A Cyclic AMP Response Element in the Angiotensin-converting Enzyme Gene and the Transcription Factor CREM Are Required for Transcription of the mRNA for the Testicular Isozyme*

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The angiotensin-converting enzyme (ACE) gene produces two mRNA species from tissue-specific promoters. The transcription start site of the mRNA for the smaller testicular isoform (ACE T) is located within an intron of the larger transcription unit that encodes the pulmonary isoform (ACE P). We have previously demonstrated that a 298-base pair DNA fragment, 5’ to the rabbit ACE T mRNA transcription initiation site, can activate the testicular expression of a transgenic reporter gene. In the current study, using the same transgenic reporter system, we identified a putative cyclic AMP response element present within this DNA fragment to be absolutely essential for transcriptional activation. Moreover, we observed that ACE P mRNA was not expressed in the testes of mice homozygous for a null mutation in the same gene through tissue-specific choice of transcription factor CREM. However, in the same mice, ACE P mRNA was abundantly expressed in the lung. Our observations indicate that ACE P mRNA expression in the testes is regulated by the putative cyclic AMP response element present 5’ to the transcription start site and the corresponding transcription factor CREM.

Angiotensin-converting enzyme (ACE) is a carboxyl-terminal dipeptidyl exopeptidase that converts angiotensin I to angiotensin II, a potent vasopressory hormone (1). The two isoforms of ACE, ACE P and ACE T, have identical enzymatic activity and are encoded by distinct mRNAs transcribed from the same gene through tissue-specific choice of transcription initiation and polyadenylation sites (2–4). The genomic organization is such that the ACE P transcription unit is nested within the ACE T transcription unit (5–7). In addition, the first exon of ACE P mRNA is unique to the ACE T transcription unit. The ACE T promoter and first ACE P exon reside in the 12th

ACE P intron, which is spliced out of the primary ACE P transcript (2, 8). Rabbit ACE P is a glycoprotein of 140 kDa and is produced by vascular endothelial cells, intestinal brush-border cells, renal proximal tubular cells, monocytes, and Leydig cells (9–14). The ACE P isoform has a molecular mass of 100 kDa and is produced exclusively in adult testis by developing sperm cells, specifically late pachytene spermatocytes (13, 15).

We have previously demonstrated that the 5’ proximal 298 bp of DNA upstream of the ACE T transcription initiation site are sufficient to provide correct tissue-specific expression of the rabbit ACE T message (8). Within this region lies a cyclic AMP response element-like site (CRET) at −52 and a TATA-like binding site at −27, which are homologous to the murine ACE T CRE-like and TATA-like sites (8). It was previously reported that the ACE T TATA sequence binds TATA-binding protein from non-testicular nuclear extracts. In addition, mutation of the ACE T promoter TATA-like element to a consensus TATA sequence did not alter the testes-specific gene expression in transgenic mice (16). These data suggest that the ACE T TATA-like site is not responsible for tissue-specific expression of ACE T mRNA, and the focus should be directed at the role of CRET in ACE T gene transcription.

Cyclic AMP response in differentiating sperm cells is mediated by the CREM gene family. All members contain a CRE binding domain and a kinase-inducible domain that is phosphorylated by cAMP-activated cyclic AMP-dependent protein kinase. CREM isoforms differ in their ability to stimulate or repress transcription due to the presence (CREMα) or absence (CREMβ) of glutamine-rich domains (17). In addition, CREM isoform transcriptional effects are gene, cell-type, and promoter specific (18). Though immature sperm cells contain both isoforms at low levels, differentiated sperm cells contain markedly increased levels of CREMα protein (19, 20). The physiological importance of CREM has recently been demonstrated through murine gene-targeting methodologies (21). Male mice devoid of all CREM isoforms are sterile due to dramatic reduction in post-meiotic sperm-specific gene expression and failed spermatogenesis. Transcription of protamine 1, protamine 2, TP1, TP2, calispermin, Krox-20, Kox-24, proacrosin, MCS, and RT7 genes was absent in CREM−/− mice (21, 22). There is no other phenotypic alteration of the physiology of these male mice nor in homozygous CREM mutant females that retain fertility (21). The ACE T CRET binds both CREMα and CREMβ proteins and directs proper cAMP stimulation of a heterologous promotor in vitro (18).

In the current study, we investigated the role of the CRET site in directing ACE T mRNA transcription by mutating this site in a transgenic reporter gene that is expressed in sperm cells. In addition, we assessed the role of the CREM family of
transcription factors, in ACE gene expression, by measuring the levels of the two mRNAs in different tissues of CREM+/− mice. Our results showed that both the cis-element CRET and the transacting factor CREM are necessary for sperm-specific expression of ACE mRNA.

EXPERIMENTAL PROCEDURES

Materials—DNA-modifying enzymes were purchased from Life Technologies. Inc. or Boehringer Mannheim. Oligonucleotides were purchased from Operon Technologies, Inc. All radiolabeled probes were purchased from NEN Life Science Products. Total RNA was isolated and prepared from tissues (brain, kidney, lung, and testes) of wild type (+/+) or homozygous (−/−) CREM mutant adult mice as described previously (21). The p-TRI-cyclophilin-mouse antisense control template was purchased from Ambion Inc. RNazol B was purchased from Tel-Test, Inc.

Plasmid Construction—The 298 ACE promoter was mutated utilizing the Muta-Gen Phagemid in vitro mutagenesis system (Bio-Rad) and the oligonucleotide rACE-CREMM (CCTGGAGTGTGCAGCATAGACCA). Mutagenesis was confirmed by sequencing. The mutated ACE promoter was cloned into PSVOCAT to yield pACE-CRENull (23). The ACE probe used for ribonuclease protection assay (RPA) was cloned by reverse transcriptase-polymerase chain reaction utilizing 15-mers of total mouse testes RNA, the mouse ACE −2A (TCTGAAGCTTCTTAGTATTGCC), and the mouse ACE-1S (ATGGGGCAAGGTGGGGCA) oligonucleotides. The polymerase chain reaction product was subcloned into pBlueScript KS to yield plasmid PM045.

Transgenic Mice—The pACE-CRENull plasmid was digested with XbaI and BamHI to release the 2.03-kilobase DNA fragment illustrated in Fig. 1. The DNA fragment was injected into C57BL/6JSXJL/J F2 zygotes by standard techniques (8). Positive transgenics and their progeny were identified by Southern blot hybridization.

Southern Blot Hybridization—Mouse genomic DNA (15 μg) was digested with HindIII and Scal or BanI. The samples were electrophoresed through a 0.8% agarose gel in TBE and transferred to HybondN+ (Amersham Pharmacia Biotech) in 0.4 × NaOH. The membrane was prehybridized in 20 ml of C buffer (0.5 × NaPO4, pH 7.2, 7% SDS, 1% bovine serum albumin, 1 mM EDTA, 250 μg/ml denatured salmon sperm DNA) for 2 h at 65 °C. The 551-bp CAT gene probe was released from PSVOCAT by HindIII and Scal digestion and radiolabeled by random-primed DNA labeling kit (Boehringer Mannheim). The denatured probe was added to 20 ml of C buffer. The membrane was hybridized for 16 h at 65 °C and then washed as follows: solution 1 (10 mM NaPO4, pH 7.2, 5% SDS, 0.5% bovine serum albumin, 1 mM EDTA) for 30 min at 25 °C and 30 min at 55 °C, and solution 2 (40 mM NaPO4, pH 7.2, 1% SDS, 1 mM EDTA) for 30 min at 57 °C. For copy number determination, the BanI-digested CAT gene probe membrane was exposed to a PhosphorImager screen, stripped, and rehybridized with the 450-bp mouse ACE promoter. The washing conditions were as follows: solution 1 for 30 min at 25 °C and 55 °C for 30 min, and solution 2 for 30 min at 58 °C. The membrane was re-exposed to the PhosphorImager screen. Each CAT mRNA signal was normalized to the ACE-specific mRNA signal using ImageQuant software.

Chloramphenicol Acetyltransferase Assay—Tissues were analyzed according to previously described methods (8, 23). Fifty micrograms of protein was assayed for 2 h at 37 °C. The samples were spotted on a protein 2 ha t 3 7° C .T h e samples were spotted on a 8M urea gel, and visualized by autoradiography. The mRNA levels were determined by PhosphorImager analysis using ImageQuant software.

Result}s

Mutation of the CRET Site—Previous reports have implicated the ACET CRE-like site (CRET) as being a putative positive regulator of ACE activity (18, 25). The 298-bp rabbit ACE promoter, which gave correct tissue-specific reporter gene expression in transgenic mice, contains a CRE-like site (CRET) (8). To study the role of this CRET site, we utilized site-directed mutagenesis to alter the normal ACET CRET site (CRET) (8). We have previously demonstrated that this mutation abolishes the binding of CREM isoforms and α to the altered CRE site in gel mobility shift assays (18). Four independent ACE-CRENull mouse lines were established by injecting the DNA fragment illustrated (Fig. 1A) into C57BL/6JSXJL/J F2 zygotes. Positive transgenics and their progeny were confirmed by Southern blot (Fig. 1B). Genomic DNA from mouse 5090 (line A), 5091 (line C), 5108 (line D), and 5066 (line E) was digested with HindIII and Scal, which releases a 701-bp CAT gene fragment.
The probe was the 551-bp HindIII → NcoI CAT gene fragment (Fig. 1A). To verify that the ACEp-CRENull promoter and CAT gene integrated intact, the genomic DNA was digested with BanI (Fig. 1A) and reprobed with the HindIII → NcoI CAT gene probe (Fig. 1C). The transgene copy number of each line was determined as described under “Experimental Procedures.” Both CAT and ACE probes had the same specific activity. The signals from the CAT hybridization were normalized to the ACE-specific signal obtained in the mouse ACE hybridization. The approximate copy number for each line was as follows: line A, 35–40; line C, 30–35; line D, 5–10; and line E, 320–330 (Fig. 1C).

**Chloramphenicol Acetyltransferase Assay on Transgenic Mice Tissue**—The testicular isoform of ACE is expressed exclusively in sperm (13, 15). Previously, we reported that the −298 ACEp promoter drives CAT gene expression confined to the testes of the mouse containing the pACEp-CAT2 transgene (8). When the ACEp-CRENull mice were assayed for transgene expression, no CAT activity was observed in the testes of any of the four lines (Fig. 2). Our positive controls were testes extract from the pACEp-CAT 2, line F (mouse 2010) (8), and purified CAT enzyme. To determine if the CRENull mutation causes ubiquitous CAT expression, we assayed CAT activity in the lung, kidney, brain (Fig. 2) spleen, and liver (data not shown). All tissues tested were negative for CAT activity.

**Ribonuclease Protection Assay on CREM Knock-out Mice**—Having established that mutation of the CRET site abolishes rabbit ACEp promoter function in transgenic mice testes, we focused on the trans-acting factors that bind to the CRET site (18). To address this point, we studied the endogenous ACE expression in a mouse devoid of all CREM isoforms. The method used to knock out the CREM gene disrupts the coding region of all CREM family members (21). Total RNA was isolated from age-matched CREM−/− and CREM+/+ mouse brain, kidney, lung, and testes. The mouse ACE T mRNA level in each of these tissues was determined by RPA utilizing a mouse ACE probe that distinguishes between mouse ACEp mRNA and mouse ACEp mRNA levels (Fig. 3). This 470-bp mouse ACE antisense RNA probe was produced by transcribing mouse cDNA containing ACET-specific exon 12T and ACET and ACEp shared exons 13 and 14. The protected ACE T message was 450 bp due to the protection of the 12T exon present in normal ACET mRNA. The protected ACEp message was 260 bp due to the absence of the 12T exon in the normal ACEp mRNA.
ACEP mRNA were highly expressed in the testes of the CREM\(^1\) mouse, whereas only the ACE P mRNA was expressed in the testes upstream of the rabbit ACET mRNA transcription start site. Relevant sperm cells cannot be cultivated in vitro, and negative, depending on the study (32). This suggests that the CRE site is necessary for transcription of the ACEP mRNA in vivo, as demonstrated in CREM\(^2\) mice (21, 22). Like several other cAMP-dependent testicular mRNAs, ACEP mRNA expression was totally absent in CREM\(^2\) cells. These results strongly suggest that CREM is the relevant physiological transcription factor that binds to the CRET site of the ACE gene and activates transcription of the ACEP mRNA.

Although the results presented here provide an understanding of the mechanism of activation of the ACEP promoter, it is still not clear why this transcription unit is not activated in other tissues. Why is the CRET site not recognized by any of the multiple cAMP-activated transcription factors (e.g. CREM, CREB, c/EBP, etc.) and the ACET mRNA transcribed? CREB is more abundant in lung and kidney than CREM and is capable of binding to the ACET CRE site to activate transcription (30). Alternatively, does active transcription of the ACEP mRNA preclude the use of the ACEP promoter in some way?

Our results revealed another apparent anomaly. ACEP mRNA is normally expressed in vascular endothelial cells and Leydig cells of the testes (9, 13). However, ACEP mRNA was poorly transcribed in the testes, but not the lung, of the CREM\(^2\) mouse. It was not due to a global deficiency in transcription because many mRNAs, including the cyclophilin mRNA that was used as an internal control in our experiment, were transcribed normally in the same tissue. In addition, CREM\(^2\) mutant mice possess normally developed Sertoli and Leydig cells (22). Does that mean that ACEP and ACET mRNA transcription in the testes are somehow coupled? This may be the case, albeit indirectly, if the decreased expression of ACEP mRNA in the CREM\(^2\) mice reflects the dependence of its expression on paracrine factors in the testis. Cultures of Sertoli cells, with and without germ cells, have suggested that spermatocytes and early spermatids may have important regulatory influences on Sertoli cells (31). In turn, there is abundant evidence that Sertoli cell factors modulate Leydig cell steroidogenesis. These influences have been thought to be both positive and negative, depending on the study (32). This suggests that although mature sperm-deficient CREM\(^2\) mice have Leydig cells that appear normal (22), perhaps their ability to produce ACEP mRNA is impaired. Therefore, ACEP mRNA levels in the testes would be more dependent on sperm cell development as a whole rather than directly on ACEP transcription itself. It is also possible that CREM proteins are directly needed for ACEP mRNA transcription as well. However, that requirement must be obviated in the lung. No clear-cut explanation emerges from the available information, and further investigations will be required for resolving these issues.

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**TABLE I**

Quantitation of ACE\(_P\) and ACE\(_P\) mRNAs in a normal and CREM\(^{−/−}\) mouse

| CREM genotype | Testes | Lung |
|---------------|--------|------|
| +/+           | ACE\(_P\) mRNA | ACE\(_P\) mRNA | ACE\(_T\) mRNA | ACE\(_T\) mRNA |
| −/−           | 21.2   | 100  | 0     | 35.6   |
| +/−           | 0      | 3.1  | 29.3  | 0      |

Five micrograms of total RNA isolated from lung and testes of a normal (+/+) and CREM knock-out (−/−) mouse were assayed as described in Fig. 3. Following electrophoresis, the gel was dried and exposed to a Molecular Dynamics PhosphorImager screen. Protected 260-bp ACE\(_P\) 450-bp ACE\(_P\), and 103-bp cyclophilin signals were quantitated with the ImageQuant software. The table indicates the relative ACE\(_P\) or ACET mRNA levels achieved by normalizing each protected probe signal to the cyclophilin signal. The highest relative mRNA level (ACE\(_P\) in testes) was set equal to 100%.

DISCUSSION

In this study, we investigated the mechanism of testis-specific expression of the ACE\(_T\) mRNA. In adult males, this mRNA is exclusively expressed in maturing sperm cells, although the ACE\(_P\) mRNA, which arises from the same gene, is expressed in many other tissues (9–15). Using tissue-cultured cells, the rabbit ACE\(_P\) transcription unit has been extensively analyzed, and the presence of several positive regulatory sites and a strong silencer element has been noted (26–28). For analyzing the transcriptional promoter of the ACE\(_P\) transcription unit, we had to resort to a transgenic assay system because the relevant sperm cells cannot be cultivated in vitro. Using a CAT reporter gene, we previously demonstrated that a 298-bp fragment upstream of the rabbit ACE\(_P\) mRNA transcription start site is capable of driving testis-specific and developmentally regulated expression of CAT (8). Among several putative regulatory sites present within this region is the CRET element, which resembles a consensus cyclic AMP response element (29). Using *in vitro* assays, we demonstrated that this site is capable of binding to the CREM transcription factors and initiating their transcriptional stimulatory effects (18). In the current study, we investigated whether the above observations are also true *in vivo* in the ACE\(_T\)-expressing adult testes. For this purpose, we used a combination of transgenic and gene knock-out mice.

Results from the CRENull transgenic mice clearly showed that the CRET site is necessary for *in vivo* functioning of the ACE\(_T\) promoter. Five independent CRENull transgenic lines (line B and data not shown) failed to express the reporter gene in adult testes. Although earlier studies have suggested that the CRET site is functional *in vitro* (16, 18, 25), the current study has demonstrated its absolute requirement in the context of the ACE\(_T\) promoter functioning *in vivo*. Because the CREM gene family of cAMP-activating transcription factors has been shown to be important for sperm gene expression, we examined the ACE mRNA expression profile in CREM\(^{−/−}\) mice (21, 22). Like several other cAMP-dependent testicular mRNAs, ACE\(_T\) mRNA expression was totally absent in CREM\(^{−/−}\) cells. These results strongly suggest that CREM is the relevant physiological transcription factor that binds to the CRET site of the ACE gene and activates transcription of the ACE\(_P\) mRNA.
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