

Identification of positive and negative transcriptional regulatory elements of the rabbit angiotensin-converting enzyme gene

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ABSTRACT

The two tissue-specific mRNAs encoding the isozymes of rabbit angiotensin-converting enzyme (ACE) are generated from the same gene by alternative choice of two transcription initiation sites 5.7 kb apart. In the current study, we have characterized the regulatory sites controlling the transcription of the larger pulmonary isozyme mRNA. For this purpose, reporter genes driven by varying lengths of upstream region of the ACE gene were transfected into ACE-producing cells. Our results demonstrated that the transcription of this gene is primarily driven by positive elements within the first 274 bp DNA upstream of the transcription initiation site. The reporter gene driven by this region was expressed in two ACE-producing cells but not in two ACE-non-producing cells thereby establishing its tissue specificity. Our experiments also revealed the existence of a strong negative element located between -692 and -610 positions. This element suppressed the expression of the reporter gene in a dose-dependent and position and orientation-independent fashion thus suggesting that it is a true silencer element. It could also repress the expression of a reporter gene driven by the heterologous strong promoter of the β -actin gene. The repressing effects of the negative element could be partially overcome by cotransfecting the isolated negative element along with the reporter gene containing the negative element. This result was possibly due to the functional removal of a limiting *trans*-acting factor which binds to this element. 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. At least part of the negative element is strongly conserved in the upstream regions of the human and mouse ACE genes.

INTRODUCTION

Angiotensin-converting enzyme (EC 3.4.15.1) 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 (1). Other biologically important peptides such as bradykinin also serve as its substrates (2). There are two isozymes of angiotensin-converting enzyme (ACE) which are structurally related (3). They are expressed in a tissue-specific fashion (4). One (ACE_P) is produced by vascular endothelial cells, intestinal brush border cells, renal proximal tubular cells, and monocytes (5–8). The other (ACE_T) is produced in adult testis exclusively by developing sperm cells (6).

Rabbit ACE_P is a glycoprotein of 140 kDa whereas, ACE_T has a molecular mass of 100 kDa (3). The two proteins have identical enzymatic activities and they are encoded by a 5 kb mRNA and a 2.5 kb mRNA, respectively. Sequence at the 5' end of the ACE_T mRNA encoding the first 72 amino acid residues is unique to it. The rest of the sequence is identical to the 3' half of ACE_P mRNA (9). The sequence of the 5' half of ACE_P mRNA is unique to it although it has strong homology with the sequence of the 3' half thereby suggesting gene duplication (9–12).

The two ACE mRNAs are encoded by the same gene (13–15). The two mRNAs originate from this gene by tissue-specific choice of two alternate transcription initiation sites which are 5.7 kb apart (14). The two transcription units also use different polyadenylation sites which are 628 bp apart (16). Thus, the ACE_T mRNA transcription unit is completely nested within the ACE_P mRNA transcription unit.

The rabbit ACE_P mRNA starts at a G residue which is preceded by an authentic TATAA box at -26 position. Within the first 300 bp upstream sequence there are also putative binding sites for SP1, AP1 and AP2 transcription factors (14). There is strong sequence conservation within this region of the mouse, rabbit and human ACE genes. The mechanism responsible for

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the tissue specific expression of the ACE_p mRNA is not known. Such regulation could be either due to the absence of positive transcription factors in the non-expressing cells or a result of the presence, in these cells, of a tissue specific negative regulatory factor (17,18). Alternatively, both positive and negative elements may contribute to the tissue specific expression of a gene (19).

Here, we report that the 274 bp upstream region of ACE_p gene can drive the transcription of a reporter gene in ACE-producing cell lines but not in ACE-non-expressing cells. In addition, our experiments reveal the existence of a strong negative element located between -692 and -610 positions. This negative element can suppress transcription from the ACE_p promoter in a position and orientation-independent fashion. Using co-transfection experiments and electrophoretic mobility shift assays, we also provide evidence for the existence of one or more trans-acting factors which can bind to this element.

MATERIALS AND METHODS

Materials

All DNA-modifying enzymes were obtained from either GIBCO Bethesda Laboratories or Boehringer Mannheim. Oligonucleotides were synthesized by the phosphoramidite method (Applied Biosystem Model 380B). [¹⁴C] Chloramphenicol, (50.10 mCi/mmol) and γ -³²P-ATP (6000Ci/mmol) were purchased from NEN Dupont, Boston, MA. Polymerase Chain Reaction (PCR) was performed using a DNA thermal cycler (Perkin-Elmer Cetus Instruments). Cell culture products were purchased from GIBCO and Cell Culture Laboratories, Cleveland.

DNA sequence analysis

A *Bam*HI-*Nco*I (-1202 to +29) fragment from the rabbit ACE genomic clone 226 (14) was cloned into the pBluescript II KS vector. It was sequenced using either vector primers or oligonucleotide primers derived from the known sequence of the ACE gene. Sequencing reactions were performed using Sequenase and dideoxy reagents purchased from United States Biochemical Corp. The sequence information was analyzed by the Beckman Microgenie sequence analysis program.

Plasmid construction

The genomic clone containing -1202 to +29 bp sequences was released with *Bam*HI and *Nco*I, blunted and cloned into pBluescript SK vector's *Eco*RV site. *Xba*I and *Hind*III sites were then used to clone it upstream of the CAT gene in the pSV0 plasmid to obtain the -1202ACE_pCAT (20). Deletion mutants -692ACE_pCAT, -609ACE_pCAT, -274ACE_pCAT, -166ACE_pCAT and -110ACE_pCAT were obtained by releasing *Xba*I-*Ap*alI, *Xba*I-*Mst*II, *Xba*I-*Nhe*I, *Xba*I-*Sma*I and *Xba*I-*Nae*I fragments from the -1202ACE_pCAT vector, blunting and religating the vector backbone. -92ACE_pCAT, -57ACE_pCAT, and -33ACE_pCAT were obtained by using PCR. -1202ACE_pCAT vector served as template with sense primers TA2N, TA3 and TA4 for -92, -57 and -33ACE_pCAT vectors, respectively. Antisense primer (TA1) was complementary to the CAT gene approximately 120 bp downstream of the ATG. All sense primers contained an *Xba*I site at their 5' ends. PCR products were restricted with *Xba*I and *Hind*III, and cloned into the *Xba*I-*Hind*III sites of pSV0CAT. All constructs were confirmed by restriction analysis and sequencing. Sequences of the primers are given below:

A1:	TGAGCAACTG	ACTGAAATGC	CTCA	
TA2N:	GCATCTAGAG	GAGCCGCGGA	GGAAGCGCTT	TGGG
TA3:	GGCATCTAGA	GGGGGTGTGT	CGGAAGGCGG	
TA4:	GCGATCTAGA	GCGGTTTAT	TATATCCGCA	GG

Internal deletion mutant Δ 716-609ACE_pCAT was obtained by digesting -1202ACE_pCAT with *Mst*II, releasing the 106 bp 715-610 fragment and religating the isolated backbone. The -1202(610-715)ACE_pCAT construct was obtained by releasing the 106 bp *Mst*II-*Mst*II fragment from -1202ACE_pCAT and religating the insert into the backbone. The direction of insertion was confirmed by restriction and sequencing. The -1202ACE_p and Δ 716-609ACE_p constructs were transferred to pBasic CAT vector (Promega) to obtain -1202ACE_pBasic CAT and Δ 716-609ACE_pBasicCAT vectors. The 106 bp *Mst*II-*Mst*II fragment (corresponding to 715-610 bp of the ACE_p gene) was isolated, blunted and cloned into the blunted *Nde*I site 200 bp downstream of the CAT transcriptional unit and 1880 bp downstream from the ACE promoter in the pBasic CAT plasmids. This strategy was used to obtain single and double copies of the 715-610 (106 bp) insert in both orientations in this position. β -actin CAT construct was obtained by cloning the 560 bp β -actin promoter (21) into *Hind*III/*Sa*I sites of the pBasic CAT plasmid. β -actin CAT N.E. \rightarrow was obtained by cloning the N.E. into the *Bam*HI/*Nde*I sites of the β -actin CAT vector.

Cell culture

Cell lines derived from American opossum kidney (OPK), human cervical carcinoma (HeLa) and human hepatoma (HepG2) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (complete medium). Bovine aortic endothelial (BAE) cells were grown in DME/F12 medium supplemented with 5% fetal bovine serum (complete medium).

DNA transient transfection assays

Plasmid DNA was purified using Qiagen columns (Qiagen, Inc.) and transfected into OPK, HeLa or HepG2 cells using calcium phosphate precipitation method (22). Transfections were carried out in 100 mm plates using 20 μ g of reporter CAT plasmid along with 4 μ g of RSV β -gal plasmid. RSV β -gal contains RSV LTR upstream of the β -galactosidase gene and is constitutively active in all these cell types except HeLa for which 4 μ g of CMV β -gal plasmid (containing cytomegalovirus promoter) was used instead. Cell cultures grown to 60% confluency received DNA-calcium phosphate precipitate in 3 ml of complete medium. Sixteen hours later, cells were shocked with 20% DMSO in DMEM for 2 minutes and then incubated in complete medium. Cells were harvested 40 hours post-transfection: Bovine aortic endothelial cells were grown to 85% confluency in six well plates. 5 μ g of reporter gene and 15 μ g of Lipofectin reagent (GIBCO Laboratories) were mixed in 1 ml of Opti-MEM1 medium (GIBCO Laboratories) and added to cells for 24 hrs. when it was replaced with complete medium. Cells were harvested for CAT assays 72 hrs. post-transfection.

Determination of CAT activity

Extracts were prepared by repeated freeze-thawing and CAT activity was assayed as described previously (22). Briefly, CAT activity was assayed using equal volumes of cell extracts from transfected cells. After incubating for 2-4 hours at 37°C,

samples were extracted with 1 ml of ethyl acetate, organic phases were collected, dried and dissolved in 30 μ l of ethyl acetate. Samples were spotted on Silica Gel 60 thin layer chromatographic (TLC) plates (Cat. no. 5748; EM Science, Gibbstown, NJ) and the plates were developed in a solvent system comprising of chloroform and methanol in a ratio of 95:5 (V/V). After autoradiography, spots corresponding to acetylated and non-acetylated chloramphenicol were excised and counted in liquid scintillation counter. Alternatively, acetylated and non-acetylated forms of chloramphenicol on the TLC were quantitated using a Phosphor Imager screen (Molecular Dynamics). Percent acetylation/unit vol. of cell extract was corrected for differences in transfection efficiencies by measurement of β -galactosidase activity/unit vol. of cell extract. β -galactosidase activities were assayed by the method described by Maniatis *et al.* (23). Samples which had β -galactosidase activities that were more than two-fold different from the mean value were dropped from the analysis. Fewer than 10% of the samples were thus excluded from the analysis.

Electrophoretic mobility shift assay

Nuclear extracts were prepared by the method described by Dignam *et al.* (24). EMSA was performed following the protocol described by Ausubel *et al.* (25). Briefly, 2 μ g of nuclear extract or BSA was incubated with salmon sperm DNA and competitor DNA (as indicated) in reaction buffer on ice for 10 minutes prior to the addition of the negative element probe which had been

labelled with 32 P by incubating with γ - 32 P-ATP and polynucleotide kinase. After 30 minutes of incubation with the probe on ice, reaction was stopped by adding non-denaturing sample buffer and loaded on a 4% non-denaturing polyacrylamide gel in TAE buffer (67 mM Tris, pH 7.5, 33 mM sodium acetate, 10 mM EDTA). The gel was dried and exposed for autoradiography.

RESULTS

Transcriptional regulation by ACE_P upstream region

We have previously reported the isolation of a genomic clone for the rabbit ACE gene (14). For analyzing the upstream region of the ACE_P transcription unit, a suitable restriction fragment spanning from -1202 to +29 was isolated and used for promoter analysis. The sequence of this fragment is shown in Fig. 1. There are many putative binding sites for known transcription factors within this region including several SP1 and AP1 sites.

For functional testing of the upstream DNA sequences of the ACE_P transcription unit, a series of reporter genes were constructed. They contained decreasing lengths of ACE_P DNA cloned in front of the CAT coding sequence in a pSV0CAT construct which uses SV40 splicing and polyadenylation signals for eukaryotic expression but is devoid of any eukaryotic transcriptional promoter. All reporter constructs contained varying lengths of ACE_P upstream region ending at the 3' side at position +29 with respect to the transcription initiation site which is designated the position +1. Most of the specific deletion points on the 5' end of the ACE_P DNA were chosen by the presence of convenient restriction site at these positions.

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-1202 GATCCAGGCTACCGGGTCACTCTTGCCTGGATGCAAGTGTCCCATCACCCACATCCGGC
      AP-1
-1142 TACCTAGCCAGCCCTGAAGTCCACTCTCTAAGCCAGGGCCGGAGAGTCCGTGGGTAC
      SP 1
-1082 CCGGGTGGGGGTGGGGAGTGGGGTGGAGGTTGTGGGAAACCAAGGGCAAGATGGAGCTG
-1022 GGAAGAGGAGCAGGGCCCTCCTCTCAAACCTCCCAACCCCAACCCGTGTTTAAATGACAATA
      AP-1
-962 CCCTGCCTCTCTCTCCCTCTTCTCCCACTCCACCCCTGCCCTACACCATGTCACTGAC
      AP-1
-902 AACTCAGCACAAGGTACACAGAGGGTACACAGCCTCTGACACAGCTCTCTCCCGCACA
      AP-1
-842 GCGAGCCAACTGTGTAGCCAGACCCCTGGACACCCAGGAGTCCCTAAGTTCCACGCGCC
-782 CCAAAGCAGCCAAACCGGTTGGGGTCTCATCAGCAACCATCTGCTAGGAGCAAGGGGCC
-722 TCCCGCTGAGGACCCCGCCCATAGCCTGGTGCACGGAGGGGACGACACCCACCCCTCC
      SP 1
-663 CCCGCCCTGTCACTTTGAAGGCCAGGTGGAGGAGAGGGAGGGTGGAGACCTGAGGC
-603 GACTTTGGAGAGGCCACGCAAGCTGTGAGGCGGAAATTTTTCACCCGATAAAAACGAG
-543 GGGCTCTCTCTCGCTTCCAGGGCTGCTAGGGACACAAAGTCTGTCTGCCAGACTGCTTA
-483 CAGGAGAGGAGAGGGCGGCGCAGCTCCCGCTCCCATACAAGCCCCACCCCAACCCGACC
-423 CCGGGTCCAGGTGAGCAGCTGCGGGCCCTCGGGCGGCCACCCACAGCGCGGGGTCA
-363 CCTGCGCACTCCCGCACCCGAGCAGCGGCCCGCCGACCGCTGGCGGAGAGGCCGCTC
-303 GGGCCACGCCAGCCCATCGCGCGCGCTAGCGGTGCTGCGGACCGACTCCCTCCCTCC
-243 CGCTGTCTCTCCGACCCCGGCAAGCCCTGGGAGCTTAGCCCGGCACAGGAGGAAGG
      AP-2
-183 GCGCGCTGCGCTCCCGGGGGAGTGTGACCCCGCCCAACCCCGCCCGCCCGCCCGCC
      AP-1
-123 GCTCGTGTGGCGCGCGCGCGCGGACTTTGAGCGCGCGGAGGAAGCGCTTTGGCGGG
      SP 1
-63 GGAAGTGGGGTGTGTGCGAAGGGCGCGCGCGGTTTATAATCCCGAGGGCGCGCGCG
      TFIIID
-3 CCGTAGGAGGACGCGCCGCGCACCAACGCCATG +29
    
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Figure 1. Sequence of the upstream region of ACE_P transcription unit. Sequence of the upstream region of ACE_P transcription unit from position -1202 to +29 (transcription start site is designated +1) is shown. Transcribed sequence is shown in bold face type. The potential binding sites for various transcription factors are indicated below the corresponding sequences. The putative negative element (see below) is underlined. The sequence data have been deposited in GenBank; accession no: L23202.

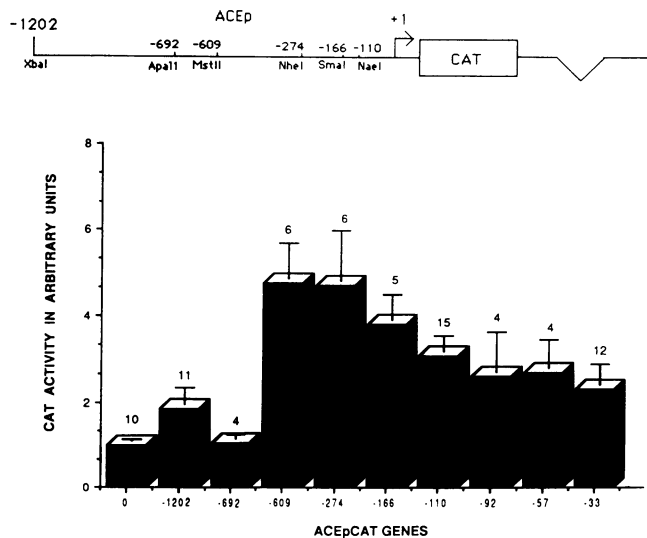


Figure 2. Transcriptional activity of ACE_PCAT genes. ACE_PCAT gene constructs containing progressive deletions ranging from -1202 to -33 lengths of the 5' upstream region of the ACE_P sequence, ending on the 3' side at +29 position, were tested in OPK cells. Results from several independent experiments, using at least two different batches of plasmid DNA and OPK cells, are presented. The observed CAT activities were normalized for experimental differences in transfection efficiencies as monitored by measuring β -galactosidase activity in transfected cell extracts. The normalized CAT activity in extract of pSV0CAT-transfected cells (bar 0) was assigned a value of 1 arbitrary unit. Bars represent mean \pm S.E. of the relative CAT activity. The number of independent replicates is shown on the top of each bar.

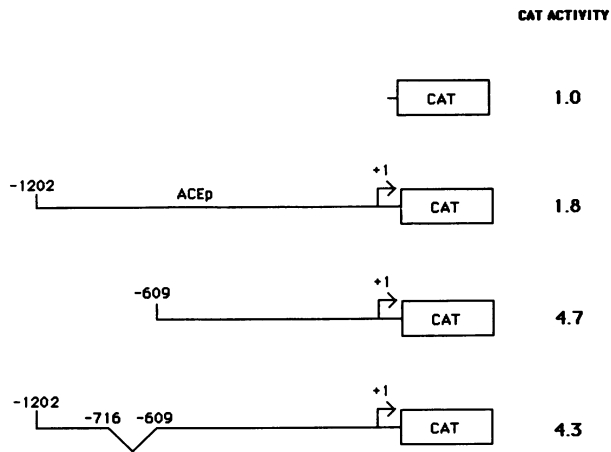


Figure 3. Expression of the internal deletion mutant Δ716-609ACEpCAT. pSV0CAT, -1202ACEpCAT, -609ACEpCAT and Δ716-609ACEpCAT are schematically represented along with their relative CAT activities in OPK cells. Values represent means of six independent transfections.

For testing the promoter strengths of this series of reporter genes, they were individually transfected into a cell line which produces ACEp in culture. American Opossum Kidney (OPK) cells originated from the proximal tubule of the kidney which is known to produce ACE *in vivo*. That the established OPK cell line produces ACE in culture was confirmed by measuring ACE enzymatic activity in cell extracts (data not shown). The calcium phosphate precipitation method was used for transfecting DNA into these cells. For comparing the transcriptional strengths of the different reporter genes several precautionary controls were built into the design of these experiments. It was ensured that all CAT enzyme assays were done at the linear range of enzyme activity which corresponded to about 5-65% conversion of the input chloramphenicol into the acetylated form. Differences in transfection efficiencies were normalized by co-transfecting a RSV-LTR-driven β-galactosidase gene with every CAT gene. β-galactosidase activity measurements were used for normalizing the CAT activities. By using mock-transfected cells it was verified that the endogenous β-galactosidase activity in the various cell lines used was extremely low, so that it was not confounding the expressed values. In every transfection experiment, pSV0CAT and pSV2CAT were included as a negative and a positive control, respectively. pSV2CAT vector contains the SV40 promoter and two copies of the SV40 enhancer cloned upstream of the CAT gene (19). The normalized CAT activity in extracts of pSV0CAT-transfected cells was considered as the background. The CAT activity above this value in extracts of cells transfected with a test CAT gene was used as an index of the promoter strength of the corresponding ACEp DNA. Finally, the transcriptional strength of every reporter gene was tested repeatedly by independent rounds of transfections of different lots of DNA. These precautions ensured that the observed differences in the promoter strengths of different reporter genes were reproducible and reliable.

Results of transfection experiments with the nine ACEpCAT genes are summarized in Fig. 2. Because the results are pooled from many experiments, they are presented in arbitrary units of CAT activity taking the mean level of pSV0CAT activity as 1. The longest piece of ACEp upstream region tested,

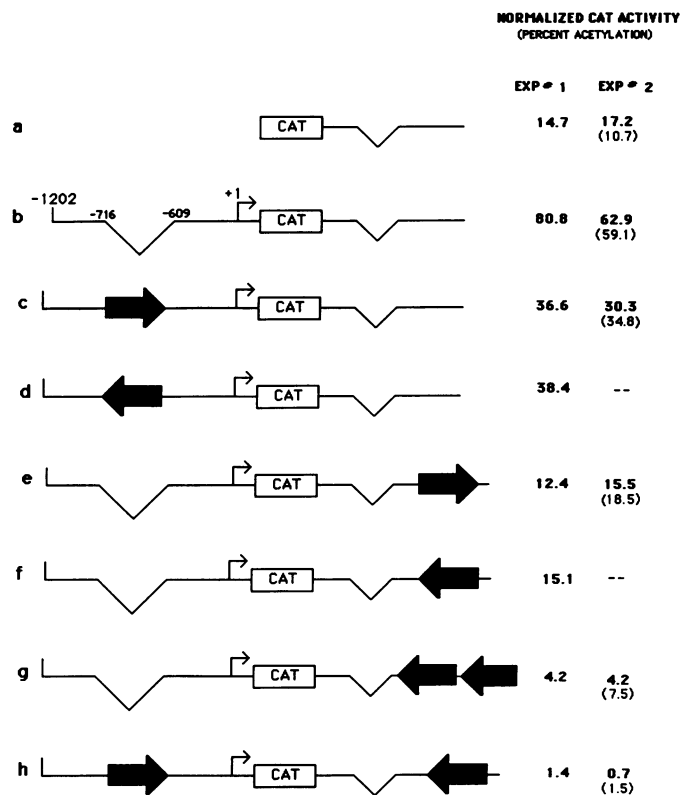


Figure 4. Function of negative element as a dose-dependent and orientation and position independent silencer. Schematically represented constructs are: pBasicCAT (a); Δ716-609ACEpBasicCAT (b); -1202ACEpBasicCAT (c); -1202 (N.E. ←) ACEpBasicCAT containing the N.E. in its native position but in the opposite orientation (d); Δ716-609ACEpBasicCAT.N.E. ← containing the N.E. cloned in 5'-3' orientation behind the CAT gene, 1.8 kb downstream of the transcription start site (e); Δ716-609ACEpBasicCAT.N.E. → similar to (e) with N.E. in 3'-5' orientation (f); Δ716-609ACEpBasicCAT.N.E. ← ← similar to (f) except that it contains two copies of the N.E. (g); -1202ACEpBasicCAT.N.E. ← contains two copies of the N.E., one in its native position and another behind the CAT gene in 3'-5' orientation (h). Normalized CAT activities from two independent experiments are presented in bold letters. Values for experiment 1 are means of duplicate transfections and values for experiment 2 are means of triplicate transfections. -- Denotes not done. Numbers in parenthesis are a representative replicate of experiment 2 presented as percent of input chloramphenicol acetylated under the assay conditions prior to normalization for the transfection efficiency of β-galactosidase.

-1202ACEpCAT, was barely 2 fold stronger than pSV0CAT. Removal of 510 bp from the 5' end (-692 ACEpCAT) reduced this activity essentially to the background. Surprisingly, removal of another 84 bp increased the promoter strength by about 5 fold (-609 ACEpCAT). This result indicated the existence of a strong suppressor element in the region between -692 and -609 of ACEp promoter. The promoter activity remained essentially unchanged between -609 and -274 ACEpCAT thereby indicating that the major positive elements driving the transcription of ACEp gene reside between -274 and +29 position. Further deletions of this region gradually diminished the level of CAT expression. The shortest gene tested, -33ACEpCAT, was 2.3 fold stronger than pSV0CAT. These results suggest that transcription of this gene may be driven by a series of positive *cis*-acting elements located in this region.

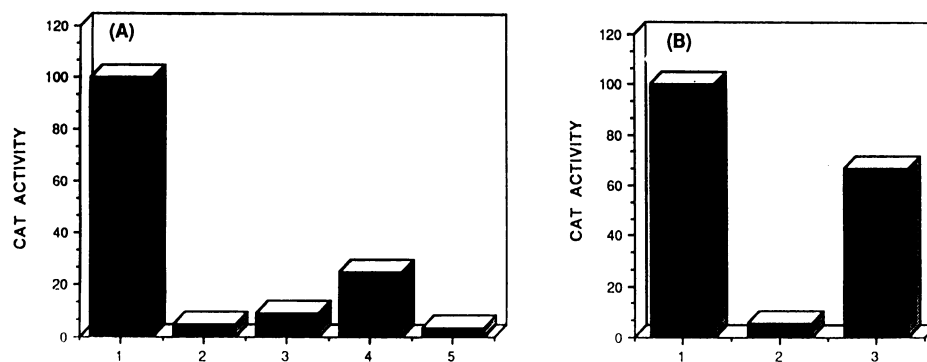


Figure 5. Competition *in-trans* with the isolated negative element. All transfections were carried out using 10 μ g of ACE_pCAT plasmids. Results are expressed as CAT activity in arbitrary units. **A** shows CAT activity from transfection of Δ 716–609ACE_pBasicCAT (*bar 1*), bars 2–5 represent Δ 716–609ACE_pBasicCATN.E. – transfected either alone (*bar 2*), or co-transfected with 1:1 molar ratio of the isolated 106 bp N.E. (715–610) (*bar 3*), co-transfected with 5 molar excess of N.E. (*bar 4*) and co-transfected with 5 molar excess of 102 bp DNA of unrelated (control) sequence (*bar 5*). **B** shows an independent experiment using different batches of cells and DNA. It shows CAT activity from transfection of Δ 716–609ACE_pBasicCAT (*bar 1*), Δ 716–609ACE_pBasicCATN.E. – alone (*bar 2*) and Δ 716–609ACE_pBasicCATN.E. – co-transfected with 8 molar excess of isolated N.E. (*bar 3*). The N.E. was obtained by restriction digestion of a pBluescript SK vector in which it had been subcloned. The N.E. and the ‘control’ fragment (comprising of the *SacI*–*KpnI* segment of the multiple cloning site of the pBluescript II KS vector) were separated from the backbone vectors by electrophoresis on low melting temperature agarose gels. Purification from the gel fragments was done by the phenol:chloroform extraction method.

Characteristics of the negative element

The putative negative element present between –692 and –609 was further examined in a series of experiments. In the experiment shown in Fig. 3, an internal deletion mutant of the upstream region was generated by deleting the sequence between –716 and –609. As expected, Δ 716–609ACE_p was as effective as –609ACE_p in driving CAT expression in transfected cells. In the next experiment, we tested whether the negative element can act as a true silencer of ACE_p gene (26). The region encompassing the negative element (N.E.) (–715 to –610) was introduced back into the Δ 716–609CAT gene in various configurations. Results of two independent experiments are presented in Fig. 4. Putting the N.E. back into its original location, but in reverse orientation, repressed CAT activity by more than 2 fold. In contrast, putting the N.E. behind the CAT gene in either orientation repressed CAT expression by about 4 to 6 fold. Two units of N.E. cloned behind the gene, reduced CAT activity by about 15–20 fold, whereas one unit of N.E. in front and another one at the back reduced the activity by about 60 fold. These results clearly demonstrated that the N.E. acts as a true silencer. It can suppress the ACE_p promoter in both orientation and position-independent fashions. Moreover, two units of the element were more effective in suppressing transcription than one unit, a common characteristic of transcriptional regulatory elements.

The transcriptional regulatory effects of both positive and negative regulatory *cis*-elements are mediated by *trans*-acting factors which specifically bind them. If the cellular concentration of such factors are limiting, their effects can be partially titrated out by co-transfecting, with the test vector, the isolated regulatory element (27). Such competition, *in trans*, for the putative N.E.-binding factor was tested in the experiments shown in Fig. 5. Results of two independent experiments are presented. In the first experiment (5A), the N.E. cloned behind the Δ 716–609ACE_pCAT gene (Δ 716–609 ACE_pCAT N.E. –) (*bar 2*) repressed CAT expression by 18 fold. Co-transfecting the isolated N.E. (–715 to –610) and Δ 716–609 ACE_pCAT

N.E. – at a 1:1 molar ratio (*bar 3*) relieved this repression by about 2 fold and co-transfection at a 5:1 (N.E.: Δ 716–609ACE_pCAT N.E. –) molar ratio (*bar 4*) relieved the repression by about 5 fold. Co-transfection of an unrelated 102 bp DNA fragment at a 5:1 molar excess (*bar 5*) had no effect on the level of expression of the Δ 716–609ACE_pCATN.E. – test vector. In the second independent experiment (5B), co-transfection at a ratio of 8:1 (N.E.: Δ 716–609ACE_pCAT N.E. –) (*bar 5*) caused a relief of repression by about 12 fold. These experiments strongly suggest that the N.E. exerts its repressing activity by binding one or more *trans*-acting factors whose cellular concentrations are limiting. As a consequence, co-transfecting isolated N.E. could alleviate the repression mediated by the *cis*-acting N.E. present in the test plasmid.

In another experiment, we tested the effect of the N.E. on a heterologous promoter. The strong cellular promoter of β -actin was chosen for this purpose. The N.E. was cloned behind the CAT gene of the β -actin CAT reporter construct. As shown in Fig. 6, β -actin promoter stimulated CAT expression in OPK cells by about 20 fold (*bar 1 vs bar 3*) but the N.E. decreased the level of CAT expression by more than 3 fold (*bar 2 vs bar 1*). These results demonstrate that the negative element of the rabbit ACE gene is not promoter-specific and it can repress a strong cellular promoter even when placed at a long distance.

N.E. binding proteins

Nuclear extracts of OPK cells were used for detecting the negative element-protein complexes using EMSA (Fig. 7). Four such complexes (A–D) were detected (lane 3). Three of these complexes, A, B and D, were specific; they were competed out by 200-fold molar excess of negative element (lane 4) but not by 200-fold molar excess of an unrelated DNA of similar length (lane 5). No complex was observed in the absence of added protein (lane 1) or in the presence of BSA (lane 2). These results demonstrate that at least three specific DNA–protein complexes can form between the negative element and nuclear proteins of OPK cells.

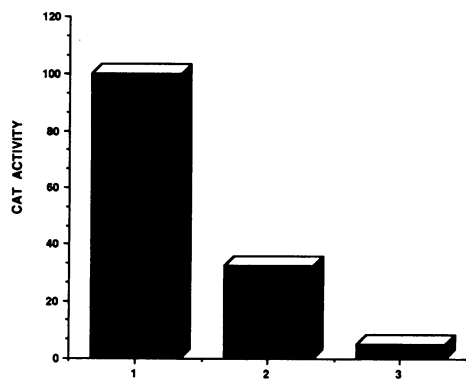


Figure 6. Repression of a heterologous promoter by the negative element. OPK cells were transfected by three reporter genes: 1) Human β -actin promoter cloned at the 5' end of the CAT gene in pBasicCAT; 2) The same construct as in 1 but carrying the N.E. \rightarrow at the 3' end of the CAT gene; 3) pBasicCAT with no regulatory element added. The normalized CAT activities are presented in arbitrary units. Values shown are means of duplicate determinations that varied by less than 5%.

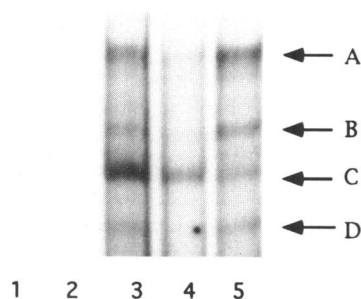


Figure 7. Electrophoretic mobility shift assay with N.E. The end-labeled 106 bp negative element sequence was used as the probe. Only the relevant portion of the autoradiogram is shown. Lanes 1 and 2 show the free probe and incubation with 2 μ g of BSA respectively. Nuclear extract (2 μ g) from OPK cells was incubated with the probe for lanes 3-5. Competition was carried out with 200-fold molar excess of unlabelled probe (lane 4) or an unrelated DNA of 145 bp (lane 5). Arrowheads A, B, C and D indicate the DNA-protein complexes.

Expression of ACE_pCAT genes in other cell lines

Since the ACE gene is expressed in a highly tissue-specific manner, we examined if the tissue specificity is maintained by the ACE_pCAT reporter genes. Several cell lines were used for this purpose. Bovine aortic endothelial cells (BAE) were tested as another ACE-producing cell type and the human cervical carcinoma cell line (HeLa), and the human hepatoma cell line (HepG2), were used as prototype non-ACE-producing cell types. Expression levels of different reporter CAT genes in these cell lines in comparison with those in OPK cells are presented in Table 1. The levels of expression of ACE_pCAT genes in BAE cells paralleled those in OPK cells. These results indicate that the positive elements and the negative element of the ACE_p promoter are functional in both ACE-producing cell types. In contrast, even the strongest ACE_pCAT gene, Δ 716-609ACE_pCAT, was inactive in HeLa and HepG2 lines thereby suggesting that the positive elements driving the ACE_p gene are ineffective in these ACE_p-non-producing cell lines.

Table 1. ACE_p promoter activity in different cell lines^a

Test genes	Cell lines			
	HeLa	HepG2	BAEC	OPKC
pSV0CAT/pBasicCAT	100	100	100	100
-1202ACE _p CAT	100	116	208	185
Δ 716-609ACE _p CAT	38	151	494	430
-609ACE _p CAT	-	173	-	475
-274ACE _p CAT	-	99	-	470
-166ACE _p CAT	123	-	-	380
-110ACE _p CAT	138	-	-	306
-33ACE _p CAT	126	-	-	232
Δ 716-609ACE _p BasicCATN.E. \rightarrow	-	-	75	88
Δ 716-609ACE _p BasicCAT(N.E.) ₂ \rightarrow	-	-	22	26
-1202ACE _p BasicCATN.E. \rightarrow	-	-	35	7

^aResults are expressed in arbitrary units assigning a value of 100 to the CAT activity present in extracts of cells transfected with pSV0CAT or pBasicCAT. Values for BAE, HeLa and HepG2 cells are means of three independent transfections from a representative experiment. Results for OPK cells are pooled from several experiments and represent between 5 and 15 transfections for different constructs. - Denotes transfection not performed.

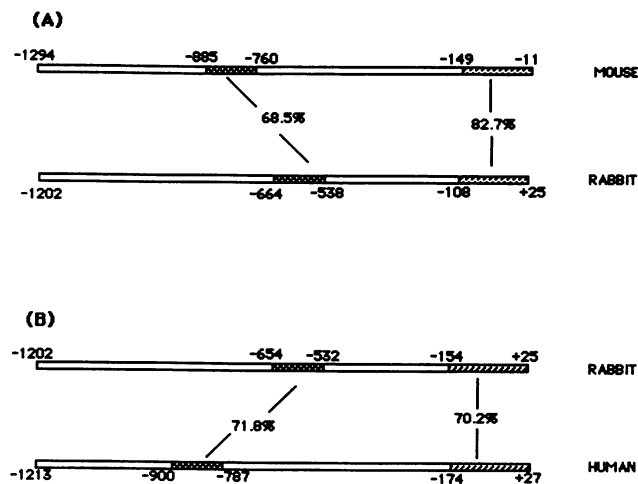


Figure 8. Comparison of rabbit, mouse and human ACE_p promoter sequences. Sequences of the upstream regions of rabbit and mouse (6A) and rabbit and human (6B) ACE_p transcription units are aligned. Shaded areas mark the two regions of overall homology. Homology search was conducted using Beckman Microgenie sequence analysis program looking for areas of at least 80% homology over a minimum length of 15 bp sequence. For the rabbit and human ACE_p sequences, +1 indicates the transcriptional start site. For the mouse ACE_p sequence, since the transcriptional start site is unknown, the translation start site has been assigned the +1 position.

These results were also confirmed by the observation that -609ACE_pCAT and -274ACE_pCAT genes were inactive in the HepG2 cells. Thus, the tissue-specific expression pattern of the resident ACE gene is mimicked by transfected reporter genes driven by the transcriptional regulatory elements of the ACE gene.

DISCUSSION

The ACE gene gives rise to two primary transcripts, the ACE_p mRNA and the ACE_T mRNA, in a tissue-specific manner. The choice of production of one transcript or the other is determined

at the level of initiation of transcription (14). In the study reported here, we analyzed the upstream region of the rabbit ACE_P transcription unit for the presence of transcriptional regulatory elements. The analysis was done in cells which express ACE_P, so that the physiological tissue-specificity was maintained. Our results demonstrated that the major promoter elements are located within 274 nucleotides immediately upstream of the transcription start site. Progressive deletion of this region resulted in a progressive diminution of the promoter strength, indicating that no single element within this region was solely responsible for driving transcription. It seems likely that transcription of the ACE_P mRNA is driven by a number of DNA elements present within this region each of which contribute partially to the cumulative strength of the promoter. An examination of the nucleotide sequence of this region reveals the presence of an AP2, an AP1, and four SP1 binding sites. It remains to be tested whether these elements play important roles in the overall strength of this promoter. A major technical difficulty for such an experimental determination is the relative weakness of the promoter. This difficulty has been a major burden throughout the analyses. To ensure that our conclusions are reliable, we performed multiple independent rounds of transfection (Fig. 2) so that the range of experimental error could be established. The observed weakness of the ACE_P promoter is not unexpected given the low copy number of this mRNA in pulmonary endothelial cells, the richest source of ACE_P (9,28). In principle, it remains possible, however, that strong enhancer elements are present further upstream of the gene or within the introns which have not been examined by us. Our experiments did not indicate the presence of strong positive elements between -274 and -1202. Instead, a strong negative element was detected within this region.

The negative element was localized between -692 and -610. There are no known transcriptional factor binding sites within this region that could account for the observed repressing effect. The negative element behaved like a true silencer when tested with its cognate ACE_P promoter. It repressed CAT expression when placed at about 600 bp upstream of the transcription start site in either orientation. When placed 1.8 kb downstream of the transcription start site, in either orientation, it was even more effective. Two units of N.E. in tandem or one in front and the other in the back of the CAT gene were more effective in suppressing transcription than one unit. As expected of true silencers, the negative element also repressed the expression of a CAT gene driven by a strong heterologous promoter (Fig. 6). This observation suggests that this particular negative element has the potential to regulate the expression of other cellular genes and it may be present in the transcriptional regulatory regions of many uncharacterized genes. Co-transfection of the isolated negative element in molar excess, partially relieved the repression of ACE_P-CAT gene containing this element. This result strongly suggests that the effect of the negative element is mediated by protein(s) which bind to it. Moreover, it appears that the cellular concentration of this protein is limiting enough that it can be effectively titrated out. The existence of negative element-binding nuclear proteins was directly demonstrated by EMSA. Further investigation of negative element-protein interactions will be needed for delineating compositions of the three specific complexes observed in the experiment shown in Fig. 7. Mutational studies using transfection assays and EMSA will lead to the identification of the specific complex whose formation is required for manifesting the biological activity of the negative

element. Such studies will also provide information regarding the physiological role of the negative element.

The issue of the basis of tissue-specific expression of ACE_P mRNA was partially addressed by testing the expression of the reporter genes in two ACE_P-expressing and two non-expressing cell lines. The expression profiles were very similar in the two expressing cell lines whereas the promoter was essentially inert in HeLa and HepG2 cells. This result suggests that at least one determinant of tissue-specific transcription of ACE_P mRNA is at the level of functioning of the promoter elements in the context of the ACE gene. Additional as yet undetermined elements may also play a role in this context. The permanently repressed state of the ACE gene in most cell types may be maintained by the absence of factors that bind to the positive elements. One can speculate that the negative element may also play a role in this phenotype in some cells due to the presence of a high level of putative repressor proteins which bind to the negative element. In ACE_P-producing cells, this repression may be partially relieved by limiting the availability of functionally active repressor protein, thereby accounting for the low level of activity of ACE_P promoter seen in these cells. To test this hypothesis we are currently assessing the negative element repression of several heterologous promoters in both ACE_P-expressing and non-expressing cell lines. Our preliminary experiments indicate that the negative element can repress the expression of β-actin CAT in cells which do not express ACE (our unpublished observation). Further detailed investigation, however, will be required for a quantitative comparison of the negative element's function in ACE-expressing and non-expressing cell lines. Such an investigation will be accompanied by measurements of the levels of N.E.-binding proteins, in the extracts of such cells, using EMSA. The physiological regulation of the level of expression of the ACE gene by hormones and other biological modulators may also be regulated by the negative element. Our results suggest that the patterns of regulation of transcription of the ACE_P mRNA are very similar in cells derived from the two major physiological sources of ACE_P synthesis: vascular endothelial cells and kidney proximal tubular epithelial cells.

Partial sequences of the upstream regions of the human and the mouse ACE genes have been published (13,29). Unlike the human and the mouse ACE genes, the analyzed region of the rabbit ACE gene (Fig. 1) did not contain any glucocorticoid response element (13). However, comparison of these sequences revealed two regions with high degrees of homology. A region encompassing the transcription start site of the rabbit gene was highly homologous to the corresponding regions of the mouse (82.7%) and the human (70.2%) genes (Fig. 8). The positive regulatory elements are located in this region. Another region, further upstream, also has strong homology. The region between -664 and -538 of the rabbit gene is 68.5% homologous to the region between -885 and -760 of the mouse gene. Similarly, the region between -654 and -532 of the rabbit gene has 71.8% homology to the region between -900 and -787 of the human gene. It is curious to note that this upstream conserved region of the rabbit gene contains part of the negative element identified by our studies. It appears therefore that the negative element may be conserved among ACE genes of different species although sequences around it are not conserved to the same degree. Such a conservation implies a functional selection pressure for maintaining the sequence. A search of the DNA sequence database in GenBank failed to reveal a significant overall homology of the rabbit ACE negative element with any described

sequence. The ACE_p negative element, therefore, may constitute a new transcriptional silencer.

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