Gene Expression, Vol. 6, pp. 73-85, 1996
Printed in the USA.

1052-2166/96 $10.00 + .00

Analysis of the Negative Transcriptional Regulatory Element in the Angiotensin-Converting Enzyme Gene

SEAN P. KESSLER, TAUQIR Y. GORAYA,[1] AND GANES C. SEN[2]

Department of Molecular Biology, Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195
Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, OH 44106

We have characterized the sequence requirements and the protein binding properties of the previously identified transcriptional negative element present in the rabbit angiotensin-converting enzyme (ACE) gene. DNase footprinting experiments revealed that within the negative element (−715 to −610) several regions interact with proteins present in the nuclear extracts of ACE-expressing and -nonexpressing cell lines. Transfection analysis using the heterologous β-actin promoter and mutated negative elements demonstrated that the SP1 site, the collagen-silencer-like sequence, and the inverted repeat elements are dispensable for their functioning. Deletion of the region between −692 to −668, however, completely eliminated the activity of the negative element, and mutation of the synapsin-silencer-like sequence present within this region vastly reduced it. This region (−692 to −668) by itself, when present in two copies, could effectively repress the activity of the β-actin promoter. The same point mutations in the silencer element that destroyed its action on the β-actin promoter greatly increased the transcriptional efficiency of the native ACE promoter. Electrophoretic mobility shift assay using the −692 to −668 ACE silencer sequence demonstrated the formation of a DNA/protein complex. UV cross-linking of the components of this complex revealed the presence of one prominent protein of approximately 21.5 kDa. This protein may be responsible for mediating the transcriptional-repressing activity of the ACE negative element. Homology between the ACE silencer and neuronal silencer consensus sequence, together with the promoter- and tissue-independent function of the the ACE silencer, suggests this element may bind a member of a large family of common negative regulatory transcription factors.

Angiotensin-converting enzyme Silencer Transcription factor Rabbit

ANGIOTENSIN-CONVERTING enzyme (ACE) is a carboxyl-terminal dipeptidyl exopeptidase that plays an important role in the regulation of blood pressure. It converts angiotensin I to angiotensin II, the biologically active hormone (32). The two isozymes of ACE, pulmonary ACE (ACE_P) and testicular ACE (ACE_T), have different molecular weights but identical enzymatic activity and are encoded by two mRNAs (5 and 2.5 kb, respectively) (16). Each mRNA is transcribed from the same gene in a tissue-specific manner through alternative choice of transcription initiation and polyadenylation sites (17,35). The sequence organization of the genome is such that the ACE_T mRNA transcription unit is completely nested within the ACE_P mRNA transcription unit (4,8,33). Rabbit ACE_P is a glycoprotein of 140 kDa and is produced by vascular endothelial cells,

Received May 30, 1996; revision accepted August 19, 1996.
[1]Current address: Department of Medicine, Washington University, St. Louis, MO 3130.
[2]Address correspondence to Ganes C. Sen, Department of Molecular Biology, NC20, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195.

intestinal brush border cells, renal proximal tubular cells, and monocytes (2,5,9,10,23). The other isoform, ACE_T, has a molecular mass of 100 kDa and is produced exclusively in adult testis by developing sperm cells (2).

We have previously reported that the transcription of the rabbit ACE_P gene is driven by positive elements located within the first 274 bp DNA upstream of the transcription initiation site (11). The reporter gene driven by this region is expressed in two ACE-producing cells, but not in two non-ACE-producing cells, thereby establishing its tissue specificity. Our experiments also revealed the existence of a strong negative element (N.E.) located between −715 and −610 positions. This element suppresses the expression of the reporter gene in ACE-expressing cell lines in a dose-dependent and position- and orientation-independent fashion. In these cell lines, the N.E. also represses the expression of a reporter gene driven by the heterologous strong promoter of the β-actin gene (11). Electrophoretic mobility shift assays revealed that the negative element can form several complexes with proteins present in the nuclear extract of an ACE-producing cell line.

Several tissue-specific genes are known to be regulated by the presence of tissue specific positive regulatory elements in their promoters (27). Cell-specific expression of other genes is under the control of silencer proteins that, by binding to specific *cis*-acting sites, inhibit their transcription in the nonexpressing cells (12). Alternatively, other genes are regulated by a combination of both the positive and negative factors (13,34). Our analysis of the ACE_P gene transcription has already suggested the presence of tissue-specific positive elements in its promoter (11). However, ACE_P N.E. function is not restricted to ACE_P-expressing tissue. We have demonstrated that the N.E. functions in ACE_P-expressing and -nonexpressing cells. Therefore, the possibility remains that the regulation of the levels of the N.E. binding protein(s) may contribute to the complete repression of the ACE_P promoter in at least some non-ACE-expressing tissues.

In the present study, we used DNase footprinting assays for identifying the specific regions in the N.E. that interact with nuclear proteins. The possible contributions of these regions to the transcriptional repression activity of the N.E. were tested by transfection analysis of appropriately mutated N.E.s. These studies identified a silencer element present within the N.E. between −692 and −668 residues. The isolated silencer, when present in two copies, could efficiently repress the β-actin promoter. It specifically bound to a 21.5-kDa nuclear protein present in both ACE_P-expressing and -nonexpressing cells. Mutation of two specific residues within the element eliminated its protein binding ability as well as the gene-repressing activity.

MATERIALS AND METHODS

Reagents

All DNA-modifying enzymes were purchased from GIBCO-Bethesda Research Laboratories (Bethesda, MD) or Boehringer Mannheim (Indianapolis, IN). [^{14}C]Chloramphenicol (50 mCi/mmol) and [γ-^{32}P]ATP (6000 Ci/mmol) were purchased from Dupont NEN (Wilmington, DE). Cell culture products were obtained from GIBCO-BRL and Cell Culture Laboratories (Cleveland, OH). Poly(dI-dC) was purchased from Pharmacia Biotech (Piscataway, NJ). Plasmid DNA was purchased from Promega (Madison, WI) or Stratagene (La Jolla, CA). Luciferase assay kit was purchased from Promega. All mutating primers were purchased from OPERON Technologies, Inc. (Alameda, CA). Computer-assisted analysis of putative binding sites present in the ACE_P N.E. was performed with the MacVector software, Eastman-Kodak, and Microgenie (24). Homology comparison between the rabbit (17), mouse, and human (31) ACE_P genes was also performed with the Microgenie DNA analysis computer program (24).

Plasmid Construction

The 105-bp ACE_P N.E., corresponding to the upstream −715 to −610 region of the ACE_P gene, was cloned into pBluescript II KS+ (SK394). The Hind III to Xho I region of this vector was subsequently replaced by a double-stranded DNA segment containing Hind III, Bgl II, Nde I, and Xho I sites. This was accomplished by annealing the following two oligonucleotide primers: HBNX sense (AGCTTAGATCTCATATGC), HBNXanti (TCGAGCATATGAGATCTA). This vector (SK394BN) served as the template for all ACE_P N.E. deletion and substitution mutagenesis. The ACE_P N.E. was also excised from SK394BN and subcloned downstream of the polyadenylation signal in the blunted BamH I site of the β-actin luciferase reporter plasmid. The β-actin luciferase plasmid (β-actinLUC) was previously prepared by cloning the 560-bp human β-actin promoter from β-actin CAT into the pGL2-Basic vector (Pro-

mega) (22). Deletion or substitution mutants of the N.E. were generated by PCR using the following primers. [All substitution mutations contained within the sequence of these primers are indicated by underlining. The 5′ restriction site and sense (S) or antisense (AS) orientation is also indicated.] 716-711S: EcoR I (CAGGAATTCGATTGAGG); 607-616AS: Hind III (GATAAGCTTGATTC AGGTCTCC); 692-674S: EcoR I (GTCGAATT CTGCACGGAGCGGACGACACC); 667-655S: EcoR I (GTCGAATTCGGCGCCTCCCCCGC GCC); 627-642AS: Hind III (CTGAAGCTTTCT CCTCCACCTGCCC); 608AGAmS: (CAGGTC TCCACCCACCCGCTCACCTCCACCT G CCC TTC); 692mS: SpeI (TGGACTAGTTGCACGG AGCGACGACACCAAACACCTCCCC C G C G CC); 607mAS: Nde I (GAGCATATGTCAGG TCTCAACCATCACTCTCTC C T C C A CCTG); 692TTmS: Spe I (TGGACTAGTTGCACGGAG CTTACGACACCACCCCC); 668-685AS: Hind III (CTGAAGCTTGGGGTGGTGTCGTCCGC T); 631-607S: EcoR I (GTCGAATTCGGAGAG AGGGAGGGTGGAGACCTGAATC); 668-679-AS: Sac I (GCGGAGCTCGGGGGTGGTGT CG). All PCR mutants were cloned into pBluescript II KS+ to facilitate cloning into the β-actinLUC vector. For consistency, all constructs were transformed into DH5α *E. coli*. Experimental sets of plasmids were purified as a group using Qiagen (Qiagen, Inc., Chatsworth, CA) columns. The same set of DNA was then transfected into the HepG2 cell line for comparison.

Substitution of the G nucleotides at postion −681, −682 in the full-length rabbit ACE_P promoter was performed by PCR utilizing −1202ACE_PCAT (11) as the template and oligonucleotides S722-673TT: Mst II(TCCCGCCT GAGGACCCGCCCCATAGCCTCGTGCACGG AGCTTACGACACC) and AS600-616: Mst II-(AGTCGCCTCAGGTCTCC). The 105-bp N.E. in the rabbit ACE_P promoter was then replaced with the Mst II-digested PCR fragment. The nucleotide substitutions and orientation of the mutated N.E. was verified by sequencing. The −1202 wild-type and mutated rabbit ACE_P promoters were then cloned into the Nhe I-Hind III site of pGL2Basic vector to generate −1202ACE_PLUC and −1202ACE_PTTLUC, respectively. The promoter activity of both constructs was analyzed by transfection into ACE_P-expressing OPK cells.

Cell Culture

Non-ACE_P-expressing human hepatoma cells (HepG2) [ATCC# HB-8065] were cultured in α-MEM medium with 10% fetal bovine serum. ACE_P expressing bovine aortic endothelial cells (BAE) were a generous gift from the lab of Paul DiCorleto at the Cleveland Clinic Foundation. ACE_P expressing American opossum kidney (OPK) [ATCC#: CRL-1840] were cultured in MEM with Earle's BSS and 10% fetal bovine serum (11). Cells were continuously cultured in 175-cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were then cultured in 100-mm tissue culture plates for transfection studies or in 150-mm tissue culture plates for preparation of nuclear extracts.

DNA Transient Transfection Assays

The standard calcium phosphate-mediated, transfection method was utilized for introduction of the ACE_P N.E.-β-actinLUC expression vectors into HepG2 cells or −1202ACE_PLUC expression vectors into OPK cells (1). Cells were cultured in 100-mm tissue culture plates to 50% confluency. Ten micrograms of reporter construct DNA was mixed with 2 μg of rous sarcoma virus (RSV) β-galactosidase vector in the precipitate as indicated in the figure legend. The precipitate remained in contact with the cells for 16 h prior to a 2-min, 20% dimethyl sulfoxide (DMSO) shock. Following 24 h of growth in complete medium, the cells were harvested. Each assay was performed in triplicate with independently precipitated reporter constructs. All data reflect correction for transfection efficiency by normalizing with β-galactosidase activity.

Reporter Assays

Cell extracts were prepared 48 h after transfection by one freeze-thaw in the Luciferase lysis buffer (Promega). Cells were spun for 5 min at 14K in a microfuge at 4°C. The supernatant fraction was collected and the β-galactosidase activity measured as described previously (26). Cell extracts were assayed for Luciferase activity by following the procedure outlined in the Luciferase Kit (Promega). Briefly, 10 μl of cell extract (prepared in 1 × Luciferase Lysis Buffer) at room temperature were mixed with 50 μGl Luciferase Assay Reagent in a microtiter plate (Dynatech, Chantilly, VA). The plate was immediately read by a Dynatech ML 1000 luminometer.

Preparation of Cellular Nuclear Extracts

Nuclear extracts were prepared according to the protocol of Dignam et al. (7). The cell pellet was

suspended in 5 volumes of buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 10 mM dithiothreitol]. Following centrifugation, the pellet was suspended in 2 volumes of the packed cell pellet in buffer A. Cells were lysed with a type B pestle and centrifuged to remove residual cytoplasmic material. The pellet, which contained crude nuclei, was suspended in buffer C [20 mM HEPES (pH 7.9), 25% (v/v) 0.42 M NaCl, 1.5 M $MgCl_2$, 0.2 M EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM dithiothreitol]. An equal volume of buffer C was added to the suspension dropwise with constant stirring. The supernatant fraction collected by centrifugation was retained and the pellet was discarded. The supernatant was dialyzed twice against buffer D [20 mM HEPES (pH 7.9), 20% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM dithiothreitol] and clarified by centrifugation. The nuclear extract was stored in 50-μl aliquots at −70°C.

Electrophoretic Mobility Shift Assay

Binding assays were performed as previously described (30). The probes were prepared by annealing two oligonucleotides and filling in with the Klenow fragment of DNA polymerase. The −692 to −668 probe utilized the S692-674 and 668-685AS oligos whereas the −692 ($T^{-682}T^{-681}$) −668 probe utilized the 692TTmS and 668-679AS oligos. T4 polynucleotide kinase was used to end label the DNA probes with [γ-^{32}P]ATP. The only difference between these probes is the substitution of a T for the G nucleotides present at positions −682 and −681. In a total volume of 20 μl, 2 μg of nuclear extract or bovine serum albumin (BSA) was incubated for 15 min on ice in electrophoretic mobility shift assay (EMSA) binding buffer [final concentration 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 2 mM dithiothreitol, 50 mM NaCl]. All samples received 2 μg poly(dI-dC) and a 200 molar excess of the unlabeled competitor. The specific competitor was the unlabeled ACE_P N.E. −692 to −668 or the −692 ($T^{-682}T^{-681}$) −668 mutant fragment. The nonspecific competitor was a 40-bp nonhomologous DNA fragment. All competitors were added to the binding buffer containing poly(dI-dC) and the specified nuclear extract or BSA prior to the addition of the indicated probe. After 30 min of incubation with the probe on ice, the reaction mixture was loaded on a 4% nondenaturing polyacrylamide gel in Tris-acetate (TAE) buffer [6.7 mM Tris-Cl (pH 7.5), 3.3 mM sodium acetate, 1 mM EDTA]. The gel was dried and autoradiographed with an intensifying screen at −70°C.

DNase I Footprinting

DNase I footprinting of the 105-bp ACE_P N.E. was performed as described with the following modifications (1). Two N.E. probes, generated by labeling at the 5′ end of either the sense or the antisense strand with T4 polynucleotide kinase and [γ-^{32}P]ATP, were purified by elution from a nondenaturing polyacrylamide gel. Sixty micrograms of BAE nuclear extract protein or DNase-free BSA (Boehringer Mannheim) was added to 2.5 μg of poly(dI-dC) in 4°C binding buffer [final concentration 20 mM HEPES (pH 7.6), 100 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol] to achieve a total reaction volume of 50 μl. Each sample remained on ice for 15 min. The sense strand or antisense strand labeled probe (10,000 cpm) was added and the reaction continued on ice for an additional 45 min. All samples were then heated to 25°C for 1 min prior to the addition of 5 μl of 100 mM $MgCl_2$, 30 mM $CaCl_2$, and 0.3-3.0 units of DNase I (Boehringer Mannheim). The amount of DNase I added was determined previously by titration with both protein samples. A 45-s digestion of the protected probe at 25°C was terminated by the addition of 150 μl stop solution [150 mM NaCl, 0.7% sodium dodecyl sulfate (SDS), 15 mM EDTA, 30 mg/ml tRNA]. All samples were purified by phenol/chloroform extraction and ethanol precipitation. Dried samples were suspended in 8 μl of formamide loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) (U.S. Biochemical). Equal quantities of radioactive counts of each sample were resolved on a 6% denaturing polyacrylamide gel containing 7 M urea in 1 × Tris borate (TBE) buffer. The gel was dried and exposed to X-ray film. The DNA sequence ladder corresponding to each probe was prepared by following the Maxam and Gilbert short protocol as described (26).

UV Cross-Linking

Cross-linking of the ACE_P −692 to −668 element to nuclear protein extracts was performed as described (1). A BrdU-substituted ^{32}P-labeled probe was prepared for this purpose. The deletion fragment was cloned into the EcoR I and Hind III sites of pBluescript II KS+ (Stratagene). The oligonucleotides 692-674S and 668-685AS were annealed and filled in with the Klenow fragment of DNA polymerase. The resulting plasmid was

restricted with Hind III followed by alkali denaturation. The T7 primer was annealed to the denatured DNA. The Klenow fragment of DNA polymerase was used to fill in the plasmid substituting BrdUTP for dTTP and [α-^{32}P]dGTP for dGTP. Following polymerization, the substituted plasmid was restricted with Sma I. The probe was then excised from a nondenaturing polyacrylamide gel and eluted in 50 mM NaCl.

The cross-linking reaction was performed in a total volume of 15 μl. Each reaction contained 2 μg of HepG2 nuclear extract or 4 μg of BAE nuclear extract or 4 μg BSA in EMSA binding buffer to which 5 μg poly(dI-dC) had been added. Where indicated, samples contained 200 molar excess of either unlabeled -692 to -668 specific competitor or a nonhomologous 40-bp nonspecific DNA competitor. All samples were placed in a Microwell® minitray (Nunc, Inc., Naperville, IL) on ice for 15 min prior to receiving the -692 to -668 probe. The samples remained on ice during the 30-min binding reaction and during exposure to 1.5 J in a Stratalinker® UV Crosslinker 2400 (Stratagene, LaJolla, CA) at a distance of 3 cm from the UV source. The amount of UV required for cross-linking was determined by titration with the N.E. -692 to -668 probe. All samples were resolved on a 10% SDS-polyacrylamide gel, dried, and exposed to film.

RESULTS

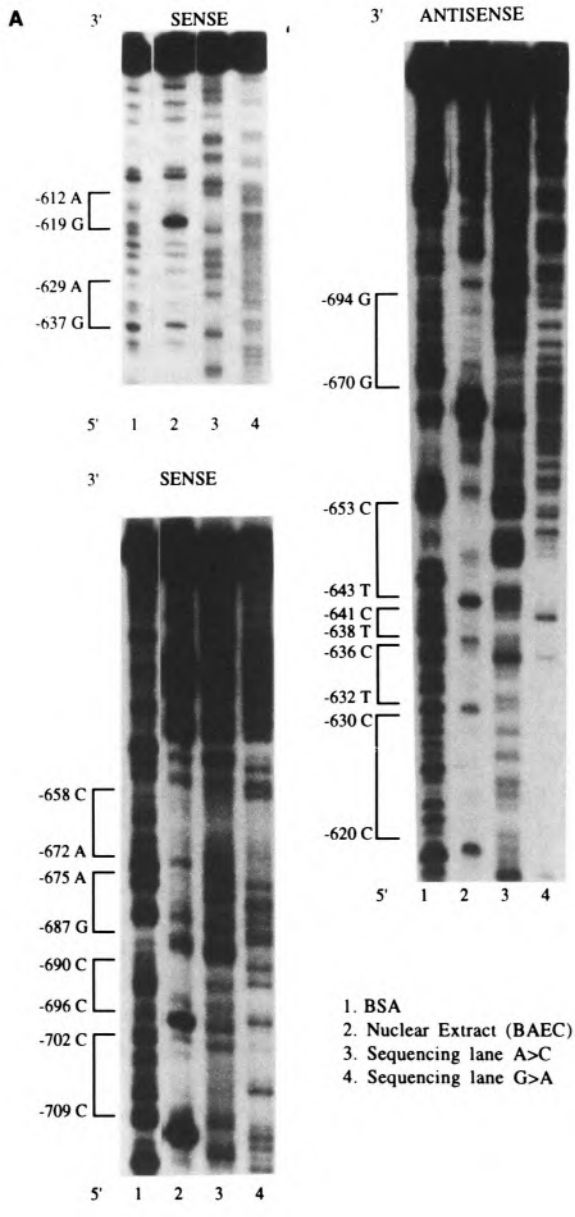
DNase Footprinting of the Negative Element

The sequence of the rabbit ACE gene negative element (N.E.), located between -715 and -610 with respect to the transcription initiation site (+1) of ACEp mRNA, is shown in Fig. 1C. It contains several interesting structural motifs: a SP1 binding site is present at -709 and an almost perfect inverted repeat sequence is present at -674 and -626. Moreover, there are two motifs that have resemblance to two known transcriptional silencers. A motif present at -690 has partial homology with a silencer present in synapsin, type II sodium channel, and other neuron-specific genes whereas another motif present at -634 has partial homology with a silencer element present in the collagen II gene (6,18,21,28,36).

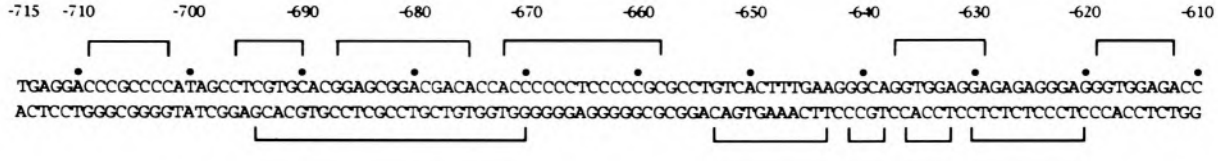
For evaluating the role of the above motifs in the transcriptional-repressing effects of the ACEp N.E., we first determined the specific regions in N.E. that interact with proteins present in the nuclear extract of BAE cells. These cells express ACEp and the N.E. is known to be functional in them (11). DNase I footprinting analysis was done on the N.E. that had been radiolabeled at the 5′ end of the sense or the antisense strand and incubated with the nuclear extract before DNase I digestion. As shown in Fig. 1A and B, six regions were protected from DNase I digestion on the sense strand and five regions were protected on the antisense strand. The two major regions protected on both strands encompassed -694 to -670 and -636 to -629 residues. These two protected regions overlap with the two silencer-like sequences present in the N.E. The same footprinting pattern was also observed when a nuclear extract of HepG2 cells, which do not express the ACE gene, was used instead of BAE cell extract (data not shown).

Functional Dissection of the N.E.

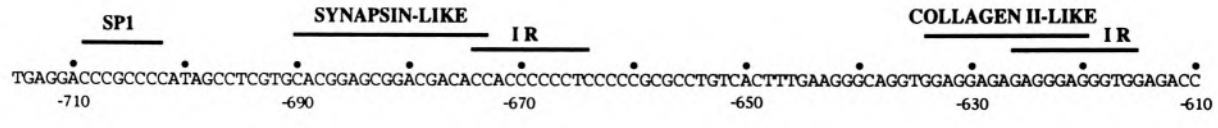
Nucleotide sequence comparison and the results of the footprinting experiments (Fig. 1) suggested that several regions within the N.E. might be important in mediating its transcriptional-repressing function. We decided to test their respective contributions by mutating them individually and testing the function of the mutated N.E.s. For this purpose, wild-type and mutated N.E.s were cloned at the 3′ end of a β-actin promoter-driven luciferase gene in an expression vector. We decided to use the β-actin promoter because it is much stronger than the cognate ACE_P promoter and it is expressed in all cell types. Because the footprinting experiments showed that HepG2 cells contain the N.E. binding proteins, we used these cells as recipients of the transfected test genes. The N.E. consistently inhibited the expression of the luciferase gene (Fig. 2). The mean degree of inhibition was 36% (Construct 1). Deletion of 715–693, the region containing the SP1 site, did not affect the function of the N.E. (Construct 2). Thus, as our previous results suggested, the SP1 site is irrelevant for the repressing activity of this element (11). Further deletion from the upstream end, however, almost completely destroyed the activity of the N.E. A truncated N.E. containing -668 to -610 inhibited transcription by only 4% (Construct 3). On the other hand, deletion of -627 to -610 from the N.E. only partially reduced its activity (Construct 4). These results indicated that a crucial motif might be present in the -692 to -668 region. The potential contribution of the inverted repeats was tested by mutating them. Residues -616, -620, and -623 and residues -68, -670, and -671 were mutated separately (Construct 5) or in combination (Construct


A
3' SENSE
-612 A
-619 G
-629 A
-637 G
5' 1 2 3 4
3' ANTISENSE
-694 G
-670 G
-653 C
-643 T
-641 C
-638 T
-636 C
-632 T
-630 C
-620 C
5' 1 2 3 4
3' SENSE
-658 C
-672 A
-675 A
-687 G
-690 C
-696 C
-702 C
-709 C
5' 1 2 3 4
1. BSA
2. Nuclear Extract (BAEC)
3. Sequencing lane A>C
4. Sequencing lane G>A

B


-715 -710 -700 -690 -680 -670 -660 -650 -640 -630 -620 -610
TGAGGACCCGCCCCATAGCCTCGTGCACGGAGCGGACGACACCACCCCCCTCCCCCGCGCCTGTCACTTTGAAGGGCAGGTGGAGGAGAGAGGGAGGGTGGAGACC
ACTCCTGGGCGGGGTATCGGAGCACGTGCCTCGCCTGCTGTGGTGGGGGGAGGGGGCGCGGACAGTGAAACTTCCCGTCCACCTCCTCTCTCCCTCCCACCTCTGG

C


SP1
SYNAPSIN-LIKE
I R
COLLAGEN II-LIKE
I R
TGAGGACCCGCCCCATAGCCTCGTGCACGGAGCGGACGACACCACCCCCCTCCCCCGCGCCTGTCACTTTGAAGGGCAGGTGGAGGAGAGAGGGAGGGTGGAGACC
-710 -690 -670 -650 -630 -610

6). These mutations destroyed the sequence homology of the repeat elements, but did not affect the function of the N.E. For testing the contribution of the collagen II silencer-like sequence, similar mutations were performed. The A at −621 was mutated to T, A at −625 was mutated to C, and A at −629 was mutated to T (Construct 7). The mutated A residues are implicated in the functioning of the collagen II silencer, and their mutations considerably reduced the homology between this region of N.E. and the collagen II silencer (28). The above mutations did not affect the functioning of the ACE N.E., thus indicating that the collagen II silencer-like sequence has no contribution. Finally, the potential contribution of the synapsin silencer-like sequence was tested by mutating the two G residues at −681 and −682 to T (Construct 8), because such mutations are known to destroy the activity of the synapsin silencer (18). The above mutations considerably reduced the activity of the N.E. (Fig. 2), indicating that the mutated residues play an important role.

Because our deletion analysis showed that sequences present near both ends of the N.E. are important for its function, in the next series of experiments we investigated if these regions by themselves can repress transcription (Fig. 3). For these experiments, two regions encompassing residues −692 to −668 and −631 to −610 were cloned in single or duplicate copies in the test vector. In another construct, single copies of both ends were ligated together. Two copies of the left end were as efficient as the N.E. (Construct 3 vs. 1), but a single copy was nonfunctional (Construct 2). Two copies of the right end had an intermediate level of activity (Construct 5), as did the ligated left and right end fragment (Construct 6). These experiments demonstrated that the most important determinant of the N.E.s repressing activity lies at the left end (−692 to −668), a region that also contains the synapsin silencer-like sequence. Because two copies of this region could repress transcription as efficiently as the N.E., further investigation was carried out with this −692 to −668 ACEp silencer element.

In the next experiments, we tested whether the silencer can function in an ACE_P-expressing kidney cell line, OPK. As shown in Fig. 4, two copies of the silencer repressed β-actin luciferase expression by about 45% in the OPK cells. Thus, the silencer functions in both ACE_P-expressing and -nonexpressing cell lines.

Function of the Silencer in its Native Context

The above results indicated that the silencer element, as present in the ACE gene at −692 to −668 position, may regulate the level of expression of the ACE_P transcription unit. To test this hypothesis directly, we measured the expression level of a reporter luciferase-gene driven by the upstream region (−1202 to +29) of the rabbit ACE gene. Because the ACE_P promoter functions only in the ACE_P-producing cells (11), we had to use a kidney cell line for this experiment. For testing the role of the silencer element in its native context, the two G residues at −681 and −682 were mutated to T. As shown in Fig. 2, these mutations diminished the effects of the silencer element when tested in the context of the heterologous β-actin promoter. The same was true for the ACE_P promoter (Fig. 5). Mutating the two specific G residues among the 1202 residues of the ACE_P promoter increased its ability to promote transcription by 2.4-fold. This experiment established the physiological relevance of the silencer element.

Interaction of ACEp Silencer With Nuclear Proteins

We have previously reported that three specific protein/DNA complexes are detected by EMSA when the whole N.E. is incubated with BAE nuclear extract (11). Similar experiments were performed using the ACEp silencer (−692 to −668) (Fig. 6). Only one prominent complex was detected both in HepG2 (lane 1) and BAE (lane 5) nuclear extracts. A close examination of this complex suggests that it may contain a doublet, which was better separated in lanes 9 and 11 of Fig. 6B. The appearance of two closely migrating com-

FIG. 1. (A) DNase I footprinting was performed with the −715 to −610 rabbit ACE_P N.E. and BAE nuclear extract. Protected regions are indicated with both brackets and nucleotide location with respect to the start of transcription of the rabbit ACE_P gene. Footprinting was performed on 5′ end labeled sense and anitsense strands. Lane 1: DNase-free BSA; lane 2: BAE nuclear extract; lane 3: Maxam and Gilbert sequencing ladder (A > C); lane 4: Maxam and Gilbert sequencing ladder (G > A). (B) Graphic illustration of protected regions observed in (A). The two regions protected on both sense and antisense strands are −694 to −670 and −636 to −629. (C) The MacVector transcription factor sequence data bank and Microgenie DNA analysis program were used to identify putative structural motifs within the −715 to −610 rabbit ACE_P N.E. The indicated sites are: SP1 (−709 to −702), synapsin (−690 to −673), collagen II (−634 to −620). The near perfect inverted repeat (IR) sequences are −674 to −664 and −626 to −615.

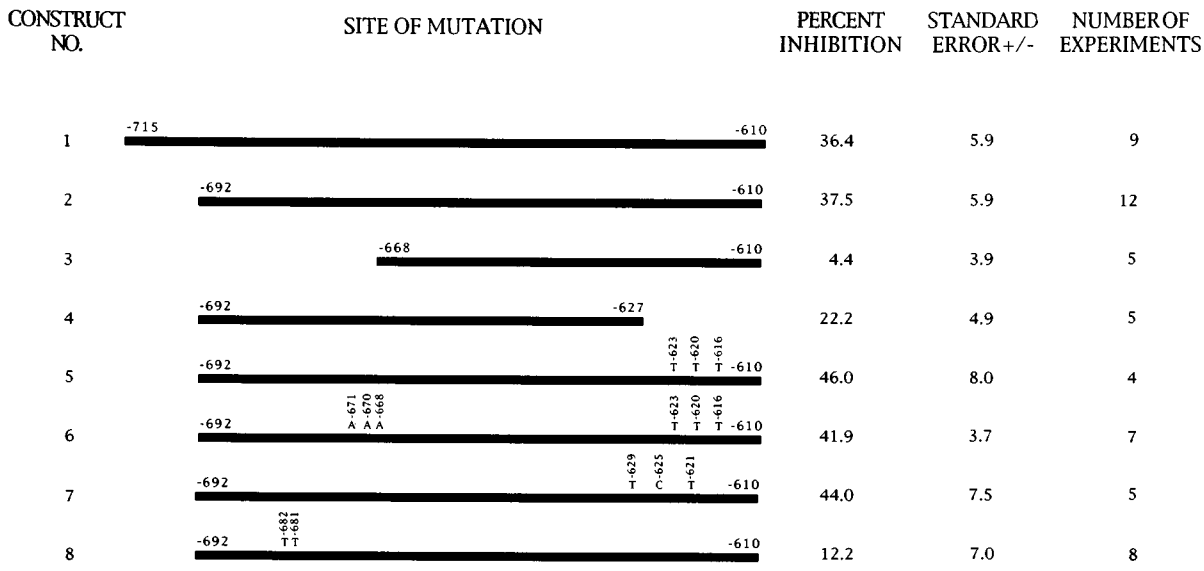


FIG. 2. Effects of mutagenesis of the ACE_P N.E. PCR was performed with oligonucleotides and the ACE_P N.E. in pBluescript II KS+ (SK394) to generate ACE_P N.E. deletion or substitution mutants. The specific nucleotide substitutions and the boundaries of the deletion mutants are both indicated above the wild-type bar. The wild-type N.E. and all mutants were cloned into the blunted BamH I site of the β-actinLUC reporter plasmid. Each reporter construct was transfected into HepG2 cells along with a RSV-β-gal gene, which served as the internal control for normalizing transfection efficiencies. Both luciferase and β-galactosidase activities of each extract of the transfected cells were measured and the luciferase activities of different cell extracts were normalized using the corresponding β-galactosidase activity values. Normalized luciferase activity of each transfectant was compared to that of cells transfected with the β-actinLUC gene without any N.E. The percent inhibition of normalized luciferase activity by the wild-type and mutant N.E.s is presented. The column labeled Number of Experiments is the number of independent transfections performed with the specific constructs using different lots of plasmid DNAs. Standard error was calculated with the Microsoft® Excel program, which divides the standard deviation of the mean by the square root of the sample size.

plexes could be due to differential posttranslational modifications of the same protein present in them. The complex was specific; unlabeled probe competed it out (lanes 2 and 6), but the same amount of a nonspecific DNA did not (lanes 3 and 7). The −682 and −681 mutations, which negatively affected the repressing function (Fig. 2), also affected the formation of the specific DNA/protein complex, as shown in Fig. 6B and C. Unlabeled mutated silencer failed to compete

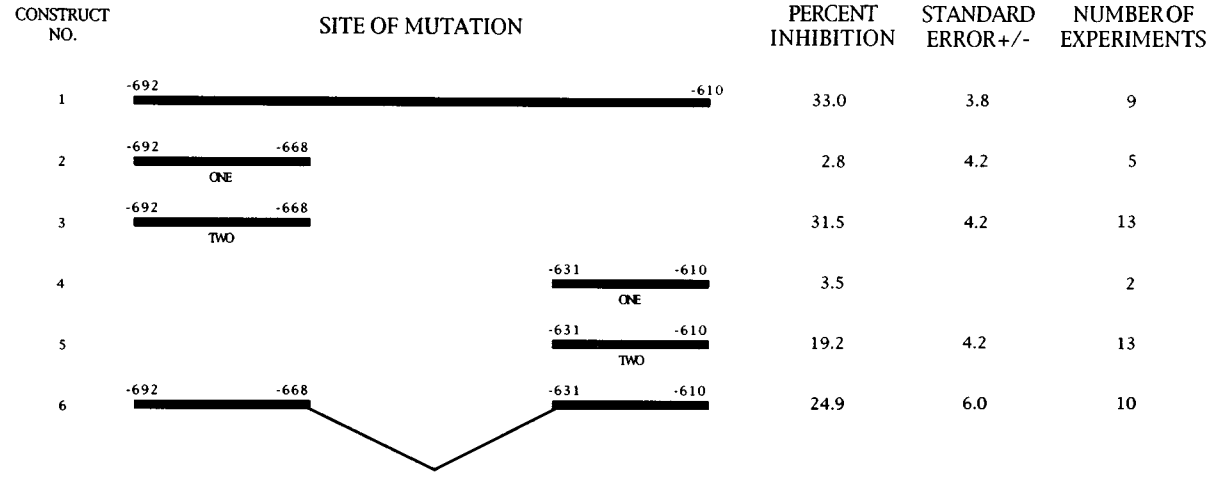


FIG. 3. Repression of the β-actin promoter by the ACE_P −692 to −668 and −631 to −610 N.E. fragment. HepG2 cells were transfected with the β-actinLUC constructs containing the ACE_P N.E. or the indicated copies of ACE_P N.E. deletion fragments −692 to −668 or −631 to −610. When two copies of a fragment were used, they were ligated head to tail. Other procedures were as described in the legend for Fig. 2.

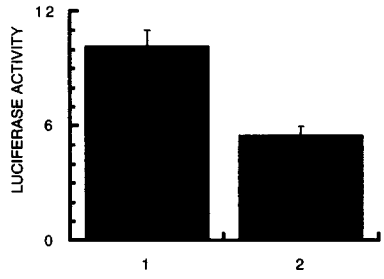


FIG. 4. Repression of β-actin promoter by the ACE_P silencer in OPK cells. The β-actin luciferase expression vector with (bar 2) or without (bar 1) two copies of the ACE_P silencer (−692 to −668) was transfected into ACE_P-expressing OPK cells. Luciferase values are means of three independent transfections normalized with β-galactosidase activity.

with the wild-type silencer (lane 11), and radiolabeled mutated silencer could not form the complex (lane 13). These results suggest that the specific DNA/protein complex detected by EMSA is involved in the silencer's functional activity.

To obtain further insight into the nature of the protein present in this complex, UV cross-linking studies were undertaken. BrdUTP-substituted radiolabeled silencer was cross-linked in solution with proteins present in the nuclear extracts of HepG2 and BAE cells, and the DNA cross-linked proteins were separated by denaturing gel electrophoresis (Fig. 7). One prominent radiolabeled band was observed in both cell extracts (lanes 2 and 5). Incubation with only bovine serum albumin did not form this complex (lane 1). An excess of the unlabeled probe (lanes 3 and 6), but not an unrelated DNA (lanes 4 and 7), blocked the cross-linked complex formation. Thus, the formation of the cross-linked complex was highly specific. The observed broad band had a mean apparent molecular weight of 50 kDa. The heterogeneity

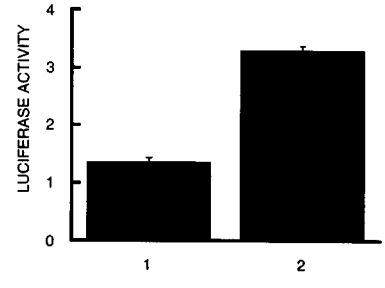


FIG. 5. Effect of silencer mutations in the context of the ACE_P promoter. OPK cells were transfected with 10 μg of −1202ACE_PLUC (bar 1) or −1202ACE_PTTLUC (bar 2) and 2 μg RSV-β-gal. All luciferase values represent means of four independent transfections normalized with β-galactosidase activity.

of the band could be due to the presence of DNA in the complex or due to the presence of a closely migrating doublet. Two other minor, but specific, DNA/protein complexes of apparent molecular weights of 67 and 29 kDa were also observed with both cell extracts. However, their intensities were much less than that of the 50-kDa DNA/protein complex. These results strongly suggest that the protein present in the 50-kDa complex could be a repressor of transcription. Molnar et al. (20) have demonstrated that a fairly accurate estimate of the molecular weight of the protein present in such a DNA/protein complex can be obtained by subtracting the molecular weight of the DNA probe from the apparent molecular weight of the complex. Followiing this method, after adjusting for the size of the DNA probe, we estimate the approximate molecular mass of the protein component of this 50-kDa complex to be 21.5 kDa.

DISCUSSION

The pulmonary isozyme of ACE (ACE_P) is expressed in only a selective set of cells such as vascular endothelial cells and renal proximal tubular epithelial cells (2,5,9,10,23). Even in these cells, the level of ACE gene transcription is low as judged by the steady-state levels of ACE mRNA and by the level of expression of transfected reporter genes driven by the ACE_P promoter (11,16,25). Our finding that this promoter contains a transcriptional negative element suggested the possibility that the low level of expression of ACE_P mRNA is mediated through this element. To be able to examine this putative regulatory mechanism(s), we undertook the current study of characterizing the negative element rigorously.

The first new information obtained from the present investigation is that the negative element can function in ACE_P-nonexpressing cell lines as well. The negative element gave similar footprinting patterns with nuclear extracts of both ACE-expressing and -nonexpressing cells, suggesting that the same binding proteins exist in both cell types. The regions protected from DNase digestion were spread over the entire length of N.E., although there were only two areas, centered around −680 and −635, which were protected on both strands of DNA (Fig. 1A, B). These data, along with the nucleotide sequence comparison information (Fig. 1C), suggested the existence of several domains that might be important for the N.E.'s function. We used transfection analysis of

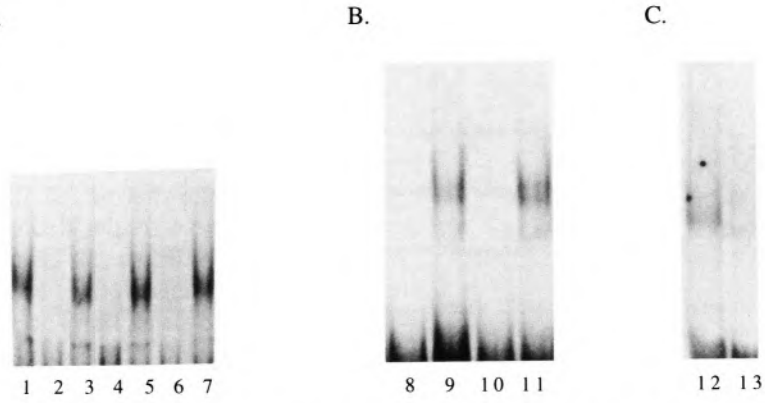


FIG. 6. Electrophoretic mobility shift assay of wild-type and mutant ACE_P silencers. EMSA was performed with both the wild-type -692 to -668 ACE_P probe and the mutated -692 ($T^{-682}T^{-681}$) -668 ACE_P probe. Samples were resolved on a 4% nondenaturing polyacrylamide gel. (A) The probe in all lanes is the -692 to -668 wild-type ACE_P silencer. Lanes 1–3 contain HepG2 extract; lane 4 contains BSA; lanes 5–7 contain BAE extract. Lanes 2 and 6 contain 200-fold molar excess of unlabeled probe and lanes 3 and 7 contain 200-fold molar excess of a 40-bp unrelated DNA. (B) The probe is the same as in (A). Lane 8 contains BSA and lanes 9–11 contain HepG2 extract. Lane 10 contains 200-fold molar excess of unlabeled probe and lane 11 contains the same amount of the mutated silencer. (C) Lanes 12 and 13 contain HepG2 extract. The probe in lane 12 is radiolabeled wild-type ACE_P silencer whereas the probe in lane 13 is radiolabeled mutated ACE_P silencer.

mutated N.E.s for testing the putative role of each of these domains.

As suggested by our previous study, the SP1 site present at the distal end of the N.E. was proven to be dispensable for its function (11). The almost perfect inverted repeats could also be mutated without a loss of the N.E.'s activity. Sequences present between -620 and -635, which partially resemble silencer sequences present in the collagen II gene and the c-*myc* gene, seem to have a minor contribution to the ACE N.E.'s activity (14,19,28). In contrast, sequences around -680 proved to be the major contributor. Mutations of two G residues in this region considerably reduced the N.E.'s activity. This region has partial homology with silencer sequences present in several neuronal-specific genes, and similar mutations are known to perturb their functions (18). Thus, site-directed mutagenesis of the N.E. identified only one functionally important region.

Deletion mapping of the N.E. confirmed that a functionally important motif is present within -692 and -668. This motif, called the ACE silencer, could by itself repress expression of the reporter gene. For manifestation of this activity, however, two copies of the silencer were required. Such a requirement of multiple copies is quite common and well documented for other transcriptional regulatory elements. When enhancers or silencers are tested by transfection analysis of the kind used here, more than one copy of the element is often needed for manifesting reproducibly large effects. The same elements, however, function adequately in single copies in the natural context of the genes where presumably they function in concert with other elements present in the neighboring region (3). Our experiments suggest that the ACE N.E. may have such an ancillary element at the proximal end between -631 and -610. Deletion of this region reduced N.E.'s activity (Fig. 2), and two copies of this region, without the rest of N.E., had substantial activity (Fig. 3). Thus, it is conceivable that in its natural context, the distal silencer element acts in concert with the element present at the proximal end of ACE N.E. Indeed, when these two elements were directly ligated in single copies, without the intervening sequence, substantial repressor activity was reconstituted (Fig. 3).

Because the silencer element was identified by using the heterologous β-actin promoter, it was important to establish its significance in the natural context. We have previously shown that deletion of the N.E., which includes the silencer element, greatly enhances the transcriptional activity of the upstream region of the ACE_P transcription unit (11). That the silencer is an important player in this transcriptional regulation was shown in the current study by introducing two point mutations in the ACE_P promoter that destroyed the functioning of the silencer. The resultant upregulation of the ACE_P promoter activity was 2.4-fold, which

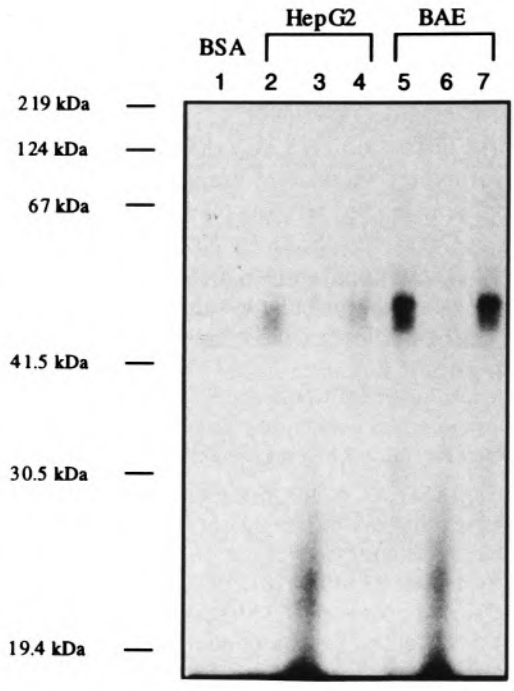


FIG. 7. UV cross-linking of the ACE_P silencer. The −692 to −668 ACE_P silencer probe was incubated on ice with either BSA, HepG2, or BAE nuclear extract and the indicated competitor prior to exposure to 1.5 J of UV light in a Stratalinker® UV Crosslinker 2400. All samples were then resolved on a denaturing 10% SDS-polyacrylamide gel. The competitors, present at 200-fold molar excess, include unlabeled probe (lanes 3 and 6) or nonhomologous double-stranded DNA fragment (lanes 4 and 7). The position of molecular weight markers is indicated on the left.

```
NEURONAL (consensus)     tTcagcacc GCGGAC agtgCC

                        -695                  -669
RABBIT ACEp              CGTGCACGGA GCGGAC GACACCACCCC

                        -912                  -893
HUMAN ACEp               gtcaCtttGA G-GGAC acgtggAtgag

                        -853                  -834
MOUSE  ACEp              gtcaCtttGA G-GGAC GggtggAggag
```

FIG. 8. Homology among the rabbit ACE_P silencer, neuronal silencers, and other ACE_P genes. The Microgenie DNA analysis computer program was used to locate regions in the upstream sequences of the mouse and human ACE_P genes, which are homologous to both the rabbit (−692 to −668) ACE_P silencer and the neuronal silencer consensus sequence. The neuronal silencer consensus sequence was derived by comparison of the silencer sequences identified in the type II sodium channel gene, the SCG10 gene, and both synapsin H-255 and synapsin R-259 genes (21). All nucleotides that are homologous to the rabbit ACE_P sequence are capitalized. The ACE_P sequences that share homology around the neuronal silencer core GG nucleotides are boldface type.

is highly significant in the physiological context. Moreover, the effect of the silencer was much more pronounced on the weak ACE_P promoter than on the strong β-actin promoter, probably because of the inherent difference in their promoter strengths. It remains to be tested whether, in addition to this silencer, another element, the promoter proximal element present between −631 and −610 (Fig. 3), also contributes to the physiological regulation of the ACE_P mRNA transcription.

The rabbit ACE_P silencer identified in this study has partial homology with similar regions present in the human and the murine ACE genes (Fig. 8). The maximum homology is in the central domain. This domain is also conserved in the neuronal silencer element found in several genes specifically expressed in neuronal cells. The neuronal element is, however, quite distinct from the ACE silencer. Its overall sequence is highly conserved among neuronal genes encoding synapsin, type II Na channel, SCG10 protein, and dopamine β-hydroxylase (13,15,21). The sequence homology with the ACE silencer, on the other hand, is restricted to the central region. The functioning of both the ACE and the neuronal elements, however, requires the two G residues present in this homologous region. It is therefore conceivable that, although distinct, the two elements may function through similar mechanisms. The nonidentity of the two elements is also manifested by the nature of the proteins that bind to them. Both human and bovine ACE silencer binding proteins have an apparent molecular mass of 21.5 kDa. In contrast, the neuronal silencer binding protein has a molecular mass of 121 kDa (6). The latter protein has recently been cloned and shown to have multiple zinc finger domains (6,29). Further studies will be needed to determine whether the ACE silencer binding protein belongs to the same family of zinc finger proteins.

The tissue-specific expression of the genes that contain the neuronal silence element is maintained due to the absence of the corresponding binding protein in neuronal cells (6). This, however, does not seem to be the case for the ACE silencer. Because the ACE silencer sequence functions in both ACE-expressing and -nonexpressing cells and the corresponding binding protein is also present in both cell types, this element, by itself, cannot be the major determinant of tissue-specific expression of ACE_P. However, it may act as a general downregulator of ACE_P transcription in all tissues. As a result, ACE_P transcription is kept low even in ACE-expressing tissues. It is conceivable that the observed enhancement of ACE expression in such tissue, in response to various physiological stimuli such as hormones, hemodynamic pressure, or tissue injury, is mediated by regulating the action of the silencer binding protein either at the

level of its DNA interaction or at the level of its interaction with other proteins of the general transcriptional machinery. Purification of the ACE silencer binding protein and generation of suitable reagents, such as antibodies, will be necessary for studying these putative regulatory processes.

ACKNOWLEDGEMENTS

We thank Paul DiCorleto for providing the BAE cells and Patricia McGuire, Austin Gurney, Poornima Janaswami, and Eleanora Scarpati for technical advice. We thank Paul T. Stanton for expert technical assistance. We thank the past and present members of the G. C. Sen laboratory for helpful discussion. We also thank Dorthy Herzberg for expert manuscript preparation. This work was supported in part by National Institutes of Health Grant HL-48258, by National Institutes of Health Training Grant DK-07678 (to S.P.K. and T.Y.G.) and Research Fellowship from the Northeast Ohio Affiliate of the American Heart Association (to S.P.K.).

REFERENCES

1. Ausebel, F. M.; Brent, R.; Kingston, R. E.; Moore, D. D.; Seidman, J. G.; Smith, J. A.; Struhl, K. Current protocols in molecular biology. Vol. 1 and 2. New York: John Wiley and Sons; 1989.
2. Berg, T.; Solner, J.; Lai, C. Y.; Soffer, R. L. Immunohistochemical localization of two angiotensin I-converting enzymes in the reproductive tract of the male rabbit. J. Histochem. Cytochem. 34:753–760; 1986.
3. Berk, A. J.; Schmidt, M. C. How do transcription factors work? Genes Dev. 4:151–155; 1990.
4. Bernstein, K. E.; Martin, B. M.; Edwards, A. S.; Bernstein, E. A. Mouse angiotensin-converting enzyme is a protein composed of two homologous domains. J. Biol. Chem. 264:11945–11951; 1989.
5. Caldwell, P. R. B.; Seegal, B. C.; Hsu, K. C.; Das, M.; Soffer, R. L. Angiotensin-converting enzyme: Vascular endothelial localization. Science 191:1050–1051; 1976.
6. Chong, J. A.; Tapia-Ramirez, J.; Kim, S.; Toledo-Aral, J. J.; Zheng, Y.; Boutros, M. C.; Altshuller, Y. M.; Frohman, M. A.; Kraner, S. D.; Mandel, G. REST: A mammalian silencer protein that restricts sodium channel gene expression to neurons. Cell 80: 949–957; 1995.
7. Dignam, J. D.; Lebovitz, R. M.; Roeder, R. G. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 11:1475–1489; 1983.
8. Ehlers, M. R.; Fox, E. A.; Strydom, D. J.; Riordan, J. F. Molecular cloning of human testicular angiotensin-converting enzyme: The testis isozyme is identical to the C-terminal half of endothelial angiotensin-converting enzyme. Proc. Natl. Acad. Sci. USA 86:7741–7745; 1989.
9. El-Dorry, H. A.; Pickett, C. B.; MacGregor, J. S.; Soffer, R. L. Tissue-specific expression of mRNAs for dipeptidyl carboxypeptidase isozymes. Proc. Natl. Acad. Sci. USA 79:4295–4297; 1982.
10. Friedland, J.; Setton, C.; Silverstein, E. Induction of angiotensin-converting enzyme in human monocytes in culture. Biochem. Biophys. Res. Commun. 83:843–849; 1978.
11. Goraya, T. Y.; Kessler, S. P.; Kumar, R. S.; Douglas, J.; Sen, G. C. Identification of positive and negative transcriptional regulatory elements of the rabbit angiotensin-converting enzyme gene. Nucleic Acids Res. 22:1194–1201; 1994.
12. Goto, M.; Tamura, T.; Mikoshiba, K.; Masamune, Y.; Nakanishi, Y. Transcription inhibition of the somatic-type phosphoglycerate kinase I gene in vitro by a testis-specific factor that recognizes a sequence similar to the binding site for Ets oncoproteins. Nucleic Acids Res. 19:3959–3963; 1991.
13. Ishiguro, H.; Kim, K. T.; Joh, T. H.; Kim, K.-S. Neuron-specific expression of the human dopamine β-hydroxylase gene requires both the cAMP-response element and a silencer region. J. Biol. Chem. 268:17987–17994; 1993.
14. Kakkis, E.; Riggs, K. J.; Gillespie, W.; Calame, K. A transcriptional repressor of c-myc. Nature 339: 718–721; 1989.
15. Kraner, S. D.; Chong, J. A.; Tsay, H.-J.; Mandel, G. Silencing the type II sodium channel gene: A model for neural-specific gene regulation. Neuron 9:37–44; 1992.
16. Kumar, R. S.; Kusari, J.; Roy, S. N.; Soffer, R. L.; Sen, G. C. Structure of testicular angiotensin-converting enzyme. J. Biol. Chem. 264:16754–16758; 1989.
17. Kumar, R. S.; Thekkumkara, T. J.; Sen, G. C. The mRNAs encoding the two angiotensin-converting isozymes are transcribed from the same gene by a tissue-specific choice of alternative transcription initiation sites. J. Biol. Chem. 266:3854–3862; 1991.
18. Li, L.; Suzuki, T.; Mori, N.; Greengard, P. Identification of a functional silencer element involved in neuron-specific expression of the synapsin I gene. Proc. Natl. Acad. Sci. USA 90:1460–1464; 1993.
19. Lobanenkov, V. V.; Nicolas, R. H.; Alder, V. V.; Paterson, H.; Klenova, E. M.; Polotskaja, A. V.; Goodwin, G. H. A novel sequence-specific DNA binding protein which interacts with three regularly spaced direct repeats of the CCCCTC-motif in the 5′-flanking sequence of the chicken c-myc gene. Oncogene 5:1743–1753; 1990.
20. Molnar, G.; O'Leary, N.; Pardee, A. B.; Bradley, D. W. Quantification of DNA–protein interaction

by UV crosslinking. Nucleic Acids Res. 23:3318–3326; 1995.
21. Mori, N.; Schoenherr, C.; Vandenbergh, D. J.; Anderson, D. J. A common silencer element in the SCG10 and type II Na^+ channel genes binds a factor present in nonneuronal cells but not in neuronal cells. Neuron 9:45–54; 1992.
22. Ng, S.; Gunning, P.; Eddy, R.; Ponte, P.; Leavitt, J.; Shows, T.; Kedes, L. Evolution of the functional human β-actin gene and its multi-pseudogene family: Conservation of noncoding regions and chromosomal dispersion of pseudogenes. Mol. Cell. Biol. 5:2720–2732; 1985.
23. Patchett, A. A.; Cordes, E. H. The design and properties of N-carboxyalkyldipeptide inhibitors of angiotensin-converting enzymes. Adv. Enzymol. 57:1–84; 1985.
24. Queen, C.; Korn, L. J. Molecular cloning and sequence analysis of the human parainfluenza 3 virus mRNA encoding the P and C proteins. Nucleic Acids Res. 12:581–599; 1984.
25. Roy, S. N.; Kusari, J.; Soffer, R. L.; Lai, C. Y.; Sen, G. C. Isolation of cDNA clones of rabbit angiotensin converting enzyme: Identification of two distinct mRNAs for the pulmonary and the testicular isozymes. Biochem. Biophys. Res. Commun. 155:678–684; 1988.
26. Sambrook, J.; Fritsch, E. F.; Maniatis, T. Molecular cloning: A laboratory manual. Vol. 1, 2, and 3. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1989.
27. Sartorelli, V.; Webster, K. A.; Kedes, L. Muscle-specific expression of the cardiac alpha-actin gene requires MyoD1, CArG-box binding factor, and SP1. Genes Dev. 4:1811–1822; 1990.
28. Savagner, P.; Miyashita, T.; Yamada, Y. Two silencers regulate the tissue-specific expression of the collagen II gene. J. Biol. Chem. 265:6669–6674; 1990.
29. Schoenherr, C. J.; Anderson, D. J. The neuron-restrictive silencer factor (NRSF): A coordinate repressor of multiple neuron-specific genes. Science 267:1360–1363; 1995.
30. Sen, R.; Baltimore, D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell 46:705–716; 1986.
31. Shai, S. Y.; Langford, K. G.; Martin, B. M.; Bernstein, K. E. Genomic DNA 5′ to the mouse and human angiotensin-converting enzyme genes contains two distinct regions of conserved sequence. Biochem. Biophys. Res. Commun. 167:1128–1133; 1990.
32. Soffer, R. L. Biochemical regulation of blood pressure. In: Soffer, R. L., ed. Biochemical regulation of blood pressure. New York: John Wiley & Sons; 1981:123–164.
33. Soubrier, F.; Alhenc-Glass, F.; Hubert, C.; Allengrini, J.; John, M.; Tregear, G.; Corvol, P. Two putative active centers in human angiotensin I-converting enzyme revealed by molecular cloning. Proc. Natl. Acad. Sci. USA 85:9386–9390; 1988.
34. Thekkumkara, T. J.; Livingston, W.; Kumar, R. S.; Sen, G. C. Use of alternative polyadenylation sites for tissue-specific transcription of two angiotensin-converting enzyme mRNAs. Nucleic Acids Res. 20: 683–687; 1992.
35. Steiner, C.; Muller, M.; Baniahmad, A.; Renkawitz, R.; Lysozyme gene activity in chicken macrophages is controlled by positive and negative regulatory elements. Nucleic Acids Res. 15:4163–4178; 1987.
36. Thiel, G.; Schoch, S.; Petersohn, D. Regulation of synapsin I gene expression by the zinc finger transcription factor zif268/egr-1. J. Biol. Chem. 269: 15294–15301; 1994.