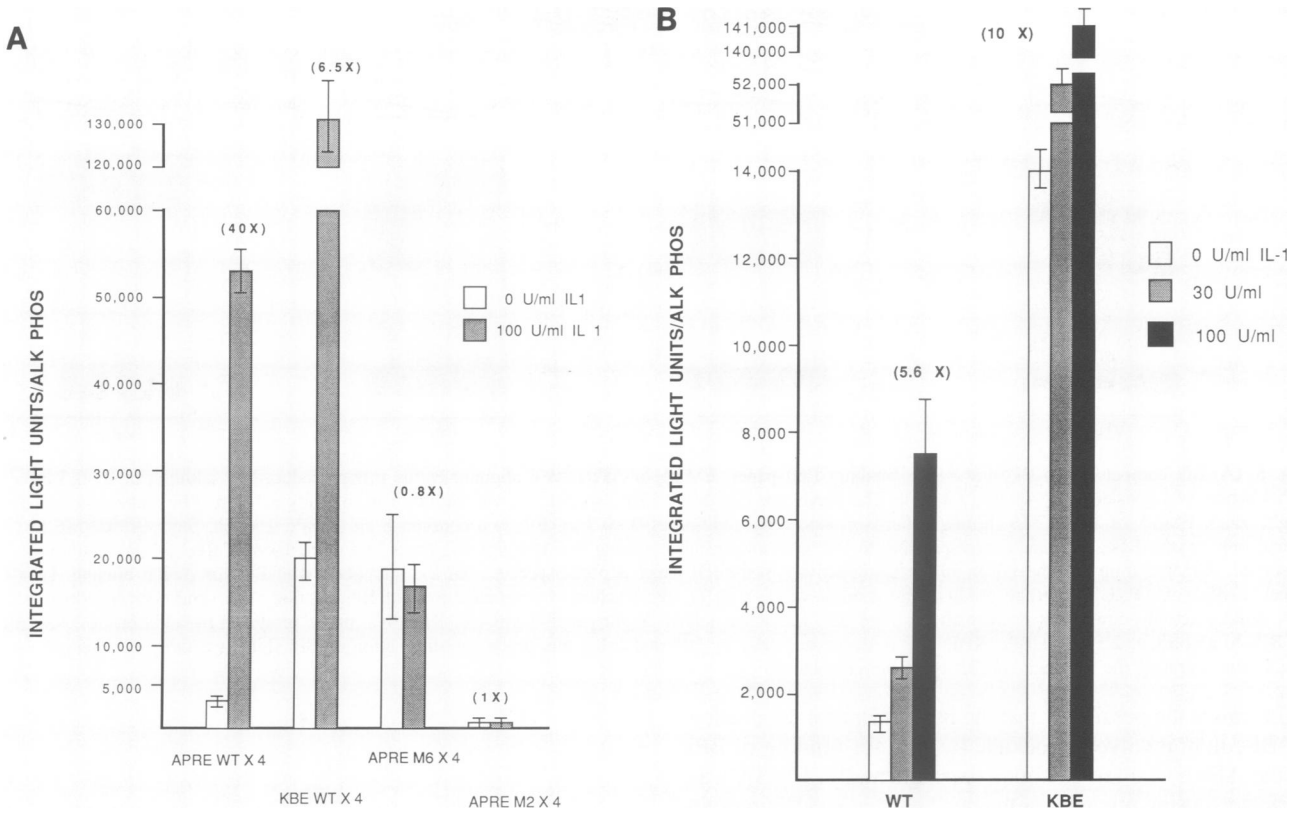


Fig. 3. (**A**) Transcriptional activity of multimerized APRE templates upstream of the angiotensinogen minimal promoter. Four copies of APRE WT, KBE WT, APRE M6 and APRE M2 were ligated upstream of the rat angiotensinogen minimal promoter (nt −59 to nt +39) linked to a firefly luciferase reporter. These plasmids were transiently transfected into HepG2 cells along with an internal control reporter plasmid encoding placental alkaline phosphatase (pSV2APAP). The cells were stimulated with 100 U/ml recombinant IL-1α for 4 h. Luciferase reporter activity normalized to alkaline phosphatase activity is presented as the mean ± SD of triplicate plates from a transfection representative of five separate experiments. Numbers in parentheses represent fold-increase of reporter activity when cells are stimulated with IL-1α compared with the activity of the unstimulated promoter.(**B**) Transcriptional activity of oligonucleotide-directed mutations within the angiotensinogen cytokine responsive enhancer. Site-directed mutations within the APRE were introduced into nt −615 to nt −526 of angiotensinogen sequences (Ohkubo *et al.*, 1986; Brasier *et al.*, 1989b) cloned upstream of the angiotensinogen minimal promoter and transfected along with pSV2APAP into HepG2 cells. Reporter activity was normalized to alkaline phosphatase activity in the absence or presence of 4 h stimulation by IL-1α. Numbers in parentheses represent fold-increase above the unstimulated levels for each plasmid. Values represent the mean ± SD of five independent experiments pooled by normalizing reporter activity in each transfection to unstimulated APRE WT activity from a representative transfection.

induced luciferase activity (18-fold) compared with APRE WT. Thus, in the context of the angiotensinogen promoter, APRE WT, permissive for binding both BPi and BPc, is a less active enhancer than KBE that binds only BPi.

Both site-directed mutations and concatenated DNA templates demonstrate that APRE WT sequences have lower basal and IL-1α induced luciferase reporter activity than does the 'pure BPi' binding template KBE WT. These results are consistent with the hypothesis that binding of BPc to the APRE attenuates the transcriptional activity mediated by the inducible BPi species.

To exclude the possibility that the difference in transcriptional activities of KBE compared with the APRE is due to a higher affinity of binding of BPi to the KBE WT compared with the APRE WT templates, gel mobility shift assays with oligonucleotide competition were performed (Figure 4). The gel shifting activity produced by nuclear extracts containing BPi was competed with a 3-fold greater affinity by APRE WT than by KBE WT. Thus BPi binds to the APRE WT sequences with a greater affinity than it binds to KBE WT sequences and the greater basal activity of the KBE binding site cannot be explained by differences in protein binding affinities.

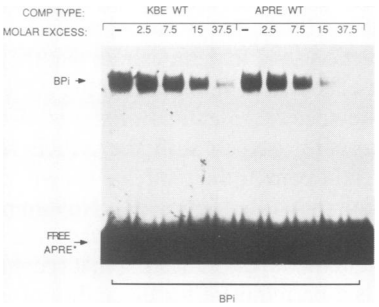


Fig. 4. Determination of relative binding affinity of BPi for KBE WT and APRE WT sequences. Fractions containing BPi (2.5 μg) were used to shift APRE WT oligonucleotide. Competition of BPi shifting was performed with 2.5- to 37.5-fold molar excess of either KBE WT (left) or APRE WT (right) oligonucleotides. APRE WT competes with a 3-fold greater affinity than does KBE WT.

BPi and BPc compete for binding to the APRE WT template in vitro

The data described above suggest that BPc acts as an attenuator of BPi mediated transcriptional activity. The observation of overlapping nucleotide contact sites for BPi and BPc within the DNase I protected footprint (Figure 2A,

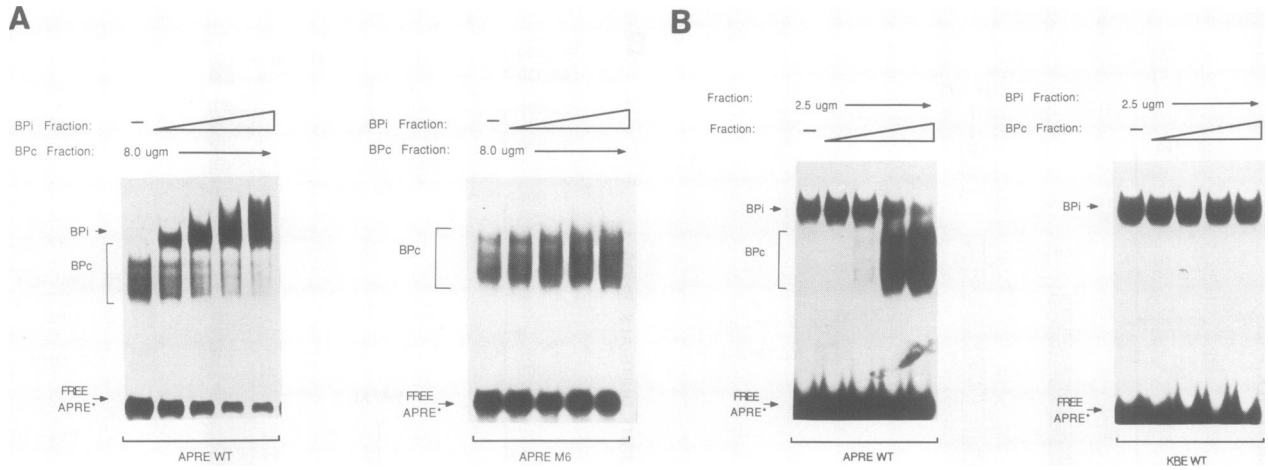


Fig. 5. (**A**) BPi competes with BPc for APRE binding. Left panel, EMSA of APRE WT oligonucleotide probe produced by addition of 0−0.3 μg BPi fraction to a constant 8 μg of BPc fraction. The bands correspnding to BPc shifting activity diminish in intensity as more BPi fraction is added. Right panel, EMSA of APRE M6 shifting patterns produced by addition of 0−0.3 μg BPi to a constant 8 μg of BPc fraction. Since APRE M6 is bound only by the BPc, the bands corresponding to BPi are not detectable. Using this BPc specific probe, no diminution of BPc shifting activity is observed, demonstrating that the fall in BPc shifting activity in the left panel is probe-specific. (**B**) BPc competes with BPi for APRE binding. Left panel, EMSA of APRE WT probe produced by addition of 0−8 μg of BPc fraction to constant 2.5 μg of BPi fraction. The band corresponding to BPi shifting activity diminishes in intensity as more BPc fraction is added. Right panel, EMSA of KBE WT probe produced by addition of 0−8 μg of BPc to a constant 2.5 μg of BPi. KBE WT is recognized only by BPi and using this probe, no diminution of BPi shifting activity is observed, again demonstrating the template specificity of the competition.

right) raises the possibility that the mechanism for attenuation by BPc may involve a blockade by BPc for binding of BPi to the APRE. To test this idea experimentally, EMSA was performed using mixtures of the purified BPc and BPi protein fractions (Figure 5). In this assay the concentration of one of the two binding proteins is fixed and increasing amounts of the competitor protein are added. Binding of both proteins occurred rapidly at room temperature; 90% of binding activity was attained within 5 min of the addition of the DNA probe (data not shown). Binding reactions were analyzed by EMSA after binding equilibrium was reached (20 min).

Addition of increasing amounts of BPi to a constant amount of BPc results in a loss of gel shifting activity contributed by the BPc species (Figure 5A, left panel). No unique intermediate or larger bands were detected, arguing against the simultaneous binding of both BPi and BPc proteins to the APRE. To exclude non-specific protein−protein interactions and to demonstrate that the competitive displacement of the binding of BPc to the APRE by BPi is DNA template specific, identical mixtures of the BPi and BPc proteins were used to shift the APRE M6 template (Figure 5A, right panel), the template shown to be a target for BPc binding only (see Figure 2B). No diminution of the binding of BPc to the APRE M6 was detectable using identical protein mixtures, indicating that recognition of the DNA probe is a prerequisite for the ability of BPi to block the binding of BPc. Conversely, the addition of BPc in excess competes the binding of BPi to the APRE WT template (Figure 5B, left panel; compare with Figure 5A, left panel). Sequence specificity for displacement of BPi by BPc in this mixture of binding proteins is shown by using the KBE WT as a probe [a template specific for BPi (Figure 5B, right and Figure 1D)]. Thus, BPi competes with BPc for binding to the wild-type APRE sequences and conversely BPc competes with BPi for binding APRE *in vitro*.

Overexpression of rat C/EBP blocks BPi mediated activation of the APRE

In view of the demonstrated similarity between BPc and C/EBP, we asked whether C/EBP overexpression can block

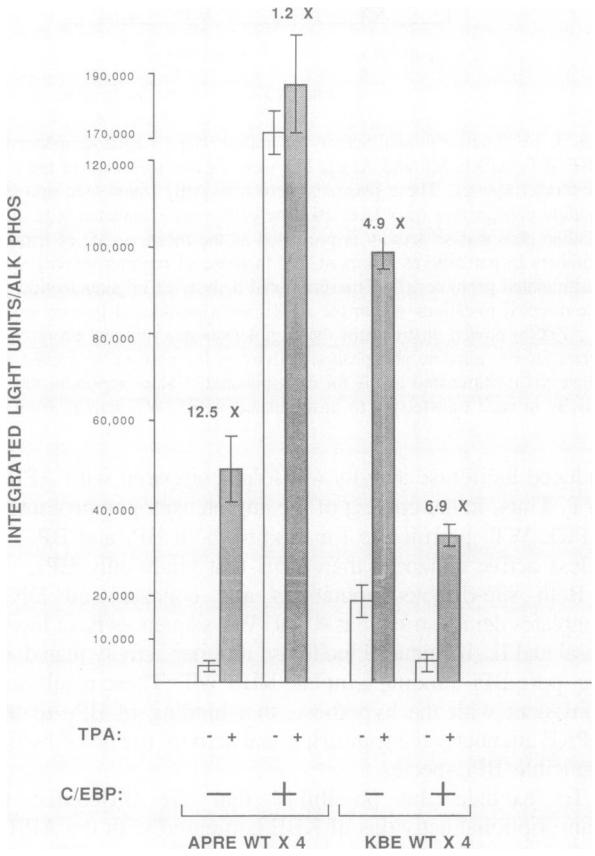


Fig. 6. C/EBP overexpression blocks BPi mediated *trans*-activation of the APRE. Four copies of APRE WT and KBE WT sequences were ligated upstream of the angiotensinogen promoter and transiently cotransfected with (10 μg) internal control reporter plasmid pSV2APAP (2 μg) in the absence or presence of C/EBP expression vector (24 μg) (MSV-C/EBP, Friedman *et al.*, 1989) into HepG2 cells. Basal and TPA (an IL-1α surrogate)-stimulated reporter activity was measured. C/EBP *trans*-activates APRE WT but not KBE WT sequences and APRE WT sequences are not further TPA inducible. Fold-induction of luciferase reporter activity relative to unstimulated value is indicated above each set of duplicate points.

BPi mediated *trans*-activation of the APRE. Multimerized APRE WT and KBE templates upstream of the angiotensinogen promoter were tested for TPA inducibility in the absence and presence of a eukaryotic expression vector for rat C/EBP (MSV−C/EBP, Friedman *et al.*, 1989). Rat C/EBP markedly increased reporter activity driven by APRE WT multimers demonstrating that C/EBP *trans*-activates the APRE (Figure 6). This result is consistent with the low levels of endogenous C/EBP observed in cultured hepatocytes (5% of the levels found in rat liver, Friedman *et al.*, 1989). As anticipated, C/EBP overexpression completely blocks the BPi mediated activation of the APRE WT sequences induced by TPA (Figure 6). Sequence specificity for these events is shown by the inability of C/EBP to activate the angiotensinogen promoter driven by multimeric KBE WT sequences and by its inability to block TPA induced BPi mediated activation of the KBE WT templates. The fall in basal activity of the KBE WT construct induced by overexpressing C/EBP is a reproducible finding which we attribute to 'squelching' in *trans* (Gill and Ptashne, 1988).

Discussion

We present evidence that two distinct transcriptional *trans*-activator proteins bind to overlapping contact sites on the functionally defined angiotensinogen APRE. The binding of these two proteins, BPi and BPc, is mutually exclusive (Figure 5). A heat stable family of nuclear proteins termed BPc contact the palindromic APRE at guanosine residues corresponding to nt −536, −540 and −542 on the non-coding strand. Gel electrophoresis and renaturation experiments indicate that BPc derived from liver consists of at least five heterogeneous proteins ranging in size from 18 to 45 kd (apparent mol. wt). Electrophoretic gel mobility shift assays of mixtures of these isolated BPc proteins show that they form heterodimers in various combinations and thus the BPc proteins share dimerization surfaces. A relationship between BPc and C/EBP is noted because both proteins recognize common DNA sequences and share common antigenic epitopes in their C termini. Dimerization of C/EBP is proposed to occur by way of hydrophobic interactions between heptad leucine repeats within an α helical domain C-terminal to the DNA binding domain (Landshulz, 1988a, 1989), and in this regard it will be interesting to examine whether BPc species can interact with other members of the C/EBP family. The existence of a C/EBP family is supported by the expression cloning of the C/EBP homolog NF-IL6, an acute-phase responsive protein that binds to the IL-6 promoter (Akira *et al.*, 1990). The precise relationship of BPc proteins to C/EBP or to NF-IL6 remains to be determined.

The IL-1α inducible protein termed BPi contacts the APRE at guanosine residues between nt −540 and nt −542. This represents an overlapping yet distinct pattern from the guanosines essential for BPc binding. We have previously demonstrated that BPi is indistinguishable from NFκB based on its binding specificity, IL-1α and TPA inducibility, cytoplasmic localization in the uninduced state and 50 kd size by UV photoaffinity crosslinking (Ron *et al.*, 1990b). Although other workers report that NFκB from HeLa cells sediments in glycerol gradients as a 120 kd heterodimeric complex of a 50 kd binding protein and a 65 kd protein that does not bind directly to DNA (Baeuerle and Baltimore, 1989), we have observed that BPi from livers of rats induced

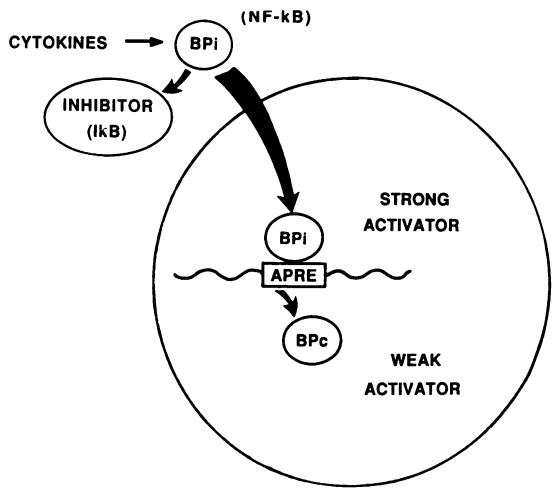


Fig. 7. Model for cytokine activation of angiotensinogen APRE. Cytokine stimulation of hepatocytes results in nuclear translocation of the NFκB-like BPi, presumably by phosphorylation of the cytoplasmic inhibitor IκB to release NFκB. BPi, which has a 5-fold higher affinity for binding to the APRE than does the constitutive nuclear protein BPc, competes with BPc for occupancy of the APRE, resulting in cytokine induction of angiotensinogen gene transcription. Transcriptional activity of the angiotensinogen APRE is attenuated by competition from the constitutive family of C/EBP-like DNA binding factors that are less potent *trans*-activators than BPi.

with LPS comigrates with a 66 kd albumin marker protein on HPLC gel filtration chromatography. This discrepancy in estimates of apparent molecular size may be due to the high ionic strength that we used to disrupt non-covalent protein−protein interactions or alternatively to different post-translational modifications of NFκB that may take place in cultured HeLa cells as compared with rat liver *in vivo*.

BPi has a 5-fold greater affinity than BPc for the APRE template *in vitro* and binds with indistinguishable affinities for both the immunoglobulin κ light chain enhancer and the angiotensinogen APRE. Because the BPcs and BPis cross compete *in vitro* for binding to the APRE, the predominant protein species occupying the APRE will be determined primarily by the relative abundance of the two proteins in the nucleus at any given time (e.g. BPc in unstimulated cells and BPi in the acute-phase). By assaying for the enhancer activity *in vivo* of oligonucleotide cassettes that are specific DNA binding sites of either BPc or BPi, we defined the transcriptional activity of these proteins. DNA elements that bind BPi only are IL-1α inducible enhancers, and elements that bind BPc only are not inducible by either cytokines or phorbol esters but do function as basal enhancers. Although the APRE sequences are recognized by BPi (NFκB) with a 3-fold greater affinity, the APRE is less transcriptionally active than is the BPi only binding KBE sequence. This holds true both under basal conditions and after IL-1α stimulation of cultured hepatocytes. These transcriptional properties demonstrate that competition for the APRE by these two proteins has functional consequences *in vivo*.

Based on these observations we propose a model for cytokine mediated activation of the angiotensinogen APRE (Figure 7). Activation of hepatocytes by IL-1α or TNF results in a signalling cascade that releases sequestered cytoplasmic NFκB (BPi) from its inhibitor, IκB (Baeuerle and Baltimore, 1988a,b; Duh *et al.*, 1989; Osborn *et al.*, 1989; Ron *et al.*, 1990b). Although the second messenger pathway is unknown, activation of protein kinase C may

be involved, because TPA is sufficient to induce nuclear translocation of NFκB in cultured non-lymphoid cells (Sen and Baltimore, 1986b), and protein kinase C is sufficient for activation *in vitro* (Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990). NFκB is translocated into the nucleus where, by mass action, it displaces the constitutive C/EBP-like binding factors (BPcs) from the APRE. Although BPc in isolation is an activator, it functions in this context as an attenuator of NFκB action by virtue of competing with a much more potent *trans*-activator than itself.

Among acute-phase response genes, hierarchies of inducibility are observed. The serum amyloid A gene is an acute-phase response gene whose level of mRNA increases by 1000-fold during the acute phase reaction. An NFκB-like protein inducible by TPA binds to a KBE-like site in the distal promoter with a methylation interference pattern identical to that of bona fide NFκB. By comparison, the angiotensinogen mRNA level increases by only a modest 4- to 5-fold during the acute-phase response. Competition between NFκB-like BPi and the constitutive BPc may dampen the transcriptional response of a subset of acute-phase response genes activated by an identical cytokine activated protein kinase C dependent pathway. Thus this system allows for cytokine activated genes to respond in a graded fashion to a common signalling event.

The functional role for BPc in the transcriptional expression of the angiotensinogen gene is not completely understood. In contrast to C/EBP binding sites within the promoter of the rat albumin gene (Gorski *et al.*, 1986; Heard *et al.*, 1987; Lichsteiner *et al.*, 1987; Friedman *et al.*, 1989) the APRE in uninduced liver does not appear to be essential for basal expression of the angiotensinogen promoter, as deletion of this element results in little if any change in transcriptional activity (Brasier *et al.*, 1989b). In spite of the fact that C/EBP overexpression in HepG2 cells activates a reporter gene containing multimerized APRE sequences (Figure 6), similar C/EBP overexpression does not change basal activity of the native angiotensinogen promoter (data not shown). We have observed that the acute-phase response element interacts synergistically with two adjacent glucocorticoid response elements during the acute-phase induction (Ron *et al.*, 1990a). A functional interaction between APRE binding proteins and activated glucocorticoid receptors may be necessary for maximal activation of gene transcription by glucocorticoids. BPc, therefore, may play a role in rendering the angiotensinogen gene fully responsive to glucocorticoids under physiological conditions in which it is desirable to activate angiotensinogen gene expression in the absence of cytokine stimulation. Along these lines we have demonstrated that complete glucocorticoid responsiveness of the angiotensinogen gene, even in the absence of cytokines, depends on an intact APRE that is permissive for the binding of BPi or BPc (Brasier *et al.*, 1990). Consistent with this model, BPc binding activity is detected in non-hepatic tissues (e.g. brain) which do not demonstrate induction of angiotensinogen mRNA during the acute-phase reaction (Kageyama *et al.*, 1985), yet both liver and brain exhibit the same fold-induction of angiotensinogen mRNA after dexamethasone administration (Kalinyak and Perlman, 1987). The observation that BPc heterodimerizes with other members of its own family implies the existence of additional protein–protein interactions that may affect transcriptional activity mediated by the APRE.

Our direct demonstration of the ability of C/EBP to block NFκB mediated activation of the APRE provides an example of the modulation of enhancer activity of NFκB binding sites by interactions with constitutive DNA binding proteins. In the H2Kb class I major histocompatibility promoter, a TNF inducible regulatory element (H2TF1) is the target for a constitutive nuclear factor H2TF1/KBF1 and an inducible NFκB-like factor. This element partially overlaps an AP-2 binding site (Yano *et al.*, 1987; Baldwin and Sharp, 1988; Israel *et al.*, 1989). In TNF-stimulated HeLa cell extracts, AP-2 binding to the H2Kb promoter sequence is detectable only when competitor oligonucleotide targets that bind NFκB and H2TF1/KBF1 are included, implying that binding of the H2TF1 sequences by the inducible NFκB excludes AP-2 binding. In a homologous regulatory element the IL-1α inducible SV40 TC-II enhanson is a target for a constitutive, apparently ubiquitous, DNA binding factor TC-IIB (indistinguishable from H2TF1/KBF1) and a cycloheximide and TPA inducible factor TC-IIA (indistinguishable from NFκB) (Kanno *et al.*, 1989; Macchi *et al.*, 1989; Espel *et al.*, 1990). In appropriate cell types, TEF-1 activity may modulate the action of NFκB by competing for target nucleotides necessary for binding by the inducible *trans*-activator. An effect of these constitutive factors on attenuating NFκB action was not directly demonstrated, although the SV40 TC-II and H2Kb enhancers exhibit marked differences in cell type and inducible enhancer activity that may reflect a difference in abundance of competing constitutive proteins in different cell types (Kanno *et al.*, 1989; Macchi *et al.*, 1989). Functional comparison of the NFκB binding sites in the κ immunoglobulin light chain enhancer to the promoter of the α chain of the interleukin 2 receptor (IL2R) has also revealed functional differences (Cross *et al.*, 1989), likely to be due to the effect of adjacent regulatory proteins binding within the IL2R sequences (Ballard *et al.*, 1989). Thus minor contextual differences within these apparently similar enhansons dictate interactions with other nuclear factors that modify the transcriptional activity of NFκB.

Competition between inducible DNA binding proteins and constitutive nuclear proteins is not restricted to NFκB. Recent evidence has been presented indicating that the human osteocalcin vitamin D responsive element is a target for binding by a Jun–Fos heterodimer and both the vitamin A and D3 steroid hormone receptors (Schule *et al.*, 1990). Thus, transcriptional activity of inducible *trans*-activators may be governed not only by the second messenger signalling pathways that are permissive for activation, but may also be governed by the expression and abundance of constitutive and inducible nuclear factors that bind to closely related or overlapping sequences.

Materials and methods

Cell culture and transfections

The human hepatoblastoma cell line HepG2 was obtained from American Type Culture Collection (ATCC) and grown in monolayers in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. Transient transfections were performed with luciferase reporter plasmids as described (Brasier *et al.*, 1989a,b), using the placental alkaline phosphatase reporter plasmid pSV2APAP as an internal control for transfection efficiency. Thirty-six hours after transfection, cells were stimulated with 100 U/ml recombinant human IL-1 (IL-1α, a gift of Steven Gillis, Immunex Corporation, Seattle, WA), or TPA (40 nM) for 4 h.

Luciferase asays were performed using the Model 1251 LKB luminometer

without modifications (Brasier *et al.*, 1989a). Alkaline phosphatase activity was measured in Triton X-100 lysis buffer as described (Henthorn *et al.*, 1988). Integrated luminescence was normalized to alkaline phosphatase activity to correct for any variation in transfection efficiency. All assays were performed in triplicate and data are reported as the mean ± SD.

Plasmid constructions

The construction of an angiotensinogen minimal promoter spanning nt −59 to nt +39 of the rat angiotensinogen gene in a pGEM3 derived luciferase reporter was described previously (Brasier *et al.*, 1989b). A unique *Bam*HI restriction endonuclease site is located upstream of the angiotensinogen promoter in the plasmid designated p59rATLUC. To generate the minimal angiotensinogen cytokine responsive enhancer sequence, the sequences nt −615 to nt −526 containing the angiotensinogen APRE (nt −531 to nt −557) on the angiotensinogen promoter were amplified using the polymerase chain reaction with primers incorporating *Bam*HI restriction endonuclease sites on both ends. This angiotensinogen fragment, sufficient to confer cytokine responsiveness on an inert promoter, was cloned into the *Bam*HI digested M13mp19 vector and mutagenized by standard techniques (Kunkel *et al.*, 1987) using primers incorporating the KBE WT, APRE M2 and APRE M6 sequences (shown in Figure 1). The reporter plasmids were purified on CsCl density gradients, and sequenced to verify correct 5′ to 3′ orientation and confirm that the correct mutation had been incorporated.

To generate reporter vectors containing multiple copies of the APRE binding sites upstream of the angiotensinogen minimal promoter, four copies of concatenated oligonucleotides encoding APRE wild-type or mutant sequences were cloned upstream of the *Bam*HI digested p59rATLUC. The sequences of the monomeric oligonucleotides are given in Figure 1A. All plasmids were purified on CsCl gradients and sequenced prior to transfection.

DNA binding assays

EMSAs were performed with double stranded oligonucleotides (APRE WT, APRE M6 or KBE WT) radioactively labeled with [^{32}P]ATP and T4 polynucleotide kinase as described (Brasier *et al.*, 1989b; Ron *et al.*, 1990a) and complexes were resolved on 6% non-denaturing polyacrylamide gels.

Dnase I footprinting reactions and methylation interference analysis was performed as described (Ron *et al.*, 1990b) using a probe labeled on the antisense strand at nt −526 after linearization by *Xho*I, dephosphorylation with calf intestine alkaline phosphatase, and labeled with T4 polynucleotide kinase and [^{32}P]ATP. The fragment was digested at nt −615 with *Sal*I, and purified on a non-denaturing acrylamide gel. The DNase I and methylation interference reactions were resolved on a denaturing 8% polyacrylamide−8 M urea gel and exposed for autoradiography at −70°C with intensifying screens.

Rat liver nuclear protein fractionation

Adult male retired breeder CD rats (Charles River Laboratories, Inc., Wilmington, MA) were injected i.p. with lipopolysaccharide (LPS) 3 mg/kg body wt to induce the acute-phase reaction. Four hours after injection, the animals were killed by CO_2 asphyxiation, the livers dissected and the nuclei prepared by centrifugation through a sucrose cushion (Gorski *et al.*, 1986). Nuclear protein was extracted and ammonium sulfate-precipitated (0.3 g/ml), and the pellet was dialyzed extensively against 20 mM HEPES, pH 7.9, 50 mM KCl, 1 mM dithiothreitol (DTT), 10% v/v glycerol and 0.1 mM EDTA. From 12 adult male rats, ~360 g wet weight liver was obtained, yielding 300−600 mg ammonium sulfate-precipitated nuclear protein. The nuclear protein was step-eluted off the heparin agarose column and dialyzed against hydroxylapatite loading buffer at 50 mM potassium phosphate (KPhos, pH 7.5), 0.01 mM $CaCl_2$, 0.01% NP-40, 1 mM DTT, 10% (v/v) glycerol. The hydroxylapatite column was loaded at 0.25 ml/min and eluted in a shallow gradient over 20 column volumes. Fractions containing gel shifting activity for BPi binding were pooled, dialyzed and lyophilized to a final volume of 0.8 ml. Subsequently, 0.2 ml of 5 M NaCl was added to the sample and the 1 ml sample was loaded onto a Pharmacia TSK 4000SW gel filtration column equilibrated and chromatographed in 50 mM KPhos, pH 6.4, 1 mM DTT, 0.1% glycerol at 0.83 ml/min. The peak BPi shifting activity was pooled and stored at −70°C. Protein determination was made by Coomassie Brilliant blue reagent (BioRad), using bovine serum albumin as a standard.

Renaturation of DNA binding activity after SDS−polyacrylamide gel electrophoresis

Crude rat liver protein (150 μg) was solubilized in reducing SDS−Laemmli buffer, heated and electrophoresed on a 10% polyacrylamide gel using discontinuous buffers between the stacking gel and running gel. The lane containing the liver proteins was cut perpendicularly into 1 cm slices and the proteins were eluted from the gel slice, acetone precipitated, denatured in guanidine/HCl, and renatured by dialysis as described (Hager and Burgess, 1980). For estimation of mol. wt markers, prestained mol. wt markers were electrophoresed in a parallel lane. Various combinations of fractions of binding proteins renatured from the SDS−gel slices were used in EMSAs with the M6 APRE probe to identify heterodimers of the BPc proteins.

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