Use of alternative polyadenylation sites for tissue-specific transcription of two angiotensin-converting enzyme mRNAs

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ABSTRACT

The pulmonary isozyme of rabbit angiotensin-converting enzyme (ACE) is encoded by an mRNA of about 5 kb. cDNA clones corresponding to different parts of this mRNA have been isolated and the complete nucleotide sequences of both the coding and non-coding regions of the mRNA have been determined. The encoded protein has 1309 residues with a 33 amino acids-long signal peptide at the amino terminus and a potential membrane-anchoring domain near the carboxyl terminus. There is a strong sequence homology between two regions of the rabbit cDNA and between the rabbit, human, and mouse cDNAs. Comparison of the nucleotide sequences of the 3' untranslated regions of rabbit pulmonary and testicular ACE cDNAs revealed that the testicular cDNA is nested within the pulmonary cDNA at the 3' end. A rabbit genomic clone encompassing this region was isolated and partially sequenced. It was shown that the gene contains two potential polyadenylation sites 628 bp apart within one exon. Northern analyses with an appropriate oligonucleotide probe confirmed that the proximal polyadenylation site is used exclusively for terminating the testicular mRNA whereas the distal one is used exclusively for the pulmonary mRNA. These results demonstrated that the transcription of the two mRNAs encoding the two ACE isozymes not only initiates at two alternative tissue-specific sites which are 5.7 kb apart but the mRNAs also get polyadenylated at two alternative sites which are 628 bp apart.

INTRODUCTION

Angiotensin-converting enzyme (EC 3.4.15.1) is a carboxyl terminal dipeptidyl exopeptidase which converts angiotensin I to angiotensin II, the biologically active hormone. It also acts upon other important peptide hormones such as bradykinin. Through its action on the above two substrates, the enzyme plays an important role in the regulation of blood pressure (1).

There are two isozymes of angiotensin-converting enzyme (ACE) which are expressed in a tissue-specific manner (2). The

pulmonary isozyme, ACE_P, is produced by the vascular endothelial cells as well as by kidney tubular epithelial cells, intestinal brush-border cells, specific cell types in the brain and macrophages (3). The testicular isozyme, ACE_T, is produced exclusively by maturing sperm cells (4). Rabbit ACE_P is a glycoprotein of 140 kDa whereas ACE_T has a molecular mass of 100 kDa (2). Rabbit ACE_T is encoded by a 2.5 kb mRNA. We have cloned and sequenced the corresponding cDNA thereby providing the primary structure of this isozyme (5). Rabbit ACE_P is encoded by a 5 kb mRNA. This paper reports the cloning and sequencing of rabbit ACE_P cDNA. The two isozymes have segmental mosaic structures. The carboxyl terminal 665 amino acid residues of ACE_P and ACE_T are identical whereas the amino terminal half of ACE_P and 72 residues at the amino terminal of ACE_T are unique to the respective isozymes (5). The cDNAs for the human and mouse isozymes have been cloned by other workers (6–9).

Recently, we have shown that the two ACE mRNAs encoding the two rabbit isozymes originate from the same gene. The sequence organization of the gene is such that the unique sequence of mRNA_P precedes that of mRNA_T which, in turn, precedes the sequence common to both mRNAs (10). Transcription of the two mRNAs initiates at two alternative sites which are 5.7 kb apart. For generation of mRNA_P, the mRNA_T-specific sequence is eliminated as an intron from the primary transcript. Transcription of mRNA_T, on the other hand, initiates within this intron at the middle of the gene. In this paper, we report that the two mRNAs also terminate at two different sites. The gene contains two alternative polyadenylation sites which are on the same exon but 624 bp apart. mRNA_T uses the proximal site exclusively whereas mRNA_P uses the distal site exclusively. The mRNA_T transcription unit is, therefore, nested completely within the mRNA_P transcription unit.

EXPERIMENTAL PROCEDURES

Isolation of rabbit pulmonary ACE cDNA clones

Rabbit cDNA for the testicular ACE which was isolated previously (5) was used as a probe to screen a rabbit lung cDNA library. The rabbit lung cDNA library in λgt11 was purchased

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from Clontech, California. About 1×10^6 recombinants were screened with a ^{32}P -labeled probe. Several positive clones were obtained from the first screen. Tertiary screening revealed 2E and 1A as positive clones. After extracting phage DNA from these clones, the DNAs were digested with EcoRI and subcloned into pGM7Z for restriction mapping and sequencing analysis. To get the cDNA clone 3, the library was rescreened with a 5', 272 bp SacII fragment of the genomic clone 226 (10). EcoRI digestion of clone 3 released two fragments of size ~600 bp and 400 bp in length. Both fragments 3B and 3S were subcloned into the EcoRI site of the pGM7Z for further analysis. Sequencing was performed by the dideoxy chain termination method with SequenaseTM (United States Biochemicals) and ^{35}S labeled ATP (10).

Isolation of a rabbit genomic clone encompassing the 3' region of the ACE gene

A rabbit genomic DNA library in the vector EMBL3 was purchased from Clontech Laboratories. Isolation and characterization of the 5' regions of the pulmonary and testicular ACE gene has been described recently (10). For isolating the 3' region of the gene, the library was rescreened with a 504 bp, PvuII-3' fragment of the testicular ACE cDNA (5). One phage called ACE-G3 was plaque purified for further analysis. After restriction digestion analysis, a 4.5 kb XbaI fragment was found to hybridize with an oligonucleotide probe specific for the 3' untranslated regions of ACE_T and ACE_P mRNA. This fragment was subcloned into pGM7Z vector for sequence analysis. The sequence was determined on both strands by priming double stranded DNA with specific oligonucleotides.

RNA isolation and Northern blot analysis

Rabbit lung and rabbit testis mRNA was isolated and analyzed according to the protocol described before (5). Two identical blots, each containing 5 µg of lung and testis poly A⁺ RNA per lane, were prepared. Lung specific probe was constructed by using an antisense oligonucleotide from 4553 to 4570 nucleotides of the lung cDNA. The common probe was prepared by using an antisense oligonucleotide from 2234 to 2263 nucleotides of the lung cDNA. Both filters were prehybridized and hybridized as previously described (5). Autoradiography was carried out at -80°C using Kodak XAR-5 films.

Sequence Analyses

The DNA sequence were analyzed for open-reading frames using the IBI MacVector Program. For dot-matrix analyses of the nucleotide sequences, Pustell DNA Matrix Method was used.

RESULTS

Isolation of ACE_P cDNA clones

Since from our previous studies it was known that lung is rich in ACE_P mRNA, a commercial λgt11 cDNA library made from rabbit lung mRNA was used for the isolation of ACE_P cDNA clones. We have reported that ACE_P mRNA is approximately 5 kb long (5). Because of this length, the corresponding cDNA was not isolated as one clone but as a series of overlapping clones. Upon screening about a million plaques with ACE_T cDNA as a probe, we isolated 200 positive plaques. Many of them represented the 3' region of the mRNA which overlaps with ACE_T mRNA. Two such clones were purified, characterized, and sequenced. Clone 2E contained 3216 nucleotides and clone

Figure 1. Alignment of cDNA clones representing different regions of rabbit ACE_P mRNA. Clone S273 was derived from the previously described genomic clone 226 (10) by digestion with SacII. Other clones are newly isolated cDNA clones whose sequences were determined. The composite mRNA_P is shown at the bottom. The boxes indicate the coding regions and the line shows the untranslated regions. The coding region is divided into distinct domains as described before (5).

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MGAAPGRRGPRLLRPFPPLLLLLLLLLLPPFPALTLDPGLPGDFADEAGARLFASSYNS 60
SAEQVLFSTASMAHDTNITAENARRQEEALLSQFAEANGRRRLSSHTRCGRSTPTQ 120
SCAGSSGLCAPWPCQAPGQAADNSLLSNMSQIYSTGRSASPTRLPAANSLDPDLNII 180
ASSRSYAMLLFANEGWHNAVIGIP LKPLYQEFTALSNEAYRQDGFSDTGAYWRSWYDPTT 240
EEDLERIYHQLEPLYLNLHAYVRRVLRHRRYGDRIINLRGPIPAHLLGNMMAQSWESIYDM 300
VVPFPDKPNLDVTSTMVQKGNATHMFRVAEEFTSLGLLPMPPEFWAESMLEKPDGRE 360
VVCHASAMDFYNRKDFRIKQCTQVTMDQLSTVHEMGEHQYLYQYKQDQVSLRRANPGFH 420
EAIQDVLALSVSTPAHLHKIGLLDHVTNDTESDINYLKMALEKIAFLPFYGLVDQNRWG 480
VFSGRTPSSRYNFDWYLRKYQGICPPVVRNETHFDAGAKHIPSVTPYIRYFVSFLVQ 540
FQFHQALCMEAGHGQPLHQCDIYQSTRAGAKLRVLAQGCSPWQEVLDKMDVASDALDAQ 600
PLLDYFQFPTQNLQEQNERNGEVLGWPEYQWRPPLPNYPPEGIDLVTDEAEASRFVEYD 660
RSFQAVWNEYAEANWNYNTNITTEASKILLQKMMQIAHNTLTGWMARRFDVSNFQATS 720
KRIKKVQDLQRAVLVPKELEEYNQILLDMETIYSVAVPCRVDSGLQLEPDLTNLMATS 780
RKYDELLWVMTSWRDKVGRAILPYFPKYVEFTNKAARLNGYVDAGDSWRSMYETPTLEQD 840
LERLFQELQPLYLNLHAYVGRALHRRHYGAQHINLEGP IPAHLLGNMMAQTSWNIYDLVAP 900
FPSASTMDATEAMIKQGWTPRRMFEEADKFFISLGLLPVPEFWNKSMLKPTDGREVVC 960
HASAMDFYNGKDFRIKQCTTVNMEDLVVHEMGEHQYLYQYKQDQVSLRRANPGFHEA 1020
IGDVLALSVSTPKHLHSINLLSSEGGGYEHDINFLKMALEKIAFIPFSYLVDEMRVRF 1080
DGSITKENYQEWWSLRKYQGLCPPAPRSQGDGFGAKFHIPSSVPYIRYFVSFIQFQ 1140
FHEALCKAAGHTGPLHTCDIYQSKAEGKRLADAMKLGYSKPWPEAMKVITGQPNMSASAM 1200
MNYFKPLMDWLLTENGRRGEKLGWPQYTWTPNSARSEGLSDSGRVNFGMNLDAQQARV 1260
GQWVLLFLGVALLLASLGLTQRLFSIRYQSLRQPHRGQFQSEVELRHS 1309

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Figure 2. Deduced amino acid sequence of rabbit ACE_P protein. The residues are numbered from the amino terminus. The signal peptide at the amino terminus is underlined as is the putative anchoring domain near the carboxyl terminus. The potential N-glycosylation sites are marked by asterisks. The two putative Zn²⁺-binding domains are boxed. The nucleotide sequence of rabbit ACE_P has been submitted to EMBL data bank (Accession number X62551).

1A contained 1335 nucleotides which were partially overlapping (Fig. 1). For obtaining cDNA clones which represent the 5' region of the mRNA, the 200 original plaques were further screened using a 5'-specific probe. This probe, S273 (Fig. 1), was generated from the genomic clone 226 (10) by digestion with SacII, and it contained sequences present at the 5' end of ACE_P mRNA. Clone 3 was isolated using this probe. It contained an internal EcoRI site and hence was cleaved into two pieces during the release of the cDNA insert from the λgt11 clone. The two pieces, 3B and 3S, were separately subcloned and sequenced. Analyses of the sequences of the four cDNA clones, 3B, 3S, 2E, and 1A, allowed us to align these clones (Fig. 1) and provided the complete sequence of ACE_P mRNA. 3B contained nucleotides 66 to 659. It overlapped at the 5' end with the previously isolated genomic clone containing the first exon. Clone 3S contained nucleotides 660 to 1064. Clone 2E corresponds to nucleotides 1065 to 4280, and clone 1A was from nucleotides

Figure 3. Dot matrix analysis of rabbit, human, and mouse ACE_p mRNAs. The rabbit ACE_p sequence is compared with itself in (A), with the mouse sequence in (B), and with the human sequence in (C). Regions of 65% or greater homology are marked. The long diagonal lines in the middle strong sequence homology. The offset diagonal lines indicate internal homologies with a cDNA (24).

3466 to 4800. Clones 3B, 3S, and 2E are contiguous with natural *EcoRI* sites at the junctions. Since 3B and 3S originated from one λ clone, it is safe to conclude that they were contiguous clones. However, the same is not true for the junction of 3S and 2E and it is possible that a bridging *EcoRI* fragment is missing. To examine this possibility, a cDNA clone was generated by reverse transcription of the mRNA followed by polymerase chain reaction using synthetic primers corresponding to the two sides of this junction. Sequencing of this cDNA confirmed that clones 3S and 2E are indeed contiguous and the bridging *EcoRI* site is present in the mRNA. The four cDNA clones and a genomic clone containing the first exon (S273) were used to construct a composite complete cDNA clone for rabbit ACE_p mRNA. It has 4800 nucleotides (EMBL accession no X62551). The translation initiation codon is at 27–29 nucleotides and the termination codon is at position 3954–3956.

Analyses of ACE_p sequence

The primary structure of ACE_p protein was derived from the nucleic acid sequence (Fig. 2). It contains 1309 residues. There is a 33 amino acids-long hydrophobic signal sequence at the amino terminal which is presumably cleaved off during post-translational processing of this protein. This signal peptide does not have any

Figure 4. Nucleotide sequences of the 3' untranslated region of the ACE_p cDNA and the corresponding region of a genomic clone. (A) The cDNA sequence is shown. The termination codon in the beginning is overlined and the two polyadenylation signals are boxed. (B) Sequence of the corresponding region of a genomic clone $ACE-G3$ is shown (EMBL Accession number X62549). The termination codon is overlined and the two polyadenylation signals are boxed. Arrows indicate the last nucleotides before the poly A tails of the two mRNAs. The GT- and TT-rich sequences are underlined.

sequence homology with the signal peptide of rabbit ACE_T (5). The sequence at the amino terminal of the mature protein is identical to the previously determined sequence of purified rabbit ACE_p (11). As was noted for ACE_T , ACE_p also contains a highly hydrophobic sequence near its carboxyl terminus. The carboxyl terminal half of the molecule beginning from residue 641 is identical to the corresponding region of the rabbit ACE_T since they are encoded by the same region of a single gene (10). As noted for ACE_p from other species (6,7), ACE_p from rabbit also has strong internal homology. There are two potential Zn^{++} binding sites around which there is very strong conservation of sequence. ACE_p contains 11 potential N-glycosylation sites. The native protein is known to be extensively glycosylated (2).

For comparing the sequence homologies among rabbit, human, and mouse ACE_p , dot matrix analyses at the nucleotide level were performed (Fig. 3). This analysis revealed strong internal homology between two domains of rabbit ACE_p sequence. Two stretches of sequences between 700 and 1700 and between 2500 and 3500 have very strong homology (Fig. 3A). Comparison of

Figure 5. Northern analyses of rabbit lung and testis RNAs. 5 μ g of poly A⁺ RNA from testis (lane 1) and lung (lane 2) was analyzed. An antisense oligonucleotide probe corresponding to nucleotides 4553 to 4570 (Fig. 4) was used in (A) and an antisense oligonucleotide probe corresponding to nucleotides 2234 to 2263 (Fig. 1) was used in B.

the rabbit sequence with the cDNA sequences of the human and mouse enzyme revealed that there is very strong sequence conservation between the three species. The rabbit sequence was virtually identical with the human sequence (Fig. 3C) but slightly less similar to the mouse sequence (Fig. 3B). The homology between the rabbit and the mouse sequence, however, was entirely absent from the 3' region. From about nucleotide 3950 to the 3' terminus there was virtually no homology. This region constitutes the 3' untranslated region of the mRNA.

Termination of ACE_P mRNA

The nucleotide sequence of the 3' untranslated region of ACE_P mRNA is shown in Fig. 4A. Comparison of the rabbit ACE_P mRNA sequence with the corresponding region of ACE_T mRNA revealed that, as expected, they share identical sequence. However, the ACE_T mRNA terminated 628 nucleotides upstream of the 3' end of ACE_P mRNA. ACE_P mRNA transcription used the polyadenylation signal present at position 4743 but not the one present at position 4123 which was used for terminating ACE_T mRNA. The fact that the 3' end of ACE_T mRNA was completely nested within ACE_P mRNA indicated that in this region there was no alternative splicing between the two mRNAs.

For the purpose of analyzing the alternative polyadenylation sites of the two mRNAs more thoroughly, we examined the corresponding region of the gene. A new genomic clone encompassing this region of the rabbit ACE gene was isolated and the relevant region was sequenced (Fig. 4B). The genomic sequence was identical to the ACE_P cDNA sequence thereby confirming the absence of any intron in this region of the gene. It appears, therefore, that for ACE_T mRNA termination the proximal polyadenylation signal is used whereas for ACE_P mRNA the distal one is used. In the experiment shown in Fig. 5, we examined if the use of the two alternative polyadenylation sites was absolutely tissue-specific. Rabbit testicular and pulmonary polyA⁺ RNAs were subjected to Northern analyses using two labeled oligonucleotide probes. One probe contained a sequence common to both ACE mRNAs whereas the second oligonucleotide corresponded to nucleotides 4553 to 4570 from the untranslated region of ACE_P mRNA. The common probe hybridized with a testicular mRNA of 2.5 kb and a pulmonary mRNA of 5 kb (Fig. 5B). The other probe hybridized only with the pulmonary mRNA (Fig. 5A), thereby demonstrating that the testicular mRNA terminates exclusively at the proximal

Figure 6. Schematic illustration of alternative transcription initiation and polyadenylation sites of ACE_P mRNA and ACE_T mRNA. The middle boxed region represents the ACE gene. The upper thick line represents the ACE_P mRNA and the lower thick line represents the ACE_T mRNA. The upward arrows indicate the transcription start sites and the downward arrows indicate the polyadenylation sites. The hatched lines indicate discontinuity. Only three regions of the gene and the mRNAs are shown: where ACE_P mRNA initiates, where ACE_T mRNA initiates and the crucial alternative splicing event takes place, and where the two mRNAs terminate and get polyadenylated at two alternative sites. The distances between the two tissue-specific initiation sites (approximately 5.7 Kb) and between the two tissue-specific polyadenylation sites (628 b) are indicated. The diagram is not drawn to scale.

polyadenylation site. It appears that the opposite is true for ACE_P mRNA since both the common probe and the discriminatory probe hybridized with only one lung mRNA. If some of the ACE_P mRNA molecules were terminated at the proximal site, we would have noticed a lower band hybridizing with the common probe but not the other probe. The underlying mechanism for this absolute tissue-specific choice of alternative polyadenylation site remains to be determined. However, comparison of the genomic sequences following the two sites revealed interesting differences. The distal site used for ACE_P mRNA transcription is followed by numerous GT and TT sequences (Fig. 4B). Such sequences have been shown, for other genes, to be important determinants of the polyadenylation event (12). They constitute, in conjunction with the AATAAA sequence, the polyadenylation signal for many transcription units. In contrast to the distal site, the proximal ATTAAG sequence was followed by only four non-contiguous GT sequences. Obviously, these or other unidentified elements are recognized as the polyadenylation signal by the ACE_T transcription unit but not the ACE_P transcription unit.

DISCUSSION

With the cloning of rabbit ACE_T (5) and ACE_P cDNAs in our laboratory and those from human and mouse in other laboratories (6–9), the structural relationship between the two isozymes has been delineated. ACE_P seems to be the product of duplication of an ancestral gene (13,14). The functional significance of the particular structures of the two isozymes remains to be understood. The testicular enzyme, although present in relatively high abundance, does not have any known physiological function. The more ubiquitous pulmonary enzyme has the same enzymatic property as the testicular enzymes (1). Recent expression studies have started to shed light on the structure-function relationships of this enzyme (15–17). Both halves of ACE_P, when separately

expressed, displayed enzyme activity although there are some differences (18). In contrast, in the native enzyme, only one enzymatic center seems to be active (19). The physiological significance of the amino-terminal half of ACE_P therefore remains an enigma. However, the strong sequence conservation in this region suggests that this region is important for some functional property. The sequence is highly conserved not only between the two halves of the molecules but among different species as well. There is about 85% amino acid identity between ACE_P of the three species. This is in contrast to the total divergence of the sequence in the 3' untranslated region. These observations suggest that there are strong evolutionary pressures to maintain the existing primary structure of ACE_P and ACE_T. It should be kept in mind, however, that both originate from the same gene. Therefore, conservation of sequences in the common regions can be maintained due to the structural needs of one isozyme or the other.

Previously, we have shown (10) that the transcription of two ACE mRNAs initiate at two sites on the gene which are separated by 5.7 kb (Fig. 6). ACE_T mRNA initiates within an intron of ACE_P mRNA. The results presented in this paper demonstrated that the two mRNAs also terminate at two different sites which are 628 bp apart. Like the choice of initiation sites, the choice of termination site was also tissue-specific and absolute. We could not detect any ACE_T mRNA which terminated at the distal site and any ACE_P mRNA which terminated at the proximal site. The interesting proposition that the choice of a specific initiation site is coupled with a specific termination site, remains to be tested. Introduction of mutated ACE genes in which one or the other termination site has been destroyed will be needed for experimentally testing this hypothesis.

Although the mechanism of tissue-specific choice of the alternative polyadenylation sites remains to be determined, inspection of the sequences around the two sites revealed important differences. The proximal polyadenylation signal is AUUAAA whereas the distal one is AAUAAA. Both are used in higher eukaryotes although the AAUAAA signal is more prevalent (12). More remarkable is the difference in G+U rich sequences after these two signals. In cell lines and tissues tested so far, these sequences have been shown to be required for proper polyadenylation (12). Two protein factors have been identified which bind respectively to the AAUAAA sequences and the G+U rich sequences (20). Both factors are usually needed for polyadenylation. In the ACE gene, G+U rich sequences are abundant in the region immediately 3' to the distal polyadenylation sites whereas there are only four isolated GU sequences after the first polyadenylation site. The distal site is also followed by a GTGTTY sequence which is absent from the proximal site. Such a sequence has also been implicated in efficient polyadenylation (21). One can speculate that in sperm cells, but not in somatic cells, polyadenylation can proceed without these sequences. Such speculation can be supported by the fact that the polyadenylation signals are not as evolutionarily conserved as the initiation signals. For example, in lower eukaryotes such as yeast, the polyadenylation signals are entirely different (12). But even if this hypothesis were true, one would expect some ACE_T mRNA terminating at the distal site, which was not experimentally observed. Use of cryptic polyadenylation sites by transcripts in testis has been reported for other genes (22). Specific secondary structures in the RNA can spatially juxtapose widely separated AAUAAA signal and GU-rich element. Such a mechanism has been shown to operate in

HTLV-1 mRNA transcription (23). Formally, this mechanism may also be responsible for ACE_T mRNA termination. Other, as yet unidentified, elements may also be crucial for polyadenylation of testicular transcripts.

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