Functional analysis of the human somatic angiotensin I-converting enzyme gene promoter

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Angiotensin I-converting enzyme (ACE) is a key enzyme in the regulation of systemic blood pressure and plays a major role in the renin–angiotensin and bradykinin–kinin systems, at the luminal surface of the vascular endothelia. To identify the promoter region, the transcription regulatory elements and the cell specificity of the *ACE* gene, five successive DNA deletions of the 5′ upstream region (−1214, −754, −472, −343, −132 bp relative to the start site of transcription) were isolated and fused in sense and antisense orientations to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene in the promoterless plasmid pBLCAT3. Promoter activities were measured in transient transfection assays using three different cell lines from rabbit endothelium (RE), human embryocarcinoma (Tera-1) and hepatocarcinoma cells (HepG2). All five fragments of the *ACE* promoter region directed expression of the *CAT* gene when transfected into the endothelial and the embryocarcinoma cells, which contain endogenous ACE mRNA and express ACE activity. In contrast only minimal levels of promoter activity were obtained on transfection into hepatocarcinoma cells in which endogenous ACE mRNA and ACE activity were not detected. Transfection of RE and Tera-1 cells demonstrated that promoter activity was defined by the length of the *ACE* promoter sequence inserted into the construct. The 132 bases located upstream from the transcription start site were sufficient to confer *ACE* promoter activity, whereas the sequences upstream from −472 bp and between −343 bp and −132 bp were responsible for a decrease of promoter activity. Furthermore, the minimal 132 bp of the *ACE* promoter contains elements which direct cell-specific *CAT* expression. In addition, the DNA transfection study in the presence of dexamethasone suggested that the potential glucocorticoid regulatory elements, located in the sequence of the *ACE* promoter, are not functional.

INTRODUCTION

Angiotensin I-converting enzyme (ACE, peptidyl dipeptide hydrolase, EC 3.4.15.1) is a zinc metallopeptidase which plays an important role in blood pressure homoeostasis (Erdös and Skidgel, 1987; Ehlers and Riordan, 1990). ACE converts angiotensin I into the potent vasoconstrictor angiotensin II and degrades bradykinin, a potent vasodilator (Yang et al., 1970). ACE displays a wide substrate specificity, hydrolysing several other natural and synthetic peptides *in vitro*.

Two isoenzymes of ACE are found in mammalian tissues having molecular masses of 170 and 90 kDa, which correspond to the expression of two mRNAs of 4.3 and 3.0 kb respectively (Soubrier et al., 1988). The larger isoenzyme is principally produced by the pulmonary vascular endothelium and by absorptive epithelia, such as the brush borders of the small intestine and kidney proximal convoluted tubules (Ward et al., 1980; Lieberman and Sastre, 1983; Bruneval et al., 1986). The larger ACE isoenzyme is also present in neuroepithelial cells, monocytes and T lymphocytes (Defendini et al., 1983; Okabe et al., 1985; Costerousse et al., 1993). The 90 kDa form is produced exclusively by germinal cells at the onset of puberty (El-Dorry et al., 1982; Lanzillo et al., 1985). The expression of the two ACE isoenzymes is regulated in a tissue-specific fashion. Furthermore, the expression of germinal ACE is developmentally regulated, whereas the synthesis of somatic ACE is induced by glucocorticoids (Krulewitz et al., 1984). The structural organization of the human *ACE* gene has been established (Hubert et al., 1991) and spans 21 kb, comprising 26 exons. The somatic ACE mRNA (4.3 kb) is transcribed from exons 1 to 26, excluding exon 13 by differential splicing. In contrast, transcription of the germinal ACE mRNA (3 kb) is initiated within the gene at exon 13 and proceeds through exons 14 to 26. The different 5′ ends of the two ACE transcripts, i.e. somatic and germinal, suggest the presence of two promoters in the gene (Hubert et al., 1991). We have recently demonstrated the germinal *ACE* promoter activity in a teratocarcinoma cell line (Tera-1) derived from primary diploid germ cells, which express somatic ACE and very low levels of germinal ACE as detected by reverse-transcription PCR (RT-PCR) assays (Nadaud et al., 1992). Alternative transcription start sites of the *ACE* gene have also been reported in both mice and rabbits (Howard et al., 1990; Kumar et al., 1991). The activity of the mouse germinal *ACE* promoter was demonstrated by injecting 698 bp of the 5′ flanking region of exon 13 linked to the β-galactosidase gene as reporter gene into a single-cell mouse embryo. The expression of β-galactosidase was selectively detected during spermiogenesis (Langford et al., 1991). The somatic *ACE* promoter contains typical sequences (TATA box and several SP1-binding sites) identified in the 5′ flanking region, but has not yet been characterized in terms of its function, either by experiments *in vitro* or by expression in transgenic animals (Shai et al., 1990; Hubert et al., 1991). To investigate the regulation of the *ACE* gene, to identify the somatic *ACE* promoter and to study the mechanism of glucocorticoid action, we have analysed the 5′ flanking region of the gene. The promoter activity of this region was tested by constructing various recombinant plasmids containing 5′ flanking fragments of the somatic *ACE* gene fused to the easily detectable chloramphenicol acetyl-

Abbreviations used: ACE, angiotensin I-converting enzyme; CAT, chloramphenicol acetyltransferase; pHGO, expression plasmid of human glucocorticoid receptor; GRE, glucocorticoid-responsive element; RE, rabbit endothelial; RSV, Rous sarcoma virus; tk, thymidine kinase; RT-PCR, reverse transcription PCR; Hip-His-Leu, hippurylhistidyl-leucine; SSC, 150 mM NaCl/15 mM sodium citrate (pH 7.0); DMEM, Dulbecco's modified Eagle's medium; WAP, whey acidic protein.

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transferase (CAT) reporter gene in the promoterless pBLCAT3. Transient transfections were tested in different cell lines: rabbit endothelial (RE) and human teratocarcinoma cells, which both express somatic ACE; and HepG2 cells, a hepatic cell line which does not express ACE. The identification of regions within the 5′ flanking sequence which influence *ACE* transcriptional activity is reported.

MATERIALS AND METHODS

Constructions of *ACE–CAT* fusion plasmids

A 1.5 kb *Pst*I–*Pst*I fragment of the human *ACE* subclone pHA6.5 (Hubert et al., 1991), containing the 5′ flanking region of the gene and part of the first exon, was isolated and digested by convenient restriction endonucleases for construction of expression vectors into the polylinker of the promoterless plasmid pBLCAT3 (Luckow and Schütz, 1987). Serial deletions were generated using *Hgi*AI, *Nhe*I, *Nla*III, *Sau*3AI, *Sma*I restriction endonucleases which created respective fragments of 1218 bp (−1214 to +4), 758 bp (−754 to +4), 493 bp (−472 to +21), 364 bp (−343 to +21), 153 bp (−132 to +21), positions all being relative to the transcription initiation site. When necessary, protruding termini were blunted with either T4 or Klenow polymerases. These fragments were subcloned in both sense and antisense orientations, into the T4 polymerase-treated *Pst*I site of pBLCAT3. The plasmids thus obtained were designated pACE1214–CAT, pACE754–CAT, pACE472–CAT, pACE343–CAT, pACE132–CAT respectively. For *ACE* glucocorticoid regulation studies two other constructs were made by insertion of two larger fragments of 3.3 kb and 2.2 kb obtained by digestion with *Mcs*I at −3.6 kb or *Rsa*I at −2.5 kb and *Not*I (position −295 bp). These fragments were inserted in the polylinker of pBLCAT2, a vector harbouring the herpes simplex virus promoter of thymidine kinase (tk) (Luckow and Schütz, 1987). These constructs were called pACE3.3tk–CAT and pACE2.2tk–CAT respectively. The deletion constructs and their orientations were confirmed by restriction mapping and dideoxy-chain-termination sequencing (Sanger et al., 1977). pMAMneoCAT (Clontech) was used as a positive plasmid control induced by glucocorticoids. The human glucocorticoid receptor plasmid (pHGO) was kindly provided by Dr. P. Chambon (Strasbourg, France).

Cell culture

An immortalized RE cell line was kindly donated by Dr. D. Paulin (Paris, France). These cells were shown to express various endothelial-cell markers (Schwartz et al., 1991) and were cultured in minimum essential medium containing 10% (v/v) fetal-calf serum. The human teratocarcinoma cells (Tera-1), obtained from the American Type Culture Collection, were cultured in McCoy medium containing 15% (v/v) fetal-calf serum. The human hepatoma cells (HepG2) from the American Type Culture Collection (ATCC HB8065) were grown in Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium in a 1:1 ratio supplemented with 10% (v/v) fetal-calf serum. All media were supplemented with L-glutamine (2 mM), penicillin (50 units/ml) and streptomycin (50 μg/ml). The cell cultures were maintained at 37 °C in an atmosphere of 5% CO_2. All media were purchased from Boehringer–Mannheim.

ACE activities

Enzymic studies were performed using hippurylhistidyl-leucine (Hip-His-Leu) (Bachem, Switzerland) as substrate as described previously (Wei et al., 1991). The detection and quantification of hippuric acid liberated from Hip-His-Leu were performed by h.p.l.c. The detection limit of hippuric acid liberated from Hip-His-Leu was 0.006% of substrate hydrolysis, corresponding approx. to 2 μ-units. One unit of activity was defined as the amount of enzyme catalysing the release of 1 μmol of hippuric acid from Hip-His-Leu/min (Cushman and Cheung, 1971). A specific ACE inhibitor, enalaprilat (1 μM), was included in the control incubation (Merck, Sharp and Dohme). Protein concentration was determined by the Bradford method (Sedmak and Grossberg, 1977). All enzymic studies were performed under initial rate conditions.

Isolation of RNA and RT-PCR

Total cellular RNA was isolated from different cell cultures using a guanidinium isothiocyanate/$CsCl_2$ procedure, as described by Chirgwin et al. (1979). RNA was quantified by measuring its absorbance at 260 nm.

Oligonucleotides, synthesized by a PCR-Mate 391 (Applied Biosystems), were designed according to the human *ACE* cDNA sequence (Soubrier et al., 1988). Antisense: 5′-GTGTTGTAGTTCCAGTTGGC, position 2030–2049 (in exon 14); and sense: 5′-GTCACCCAGTGGCTGCAGGA, position 1838–1857 (in exon 12) fragments were used as primers for PCR.

Single-stranded cDNA was synthesized from cellular RNA (5 μg) in the presence of 5 μM random hexanucleotide, 20 units of RNAase inhibitor (Promega), 1.25 mM of each dNTP and 200 units of Moloney murine leukaemia virus reverse transcriptase (Bethesda Research Laboratories) in 20 μl of PCR buffer from a Bethesda Research Laboratories kit, for 60 min at 37 °C. DNA was amplified in a volume of 50 μl with PCR buffer in the presence of 10% (v/v) dimethyl sulphoxide, 0.5 μM of antisense and sense primers, 1.25 mM of each dNTP, 5 μl of reverse transcriptase product and 2 units of Taq polymerase (Boehringer–Mannheim) for 30 cycles by a step programme (95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min), followed by a final 10 min extension at 72 °C. Amplification products were analysed by using 2% (w/v) agarose gel electrophoresis and ethidium bromide staining. These products were then transferred to nylon Hybond N^+ membrane (Amersham) and hybridized with a labelled oligonucleotide located within exon 14 of the *ACE* gene. Hybridization was carried out at 40 °C for 16 h in 6 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0)/50 mM NaH_2PO_4/0.1% SDS/5 × Denhardt's solution/100 μg/ml of denatured salmon sperm DNA. The membrane was washed at 45 °C in 2 × SSC/0.1% SDS and autoradiographed using Kodak X-Omat film.

Cell transfections

Cell transfection of plasmid DNA was carried out by the calcium phosphate precipitation technique (Graham and Van der Eb, 1973). Cells were plated on to 100-mm-diam. Petri dishes 24 h before transfection. The culture protocol was designed to transfect sub-confluent cells, to obtain confluent cultures when the cells were harvested. The medium was changed 3 h before transfection and replaced by DMEM medium (Gibco) containing sodium pyruvate and 10% (v/v) fetal-calf serum. A total amount of 25 μg of DNA was added into each plate: 15 μg of tested plasmid and 10 μg of pRSVβ-galactosidase, which was used as an internal control to monitor transfection efficiency (Edlund et al., 1985). For RE and HepG2 cells the calcium phosphate precipitates were left for 15 h at 37 °C, then the cells were washed and fresh medium was added. The Tera-1 cells were incubated

for 4 h at 37 °C in the presence of DNA–calcium phosphate. After treating with 15% (v/v) glycerol for 2 min, the cultures were rinsed with DMEM and refed with growth medium. Cells were harvested by scraping 48 h after transfection. Transfections with pRSVCAT (Gorman et al., 1982) were performed as a 100% positive control for expression in each cell type. For induction by glucocorticoids, after transfection cells were washed and refed with serum-free GT medium (Biochrom, Angoulême, France) supplemented with 4×10^{-8} M selenium and 5×10^{-7} M dexamethasone (Sigma) for 48 h. Each transfection experiment was repeated at least three times with at least two different plasmid preparations for each construct.

CAT assays

At 48 h after transfection, cells were harvested, resuspended in 250 mM Tris/HCl, pH 7.8, and lysates were prepared by three cycles of freeze–thawing. Cellular debris was removed by centrifugation. The resulting supernatants were assayed for β-galactosidase and CAT activities. The β-galactosidase activity used to monitor transfection efficiency for each dish was assayed as described by Herbomel et al. (1984). The β-galactosidase activity was expressed as $10^{-2} \times A_{420}$, extrapolated to 1 h of reaction time, for a total 200 μl of extract. The different extracts, containing equivalent β-galactosidase units, were assayed for CAT activity after incubation at 65 °C for 10 min (Crabb and Dixon, 1987). The extracts used for CAT assay were analysed for acetylation of [^{14}C]chloramphenicol (55 mCi/mmol, Amersham Corp.) in the presence of 4 mM acetyl-coenzyme A (Boehringer, Mannheim, Germany) and reactions were stopped by the addition of ethyl acetate. The acetylated and non-acetylated forms of chloramphenicol were separated by ascending chromatography on silica-gel plates using chloroform/methanol (95:5, v/v) as the mobile phase and were revealed by autoradiography as described by Gorman et al. (1982). The extent of conversion of substrate into products was quantified by excising labelled spots and counting radioactivity in a liquid β-scintillation counter. For quantification of CAT activity, the extract concentrations and the reaction times were selected to ensure linear conversion of the chloramphenicol into acetylated forms.

RNAase-protection assay

A fragment of 319 bp from the pACE472–CAT, containing 46 bp of the *ACE* gene and 273 bp of the *CAT* gene, was amplified by PCR using 20-base-long forward and reverse primers. This fragment was subcloned into the plasmid vector Bluescript SKII, and sequenced in its entirety to confirm the absence of PCR-generated misincorporation. After linearization of the plasmid with *Hin*dIII, transcription was performed *in vitro* in the presence of [α-^{32}P]UTP and T3 RNA polymerase to synthesize labelled antisense RNA (Ausubel et al., 1987). The radioactive probe was purified by gel electrophoresis and hybridized at 45 °C overnight to RNA (50 μg) prepared from cells transfected as described above. Unprotected RNA was digested by ribonuclease A (50 mg/ml) and T1 (1500 units/ml) and then applied to a 5% (w/v) acrylamide/7 M urea sequencing gel.

RESULTS

ACE activity and detection of somatic ACE mRNA by RT-PCR

To study the suitability of cell lines for testing *ACE* promoter experiments in transfection assays, ACE activity was measured in immortalized RE, Tera-1 and HepG2 cell lines. ACE activity was determined using a synthetic substrate (Hip-His-Leu) in the absence and presence of 10^{-6} M enalaprilat to confirm the specificity of the reaction.

ACE activity was detected in the RE and Tera-1 cell lines. In contrast, ACE activity was not detected in HepG2 cells (Figure 1a). The expression of endogenous ACE mRNA in each cell line was determined by an RT-PCR amplification method. Amplified fragments of the transcripts corresponded to the expected size (212 bp) for the *ACE* cDNA (Figure 1b). The fragment was specific to somatic ACE, since the primers used to amplify the cDNA were specific to the somatic transcript. This fragment hybridized to an oligonucleotide designed from the sequence of exon 14 (Figure 1b). ACE mRNA was detected in RE and Tera-1 cells but was absent in HepG2 cells, consistent with the ACE activity assays.

Identification of the functional somatic *ACE* promoter region in the immortalized endothelial cells

To define further potential regulatory elements required for promoter activity, a series of mutants containing deletions between −1214 and −132 bp was constructed. A schematic diagram of these constructs is shown in Figure 2(a). These constructs were transiently transfected into the RE cell line. Transfection efficiency was monitored by co-transfection with the pRSVβ-galactosidase plasmid to normalize the CAT activity. The pRSVCAT plasmid, a strong promoter active in most cells, was used as reference and was defined as 100% of relative CAT activity. The results of these transfections are presented in Figure 2(b). The highest level of promoter activity was obtained with the fragment containing 472 bp of DNA upstream from the transcription start site of the *ACE* gene. Addition of a further 282 bp upstream (pACE754–CAT) clearly reduced the activity to 56% of the maximal promoter activity. The inclusion of an additional 460 bp in the 5′ flanking sequence had no further effect on promoter activity compared with the 754 bp construct. The construct shorter than 472 bp, the 343 bp fragment, showed a

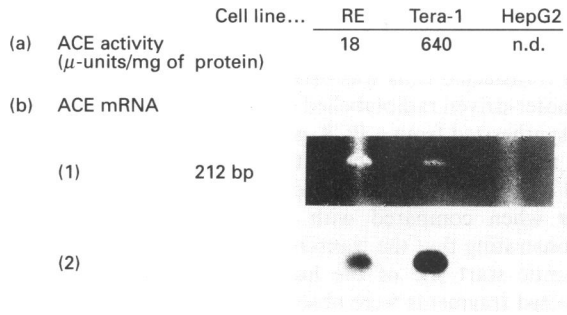
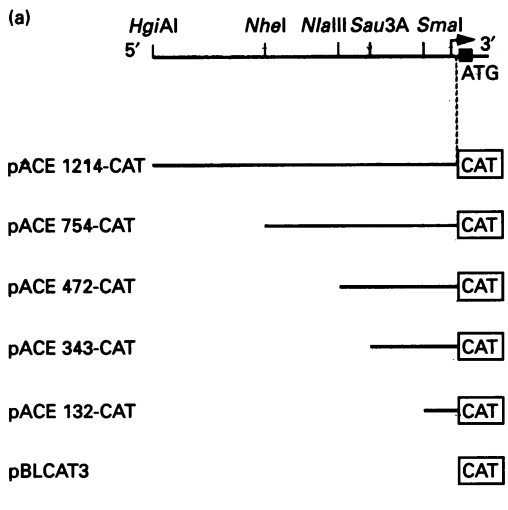


Figure 1 Expression of ACE activities and *ACE* transcripts in RE, human embryonal carcinoma (Tera-1) and human hepatocarcinoma (HepG2) cell lines

(**a**) Confluent cultures were fed overnight without fetal-calf serum and washed extensively to eliminate serum ACE. The cells were harvested and membrane proteins were solubilized by treatment with CHAPS detergent. The resulting supernatants were assayed for ACE activity as described in the Materials and methods section, using Hip-His-Leu as substrate under initial rate conditions. Abbreviation: n.d., means no detectable activity, i.e. value was under the sensitivity of the dosage. (**b**) RT-PCR amplification of cDNA generated from the three different cell lines was performed using specific primers to the somatic *ACE* cDNA as described in the Materials and methods section. (1) Ethidium bromide staining of PCR products separated by 2% (w/v) agarose gel electrophoresis. The 212 bp products are indicated. (2) Autoradiography of the PCR products after hybridization with an α-^{32}P-labelled probe corresponding to an oligonucleotide, in exon 14, located within the amplified fragment.



(b)

Relative CAT activity (%)

	RE	Tera-1	HepG2
pACE 1214-CAT	7.9 ± 1.4 (6)	19.5 ± 2.3 (4)	3.3 ± 1.6 (5)
pACE 754-CAT	6.8 ± 2.5 (5)	12.3/14.7 (2)	1.2 ± 0.6 (5)
pACE 472-CAT	15.5 ± 3.9 (8)	36.2 ± 6.4 (5)	4.1 ± 0.9 (5)
pACE 343-CAT	8.4 ± 0.7 (5)	11.8/20.3 (2)	1.7 ± 0.2 (3)
pACE 132-CAT	11.4 ± 4.4 (5)	47.1 ± 0.8 (3)	4.1 ± 2.9 (4)
pBLCAT3	1.0 ± 0.1 (6)	4.8 ± 2.0 (3)	0.9 ± 0.2 (5)

Figure 2 *ACE* promoter constructs and their respective CAT activities in RE, Tera-1 and HepG2 cells

(**a**) *Top*, partial restriction map of the 5′ flanking region of the *ACE* gene showing the sites used for the *CAT* constructs. The arrow indicates the transcription start site of the *ACE* gene. *Bottom*, representation of the pACE–CAT plasmid constructs obtained by insertion of the 5′ flanking sequences of the *ACE* gene into the promoterless pBLCAT3. Names designated to each construct, shown on the left, are indicated in base pairs relative to the initiation of the somatic *ACE* transcript. (**b**) The values of CAT activities of the various constructs have been normalized with the β-galactosidase activity generated by the RSVβ-galactosidase plasmid co-transfected with the tested constructs, and are expressed as a percentage of the CAT activity of the RSV–CAT plasmid taken as 100%. The data are expressed as relative CAT activity ± S.D. When two assays were performed for the constructs, the individual values are mentioned in the table. The numbers in parentheses are the number of independent transfections performed for each construct. When the plasmids were tested in the antisense orientation the relative CAT activities displayed the same values as with pBLCAT3.

substantial loss of activity of 46%. Further deletion of 211 bp, leading to the pACE132–CAT, produced an equivalent relative CAT activity compared with the 472 bp fragment. The 132, 472 and 1214 bp fragments indicated relative CAT activities of 0.9%, 1.2%, 0.8% respectively, when inserted in the reverse orientation, and these values were close to the pBLCAT3 expression. Therefore, the major elements required for somatic *ACE* promoter activity in endothelial cells are located within the 132 bp upstream from the transcription start site.

To elucidate whether RNA transcription from the pACE472–CAT construct involved the same initiation site as the *ACE* gene in the endothelial cell, a ribonuclease-protection assay was performed with the transfected *ACE–CAT* fusion gene. RNA from transfected cells was extracted and hybridized with a T3-promoter-driven radiolabelled antisense RNA probe. This probe was synthesized from a PCR-generated fragment (319 bp) spanning the transcription start site. The protected RNA fragment from transfected cells exhibited the expected size of approx. 294 bases when compared with DNA molecular-mass markers, demonstrating that the transcription fusion gene initiated at the authentic start site of the human *ACE* gene (Figure 3). No protected fragments were observed when yeast tRNA was used, thus confirming the specificity of the detection.

Cell specificity of the somatic *ACE* promoter

The ability of the *ACE* promoter region to confer cell specificity was tested by transfection of the *CAT* constructs into both Tera-1 and HepG2 cells. These cell lines were used since the Tera-1 cells express high levels of endogenous ACE activity, whereas ACE activity and ACE mRNA cannot be detected in the HepG2 cells (Figure 1). The transfections of the constructs in Tera-1 cells displayed an overall high promoter activity, since the pACE132–CAT expression was 4-fold higher than in RE cells (47% versus 11.4%). The pACE132–CAT construct corresponded to the maximal promoter activity in Tera-1. The addition of a fragment of 340 bp (pACE472–CAT) produced a decrease of 23% of promoter activity and the further addition of 282 bp (pACE754–CAT) or 742 bp (pACE1214–CAT) indicated a continuous diminution in activity by 29% and 41% respectively of the maximal activity (Figure 2b). All the reverse-orientation inserts gave similar results to those of the promoterless pBLCAT3. Thus, in a human cell line expressing a high level of ACE activity and ACE mRNA, a high promoter activity is present in all fragments studied.

In HepG2, the maximal activity compared with the pRSVCAT was only 4% compared with 11% and 47% in RE and Tera-1 cells respectively. This value was less than the lowest CAT activity measured in endothelial cells (Figure 2). For all constructs, the relative CAT activities were close to the basal pBLCAT3 value.

No transcriptional response to glucocorticoids in fragments up to 3.6 kb in the 5′ flanking region of the *ACE* gene

Within the sequence of 1214 bp of 5′ flanking region of the *ACE* gene, we noticed three potential glucocorticoid-responsive elements (GREs) at positions −935, −794 and −686 bp from the transcription start site (Hubert et al., 1991). To test the functionality of the GRE sequences in RE cells, preliminary experiments were carried out with the plasmid pMAMneoCAT. This plasmid contains the GRE of the mouse mammary tumour virus long terminal repeat, which drives the expression of the *CAT* gene. RE cells were co-transfected with pMAMneoCAT and the expression vector of the human glucocorticoid receptor (pHGO) which gave a 18.7-fold induction by glucocorticoid (Table 1). The effect of dexamethasone (5×10^{-7} M) in a serum-free medium on the expression of the chimeric gene pACE1214–CAT was studied using glucocorticoid-induced RE cells. To minimize the variations of *CAT* expression between two separate calcium phosphate precipitates, the same DNA precipitate was used; one in the absence and one in the presence of gluco-

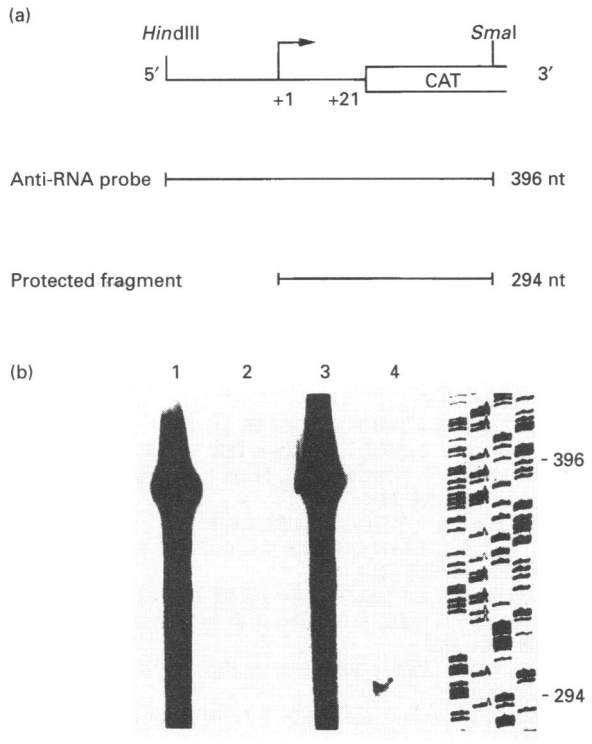


Figure 3 RNAase mapping of transcription initiation in RE cells transfected with the pACE472–CAT construct

(**a**) Schematic representation of the cloned DNA fragment of the hybrid *ACE–CAT* gene included 26 bp upstream and 21 bp downstream of the *ACE* transcription start site and segment of the *CAT* coding sequence (open bar). The fragment was linearized by *Hin*dIII of the polylinker SKII vector to synthesize an antisense RNA probe of 396 nucleotides which included 77 bp of the polylinker. The probe was hybridized with transfected endothelial RNA. After RNAases $A+T_1$ treatment the protected fragment comprises 294 nucleotides. (**b**) Autoradiogram of ribonuclease protection assay: 50 μg of tRNA were hybridized with the anti-RNA probe as mentioned in (**a**), followed by no (lane 1) or $A+T_1$ (lane 2) ribonuclease treatment. ACE–CAT mRNA (50 μg) was hybridized with the same probe followed by no (lane 3) or $A+T_1$ (lane 4) ribonuclease digestion. The sequence of M13mp18 DNA is shown on the right.

Table 1 Effect of dexamethasone on the expression of pMAMneoCAT, pPBLCAT2 and different *ACE* promoter constructs transfected into RE cells

CAT activites are quoted as the means of four transfection experiments. They indicate the chloramphenicol acetylation percentage normalized to a constant β-galactosidase unit. Samples (2 μg) of an expression vector of the glucocorticoid receptor (pHGO) were co-transfected as mentioned (+) with 15 μg of the test plasmid and 10 μg of β-galactosidase plasmid. After co-transfection, the cells were refed with a serum-free medium in absence or presence of 5×10^{-7} M dexamethasone (Dex) for induction assays.

		Relative CAT activity (%)				
	pHGO...	−	−	+	+	Induction
Plasmid	Dex...	−	+	−	+	(fold)
pBLCAT2				3.0	3.1	1.0
pMAMneoCAT		1.1	1.1	1.5	28.0	18.7
pACE 1214–CAT				2.1	2.2	1.1
pACE 2.2 tk–CAT				3.1	3.1	1.0
pACE 3.3 tk–CAT				3.4	5.0	1.5

corticoids. No dexamethasone induction was detected with plasmid pACE1214–CAT. The GRE could be located within the region upstream of the 1214 bp fragment already studied. Therefore two other DNA fragments, −3.6 and −2.5 kb upstream from the transcription start site, were analysed. Neither of these two fragments (−3.6 kb to −295 bp and −2.5 kb to −295 bp) in front of the *tk* promoter were stimulated by dexamethasone under conditions where the control, pMAMneoCAT, was induced.

DISCUSSION

The human *ACE* cDNA was previously isolated from an umbilical-vein-endothelial-cell cDNA library (Soubrier et al., 1988). However, primary cultures of endothelial cells, as well as available human endothelial cell lines, do not transfect efficiently (P. Testut, unpublished work); therefore cell cultures from different species, for example bovine aortic endothelial cells, have been used to study the human preproendothelin-1 gene (Lee et al., 1991) or the human transcription factor GATA-2 protein (Dorfman et al., 1992). The RE cell line has previously provided a useful model for biochemical studies of another zinc metalloprotease, neutral endopeptidase (EC 3.4.24.11) (Llorens-Cortes et al., 1992). To evaluate if this efficiently transfectable cell line was suitable for the study of the *ACE* promoter, the presence of ACE mRNA and ACE activity was first confirmed. The transcription start site was analysed using transient transfection of a human *ACE* promoter linked to a *CAT* gene construct. A RNAase-protection assay confirmed that the site of the *ACE–CAT* fusion transcript was identical to the ACE mRNA initiation site in human endothelial cells (Hubert et al., 1991).

Transcription is one critical step at which regulation of gene expression might occur. To analyse the transcriptional regulation of the *ACE* gene, a series of 5′ deletions of the somatic *ACE* promoter were performed and inserted upstream of the *CAT* reporter gene. Promoter activities were measured in transient transfection assays. In endothelial cells the 5′ region of the *ACE* gene directed the transcription of the reporter *CAT* gene. The strength of the promoter activity depended on the length of the promoter inserted into the construct, which enabled us to identify various DNA regulatory regions. There are at least two positive and two negative cis-acting regions involved in the *ACE* promoter. The proximal region, within the first 132 bp upstream from the transcription start site, is sufficient to significantly produce *CAT* expression over the control promoterless vector. The orientation of this fragment was crucial, since transfection in the reverse direction resulted in a promoter activity equivalent to the pBLCAT3 plasmid. The activity of the proximal fragment was made possible by the presence of (a) a canonical TATA box located 41 bp upstream from the transcription start site and (b) three potential binding sites for the SP1 factor (Shai et al., 1990; Hubert et al., 1991). The SP1 factor is involved in the transcription of a large number of genes (Dynan and Tjian, 1985; Gumucio et al., 1991; Li et al., 1991; Meroni et al., 1991). In the *ACE* promoter it should be noted that two SP1-binding sites are in phase on the same side of the helix which appeared to influence transcription of the adenovirus 2 early1B promoter more efficiently in HeLa cells (Segal and Berk, 1991).

ACE activity in Tera-1 cells was much higher than in the RE cell line, consistent with the higher overall promoter activity achieved in Tera-1 cells. However, the pattern of *CAT* expression was almost parallel, except for the first 132 bp construct where a marked enhancement of transcription activity was observed. This increased activity may be due to the presence of transactivating factors expressed at higher levels in Tera-1 cells, the activation of alternative elements in the two cell types, or to a better recognition of nuclear proteins in a homologous human system (Somma et al., 1991; Paulweber et al., 1991).

In order to test the cell specificity of the *ACE* promoter, HepG2 cells were used as a model cell line which does not express *ACE*. We first confirmed that these cells do not contain measurable ACE activity or ACE mRNA after RT-PCR amplification. In transfection assays, irrespective of the length of the promoter tested, CAT activities were detected close to basal transcription, as commonly observed in experiments *in vitro*. The markedly reduced expression in HepG2 cells contrasts with the expression observed in RE cells, suggesting that cell-specific regulatory elements located in RE cells are within the first 132 bp upstream from the initiation start site. This is consistent with the hypothesis of the cell-specificity mechanism.

The potential GREs shown within the sequence of the *ACE* gene studied are not functional in transfection assays. Up to 3.6 kb from the transcription start site, no transcriptional enhancement was observed in the presence of dexamethasone. This result is in contrast with earlier reports (Krulewitz et al., 1984). Two hypotheses could explain this discrepancy. First the GRE might be located elsewhere in the *ACE* gene, i.e. further upstream as described for a milk protein, whey acidic protein (WAP), where important hormonal regulatory elements are located between −6300 and −3000 bp upstream from the WAP transcription start site (Devinoy et al., 1991); or alternatively, the GRE might also be located within the gene, as for the 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase where it was located in the first intron of the gene (Lange et al., 1992). Secondly, in experiments *in vitro* the observed induction by glucocorticoids of the ACE protein could be the consequence of the stabilization of ACE mRNA as established for other transcripts such as human growth hormone or phosphoenolpyruvate carboxykinase (Paek and Axel, 1987; Petersen et al., 1989).

This is the first study to demonstrate that the human somatic *ACE* promoter is controlled by a combination of both positive and negative regulatory elements located within the 5′ flanking region, as has been previously described for several other genes, for example, the mouse multidrug resistance 1b (*mdr 1b*) gene, the rat ceruloplasmin and the human blood coagulation factor X (Cohen et al., 1991; Fleming and Gitlin, 1992; Huang et al., 1992). Our results indicated that 132 bp upstream from the transcription start site of the *ACE* gene is sufficient for the promoter activation co-ordinated in an endothelial cell line. Further characterization of the *ACE*-promoter region and study of its interactions with nuclear transcription factors will increase our understanding of the genetic mechanisms that govern the expression of *ACE* in different cell types.

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