

Selective Restoration of Male Fertility in Mice Lacking Angiotensin-converting Enzymes by Sperm-specific Expression of the Testicular Isozyme

Pandurangan Ramaraj,* Sean P. Kessler,* Clemencia Colmenares,† and Ganes C. Sen*

*Department of Molecular Biology and †Department of Cancer Biology, The Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195

Abstract

Although angiotensin-converting enzyme (ACE) has been studied primarily in the context of its role in blood pressure regulation, this widely distributed enzyme has many other physiological functions. The ACE gene encodes two isoforms. The somatic isozyme is expressed in many tissues, including vascular endothelial cells, renal epithelial cells, and testicular Leydig cells, whereas the testicular or germinal angiotensin-converting enzyme is expressed only in sperm. The ACE gene knockout mice lack both isoforms and they exhibit low blood pressure, kidney dysfunctions, and male infertility. Here, we report the use of a sperm-specific promoter and interbreeding of transgenic and gene knockout mice for generating a mouse strain that expressed ACE only in sperm. The experimental mice maintained the kidney defects of ACE^{-/-} mice, but unlike the knockout strain, the males were fertile. Thus, we established that the role of ACE in male fertility is completely dependent on its exclusive expression in sperm. Our study clearly demonstrated how transgenic and knockout techniques can be combined for ascribing a specific physiological function to the expression of a multifunctional protein in a given tissue. (*J. Clin. Invest.* 1998. 102:371–378.) Key words: transgenic • gene knockout • tissue-specific promoter • kidney structure • blood pressure

Introduction

Angiotensin-converting enzyme (ACE),¹ a dipeptidylcarboxypeptidase, is an important enzyme of the renin-angiotensin system that regulates blood pressure (1–3). The two most well-characterized physiological substrates of ACE are the peptides angiotensin I and bradykinin. Action of ACE on both of these peptides causes vasoconstriction; thus, elevated ACE activity

leads to hypertension. Inhibitors of ACE are effective in treating hypertension and congestive heart failure.

There are two isoforms of ACE (4–8). The larger protein, the somatic isoform (sACE; also known as ACE_s), is expressed widely in the body. Vascular endothelial cells and epithelial cells in the proximal tubules of the kidney, brain, intestinal brush border, and epididymis express sACE. Macrophages and Leydig cells in the testis also express this isoform. In contrast, the germinal isozyme (gACE, also known as ACE_t), is expressed exclusively in developing sperm cells (9–12). The two isoforms have similar enzymatic activities and the proteins are structurally related. Both are type I ectoproteins anchored in the plasma membrane by a hydrophobic region present near their carboxyl-terminals. A regulated membrane-bound protease activity can cleave the extracellular domain of ACE producing an enzymatically active soluble form of the protein in the serum (13–16).

sACE and gACE are encoded by 5.0- and 2.5-kb mRNA, respectively. Both mRNAs are transcribed from the same gene by a tissue-specific choice of two alternative transcription initiation sites and two alternative polyadenylation sites (17–20). The gACE promoter has been characterized using transgenic reporter gene expression in sperm as an assay. A cAMP-response element and the corresponding transcription factor CREM have been identified as the crucial determinants of expression of the gACE mRNA (21–26). On the other hand, several positive elements and a strong silencer element dictate the expression of the sACE mRNA (27, 28). For neither mRNA, however, have the crucial elements responsible for their tissue specificity been identified. Functioning of the gACE promoter, but not the sACE promoter, is also regulated developmentally. gACE mRNA is expressed only during a postmeiotic phase of sperm differentiation, but the protein remains associated with mature sperm. The specific function of ACE in sperm physiology and the nature of its substrate in the reproductive system remain to be determined.

Disruption of the mouse ACE gene has revealed the involvement of ACE in multiple physiological regulatory systems (29–32). The ACE^{-/-} mice had, as expected, lower blood pressure. But, they also had severe kidney structural and functional abnormalities: atrophy of the renal cortex, thickened arteries, and infiltration of inflammatory cells. These animals do not effectively concentrate urine or maintain normal urinary salt balances. The male ACE^{-/-} mice, in addition, sire very few pups, though they have normal testis structure, sperm count, morphology, and motility.

We are interested in assigning specific physiological functions of ACE to its expression in specific cell types of different tissues. In the context of this general interest, in this study, we have investigated whether expression of ACE_t in sperm, in the absence of ACE expression elsewhere in the body, is sufficient for maintaining normal male fertility. To experimentally approach this problem, we used a sperm-specific transcriptional promoter to generate transgenic mice that express a transgenic

Address correspondence to Ganes C. Sen, Ph.D., Department of Molecular Biology, The Cleveland Clinic Foundation, 9500 Euclid Avenue, NC20, Cleveland, OH 44195. Phone: 216-444-0636; FAX: 216-444-0512; E-mail: seng@cesmtp.ccf.org

Received for publication 27 March 1998 and accepted in revised form 21 May 1998.

1. *Abbreviations used in this paper:* ACE, angiotensin-converting enzyme; gACE, testicular or germinal angiotensin-converting enzyme; PGKII, phosphoglycerate kinase II; RT, reverse transcription; sACE, pulmonary or somatic angiotensin-converting enzyme.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.
0021-9738/98/07/0371/08 \$2.00

Volume 102, Number 2, July 1998, 371–378

<http://www.jci.org>

gACE only in sperm. Upon cross-breeding of those transgenic mice with ACE^{-/-} mice, we generated a mouse strain devoid of sACE, yet expressing gACE exclusively in the developing sperm cells. Our experimental mice retained the kidney defects present in ACE^{-/-} mice, but the male fertility was restored to that of the wild-type mice. Thus, our study demonstrated that male fertility requires the expression of ACE in sperm, but not in the Leydig cells, epididymis, or the vasculature of the testes.

Methods

Generation of transgenic mice. The EcoRI fragment of the rabbit testicular ACE cDNA clone (6) was cloned at the EcoRI site of pCDNAIII expression vector in the right orientation. The CMV promoter in that vector was removed by cutting with BglII and HindIII and replaced by the 515-bp human phosphoglycerate kinase II (PGKII) promoter released from the vector pPM046 (33) with BamHI and HindIII. The transgene containing the PGKII promoter, the ACE cDNA, and the bovine growth hormone poly A signal was excised from the vector by digestion with SmaI and NaeI restriction enzymes. The 3.5-kb fragment was purified by gel electrophoresis and used for microinjection into fertilized eggs of FVB mice (34). The injected eggs were implanted into the uteri of several pseudopregnant mothers. The microinjection and egg implantation was carried out at the Transgenic Facility of The Ohio State University (Columbus, Ohio).

15 pups were born and their DNA was analyzed for the presence of the transgene. Five out of the 15 mice were found to be positive. For DNA isolation, 0.5–1.0-cm tails were biopsied from 10–15-d-old mice. The samples were minced and digested in a buffer containing 100 mM Tris Cl, pH 8.5, 5 mM EDTA, 1% SDS, 200 mM NaCl, and 100 µg/ml proteinase K at 55°C overnight. After centrifugation, the supernatant was extracted with phenol-chloroform and the DNA was precipitated from the aqueous layer by adding equal volume of isopropanol. The precipitate was washed with 70% ethanol and dissolved in Tris/EDTA buffer (10 mM Tris Cl, pH 8.0, 0.1 mM EDTA). 10–15 µg of DNA was digested overnight with EcoRI or SacI and used for Southern blotting. The DNA was electrophoresed and blotted to Hybond N⁺ membrane (Amersham Corp., Arlington Heights, IL). The probe was prepared by labeling the 2.5-kb ACE cDNA by random priming using α³²P]dCTP. Hybridization was done at 65°C overnight in a buffer containing 500 mM sodium phosphate, pH 7.2, 7% SDS, 1% BSA, 1 mM EDTA, and 200 µg/ml denatured salmon sperm DNA. Washing was done first with a buffer containing 5% SDS, 40 mM sodium phosphate, pH 7.2, 1 mM EDTA, and 0.5% BSA at room temperature for 30 min, and at 58°C for 30 min. The second washing was with a buffer containing 1% SDS at 63°C for 30 min. The membrane was dried and exposed to x-ray film.

Establishment of transgenic lines. The transgenic founder mice, four females and one male, were mated with wild-type FVB mice and the pups were examined for the presence of the transgene. All five founder mice transmitted the transgene and five lines were established by brother/sister mating of the progeny. Mice homozygous for the transgene were identified by Southern blotting, and mating pairs were chosen for maintaining the transgenic lines.

Expression of the transgene. Reverse transcription PCR (RT-PCR) was used for detecting the transgenic rabbit ACE mRNA expression in different tissues. Tissue RNA was isolated using RNAzol and DNase digestion and used as templates for RT-PCR. The tissue was homogenized in RNAzol B (Tel-Test Inc., Friendswood, TX) using 2-ml solution per 100 mg tissue. 0.2 ml of chloroform was added to the homogenate and centrifuged at 12,000 g for 15 min. RNA was precipitated by adding an equal volume of isopropanol, centrifuged, dried, and dissolved in diethylpyrocarbonate-treated water. It was then incubated with RNase-free DNase I at room temperature for 15 min in DNase buffer. DNase was inactivated by heating to 65°C for 15

min. RT-PCR was carried out as recommended in the GIBCO BRL (Gaithersburg, MD) instruction manual. For denaturation, RNA and random hexamer primers were incubated at 70°C for 10 min. For annealing, all components except the enzyme were added to the denatured mixture and incubated at room temperature for 5 min. The enzyme superscript II reverse transcriptase was added and incubated at room temperature for 10 min, followed by incubation at 42°C for 50 min. The reaction was stopped by incubating at 70°C for 15 min. The RNA was removed by digestion with RNase H at 37°C for 20 min and the sample was ready for the PCR using specific primers. The rabbit ACE-specific primers were chosen from near the 5' end of the coding region, which has many mismatches with the corresponding region of the mouse cDNA. The sense primer was 5' CCA GCA TCC GGG CAA CCA CC 3' and the antisense primer was 5' ATC TGG TTG TAC TCC AGC TCC TTG ACG 3'. The PCR conditions were initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 60 s. The final extension was at 72°C for 5 min. The product of 365 bp was detected by electrophoresis in 1% agarose gel. The specificity of the method was verified by using mouse testicular RNA which gave no product with these primers. Similarly, no product was produced in the absence of RT ensuring that all genomic DNA had been removed from the RNA sample. Two mouse-specific primers were used for detecting mouse gACE mRNA. The sense primer was 5' CTG AGT CAG GTG GCC ACT GA 3' and the antisense primer was 5' GGG CAT ACA AGT GCC ATT TG 3'. The corresponding mouse PCR product was of 496 bp. Thus, the two sets of primers, the rabbit-specific and the mouse-specific, could be used in the same RT-PCR reaction with transgenic mouse RNA as the template and the two products of 365 bp and 496 bp could be distinguished by their different mobilities in gel electrophoresis.

For detecting rabbit gACE protein in tissue extracts, Western blotting was done with a rabbit ACE-antiserum that does not cross react with mouse ACE. Testis was decapsulated and minced in 1 ml of extraction buffer (50 mM sodium borate, pH 8.3, 150 mM NaCl, and 0.5% Triton X-100), followed by homogenization. Debris were removed by centrifugation and the supernatant was used for Western analysis using 1:1,000 dilution of rabbit ACE antibody (14). The blots were developed by enhanced chemiluminescence reaction.

Generation of experimental mice. An ACE^{+/-} male C57Bl/6 mouse was obtained from Dr. Oliver Smithies (University of North Carolina, Chapel Hill, NC) and used for establishing a C57Bl/6 colony. The colony was maintained as heterozygotes for the ACE gene. For detecting the normal and the disrupted alleles of the mouse ACE gene, tail DNA was digested with SacI and Southern blotted using the mouse testis specific exon 12T (210 bp) as the probe (29). The normal gene gave rise to a 6.6-kb fragment and the disrupted allele gave rise to an 8.4-kb fragment. For generating experimental mice, transgenic ^{+/+} female mice were mated with ACE^{+/-} male mice, the transgene ^{+/-}, ACE^{+/-} progenies were identified and interbred for producing transgene ^{+/+}, ACE^{-/-} mice. The control mice for phenotype analysis were littermates of appropriate genotypes from these crosses.

Histology and immunohistochemistry. Tissues were preserved in Histochoice[®] (Sigma Chemical Co., St. Louis, MO) before sectioning and embedding in paraffin and stained with hematoxylin and eosin. For antibody-staining, paraffin was removed from the sections by sequential washings in xylene, alcohol, and PBS. The sections were blocked by incubating in horse serum for 1 h, washed and incubated with anti-ACE antibody (1:1,000 dilution) for 1 h at room temperature, washed again, and incubated with FITC-conjugated secondary antibody for 1 h at room temperature. After washing, the sections were mounted and viewed through a fluorescence microscope.

Fertility analysis. Experimental and control male mice were mated with wild-type female FVB mice. The females were allowed to deliver the pups, which were counted after birth. The females that failed to become pregnant when mated with ACE^{-/-} mice were later tested to be fertile by mating with normal males.

Results

Generation of transgenic mice. The transgene used for these experiments contained a 515-bp promoter region, a 2,500-bp coding region, and a 213-bp polyadenylation region. The promoter was from the human phosphoglycerate kinase-2 gene and had been used previously for sperm-specific expression of a transgene (33). The coding region was from the rabbit gACE cDNA that encodes a protein containing 737 amino acid residues (6) and the polyadenylation signal was from the bovine growth hormone gene. The transgene was excised from the cloning vector by digesting it with *Sma*I and *Nae*I (Fig. 1 A) and purified by gel electrophoresis. The DNA was microinjected into fertilized eggs of FVB mouse and the eggs were implanted in the uterus of a pseudopregnant mother. Tails were biopsied from 2-wk-old pups and DNA was extracted. Tail DNA was digested with *Eco*RI and analyzed by Southern blotting using the 2,500-bp *Eco*RI fragment of the transgene as the probe (Fig. 1 B, lane 1). Among the 15 mice analyzed, five carried the transgene. As shown in Fig. 1 B, DNA for mouse number 6, 10, 11, 12, and 13 (lanes 4, 5, 6, 7, and 8, respectively), contained the 2.5-kb transgenic DNA. One nontransgenic sibling is shown in lane 3. DNA of all transgenic mice also contained DNA fragments of molecular masses > 2.5 kb that hybridized with the probe. Those could be the products of incomplete digestion of concatemers of the transgene or rearranged transgenes. It was apparent that different lines contained different amounts of the transgenic DNA. For example, line 13 (lane 8) contained an extremely high amount of transgenic DNA.

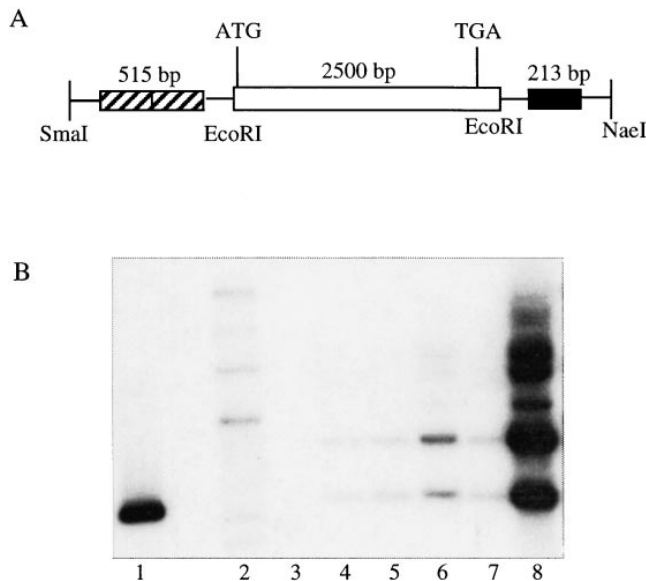


Figure 1. Identification of transgenic founder mice. (A) The transgene consisted of a 515-bp promoter fragment from the human PGK II gene, a 2,500-bp rabbit gACE cDNA fragment, and a 213-bp fragment containing the polyadenylation signal of the bovine growth hormone gene. (B) Southern blot of mouse genomic DNA digested with *Eco*RI. The 2.5-kb *Eco*RI insert of the transgene was used as the probe. Lane 1, the probe itself; lane 2, molecular mass markers; lane 3, DNA from mouse No. 2 (nontransgenic); and lanes 4–8, DNA from transgenic mice No.s 6, 10, 11, 12, and 13, respectively.

Expression of the transgene. The five transgenic founder mice were used to establish lines. Since the founder mice were of FVB strain, the lines were also established in the same strain by breeding with Wt FVB mice of the opposite sex. The transgene was transmitted by all of them to their progeny. Once the transgenic lines were established, male adult mice were tested for expression of the transgene in their testes. A RT-PCR-based assay was developed for the detection of the transgenic rabbit gACE mRNA (Fig. 2 A). In the same assay, using a different set of primers, we could measure the level of the mouse gACE mRNA. The primers used for detecting the mouse gACE mRNA did not detect the transgenic transcript (lane 3) and similarly the primers for the rabbit transgene did not detect the mouse mRNA (lane 4). When both sets of primers were used together, both mRNAs were detected (lane 5). The reaction was reverse transcriptase-dependent (lanes 1 and 2). In the gel system used, the product for the murine gACE mRNA (*m*) separated well from the product of the transgene (*r*) which, in turn, separated from the primers (*p*). Thus, this detection system was convenient and specific for simultaneously estimating the levels of the endogenous mouse gACE mRNA and the transgenic rabbit gACE mRNA. As shown in Fig. 2 B, all five transgenic lines expressed the transgene in the testes. As expected, no transgene expression was detected in

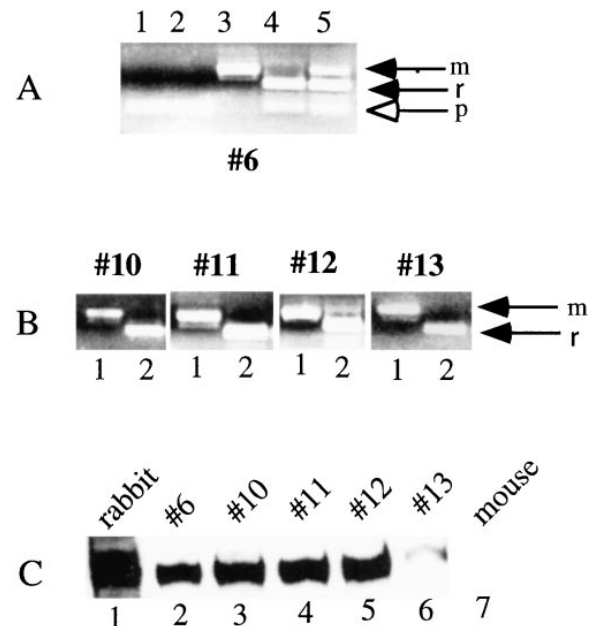


Figure 2. Expression of the transgenes in five transgenic lines. (A) RT-PCR analysis of testis RNA from a mouse of line No. 6. Mouse gACE-specific primers were used in lanes 1 and 3, rabbit gACE-specific primers were used in lanes 2 and 4, and both primers were used in lane 5. No reverse transcriptase was added to samples in lanes 1 and 2. *m*, the mouse gACE product; *r*, the rabbit transgene product, and *p*, shows the primers. (B) RT-PCR analysis of testes RNA from mice of lines 10, 11, 12, and 13. Lane 1, mouse specific primers; lane 2, rabbit specific primers. (C) Western blot of testes extracts. A rabbit ACE antibody was used. Lane 1, purified rabbit gACE; lane 2, transgenic line No. 6; lane 3, transgenic line No. 10; lane 4, transgenic line No. 11; lane 5, transgenic line No. 12; lane 6, transgenic line No. 13; lane 7, nontransgenic mouse.

the testes of nontransgenic littermates (not shown). In the next experiment, the presence of the protein product of the transgene was examined. For this purpose, testes extracts were subjected to Western analysis using an anti-rabbit ACE antibody. This antibody reacted strongly with rabbit gACE (Fig. 2 C, lane 1) but not with mouse gACE (Fig. 2 C, lane 7). The transgenic protein was present in the testes of mice of all five transgenic lines (Fig. 2 C). The levels of expression were very similar in lines 6, 10, 11, and 12, but the line 13 mice expressed much less transgenic protein.

The transgene was designed to be expressed only in the testes. However, because ectopic expression of transgenes is frequently encountered in transgenic mice, we examined the tissue-specificity of expression of the gACE transgene. The RT-PCR assay was used to measure the levels of the transgenic transcript in various tissues of an adult male mouse of line 10 (Fig. 3 A). Transgenic gACE mRNA was not expressed in liver, lung, kidney, brain, or intestine of the mouse but was present in the testis. In a female littermate harboring the transgene, there was no expression of gACE mRNA in ovary or uterus. The absence of the transgene expression in the kidney was confirmed by the Western analysis. Increasing amounts of

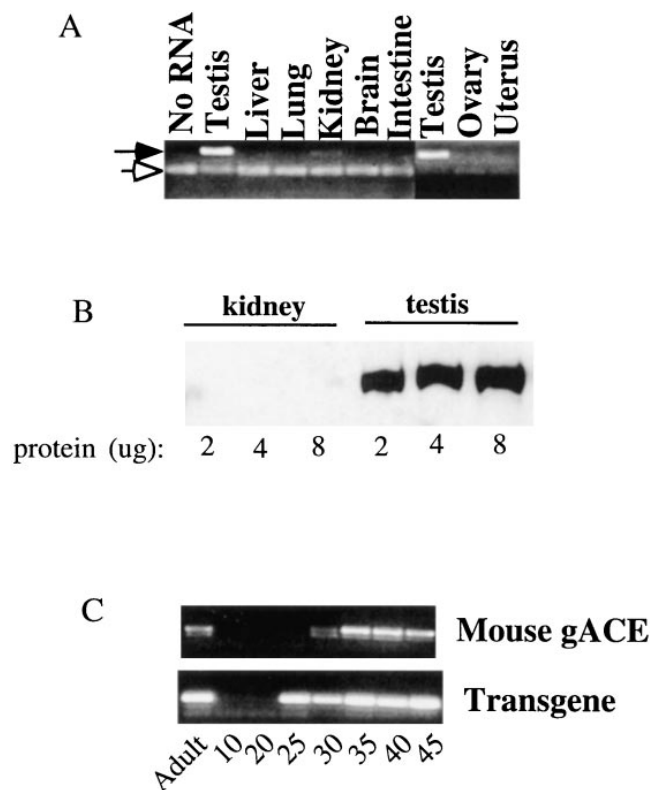


Figure 3. Expression pattern of the transgene. (A) RT-PCR analysis of RNA from different tissues of male and female transgenic mice. The solid arrow shows the product of the transgene rabbit gACE mRNA and the empty arrow shows the primers. (B) Western analysis of increasing amounts of kidney and testis extracts for transgenic gACE protein expression. (C) Developmental profile of transgene expression. Mouse-specific primers were used to detect the endogenous mouse gACE mRNA and rabbit-specific primers were used for detecting the transgene mRNA in the testis. The numbers at the bottom indicate the ages of the mice in days.

testis extract gave increasing signals for the transgenic protein, but it was totally absent in the kidney extract (Fig. 3 B).

In the next experiment, we examined the developmental profile of the transgene expression in the testes. RNA was isolated from the testes of mice of different ages and the presence of the transgenic mRNA and the endogenous mouse gACE mRNA was assayed for, using the corresponding sets of primers. As shown in Fig. 3 C, both mRNAs were expressed in the adult testes and neither was expressed in the testes of the 10-d- and 20-d-old mice. Transgene expression was, however, readily detectable in the 25-d-old mouse, whereas the endogenous mRNA was detectable in the 30-d-old mouse. These results demonstrated that the developmental profile of transgene expression was similar to that of the resident mouse gene.

Generation of experimental mice. Once we confirmed that the transgene was expressed with the appropriate tissue-specificity and the right developmental profile, we wanted to transfer it to the ACE-null background in order to generate the experimental mice of the genotype ACE⁻/ACE⁻ (a/a), transgene⁺/transgene⁺ (B/B). For this purpose, we first interbred the transgenic (No. 10) mice to create a line homozygous in the transgene locus (B/B). The ACE-null mice were obtained in the C57Bl/6 strain from Dr. Oliver Smithies' laboratory. A colony of these mice was established in our laboratory, and ACE^{+/-} (A/a) male mice from this colony were used for mating with our FVB transgenic (B/B) female mice. The F1 mice from this breeding were genotyped and further interbred for generating the experimental mice (a/a:B/B). The presence or the absence of the normal ACE allele or its disrupted allele and the transgene in mice of various genotypes are shown in Fig. 4.

The expression of the transgene in the testes of the mice of mixed genetic background were verified in the experiments shown in Fig. 5. As expected, in the A/A:b/b mouse the murine gACE mRNA was expressed (Fig. 5 A, lane 5), but the rabbit transgenic mRNA was not (Fig. 5 A, lane 6). In the a/a:b/b mouse, neither mRNA was expressed and in the experimental a/a:B/B mouse, only the transgenic rabbit mRNA was expressed (Fig. 5 A, lane 6). Western blotting confirmed the expression of the transgenic protein in the testes of the experimental (a/a:B/B) mouse (Fig. 5 B, lane 4).

Phenotypes of the experimental mice. Histologically, the adult testes of the experimental (a/a:B/B) mice and the control wild-type (A/A:b/b) mice were very similar (Fig. 6, A and B). It was reported previously (29, 30), that the absence of the mouse ACE_T does not affect the architecture of the testes. We observed that the same was true for our experimental mice

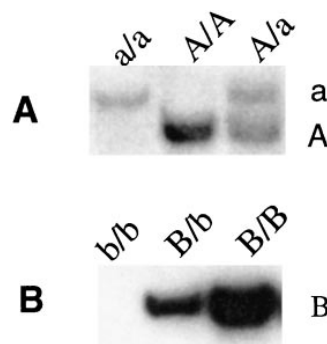


Figure 4. Genotype analysis of the experimental mice. Representative Southern blots of DNA from mice of different genotypes are shown. (A) A, the ACE gene; a, the disrupted ACE gene. (B) B, the transgene; b, the transgene's absence.

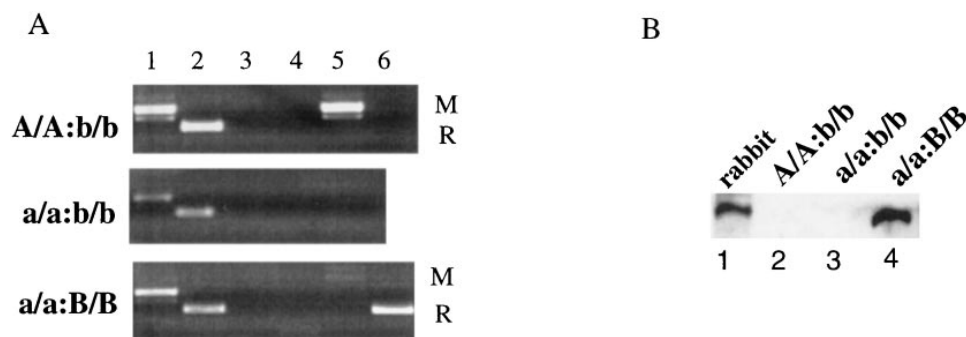


Figure 5. Transgene expression in the testes of experimental mice. (A) RT-PCR analysis of testis RNA. M, the mouse gACE specific product; R, the rabbit gACE-specific product. In lanes 1, 3, and 5, mouse-specific primers were used, and in lanes 2, 4, and 6, rabbit-specific primers were used. Lane 1 contained testis RNA of adult normal FVB mouse and lane 2 contained testis RNA of adult normal rabbit. Other lanes contained testis RNA of adult

mouse littermates of the genotypes A/A:b/b (top), a/a:b/b (middle), and a/a:B/B (bottom). Lanes 3 and 4 did not contain reverse transcriptase, others did. In the genotypes shown on the left, A stands for the mouse ACE gene and B stands for the transgene. (B) Western analysis for transgene expression in the testes. Lane 1, rabbit testes; lane 2, testis of wild-type mouse; lane 3, testis of ACE^{-/-} mouse; lane 4: testis of the experimental mouse.

which expressed the transgenic gACE, but not the endogenous mouse gACE. Immunohistochemical analysis, using a transgene-specific antibody showed a high level of expression of the transgenic protein in the experimental testes (Fig. 6D) but not the control testes (Fig. 6C). The expression pattern indicated the localization of the transgenic protein to the seminiferous tubules where sperm maturation occurs.

The ACE-null mice have major structural defects in their kidneys. In the experiments shown in Fig. 7, we inquired whether these defects were repaired in our experimental mice. Histological analysis of kidneys of experimental mice (a/a:B/B) and control mice (A/A:b/b) demonstrated that the structural defects of the null mice are inherited by the experimental

mice. There was thickening of the arteriole walls (Fig. 7B) and cellular infiltration of Bowman's capsule (Fig. 7D) in the kidneys of the experimental mice.

The above investigation established that in our experimental animals, no endogenous mouse ACE was expressed, the transgenic rabbit gACE was expressed exclusively in the developing sperm, and the kidney defects associated with the ACE-null genotype were retained. In the final experiment, we investigated the effect of transgene expression on male fertility (Fig. 8). Adult male mice of a similar age with genotypes of wild (A/A:b/b), null (a/a:b/b), and experimental (a/a:B/B) were mated with wild-type female mice. The pregnancies were brought to term and the number of pups were counted. In nine

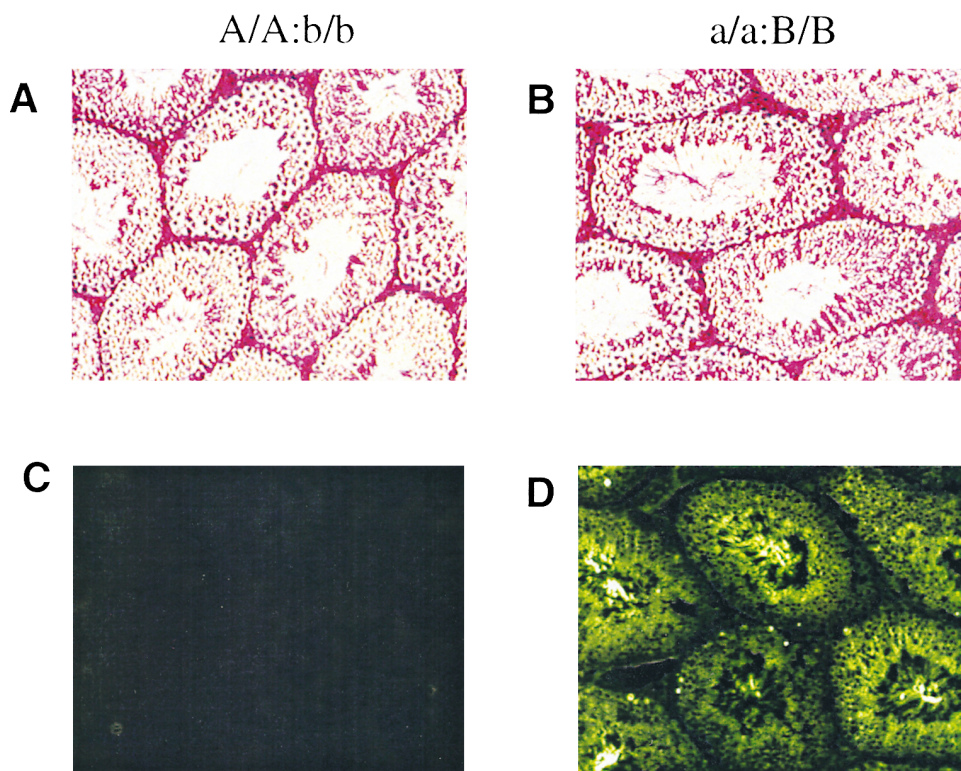


Figure 6. Testis structure and sites of transgene expression. Sections of testes of wild type (A/A:b/b) and experimental (a/a:B/B) mice were subjected to histological (A and B) and immunocytochemical analysis using rabbit ACE antiserum (C and D). A and C were from the wild-type mouse and B and D were from the experimental mouse.

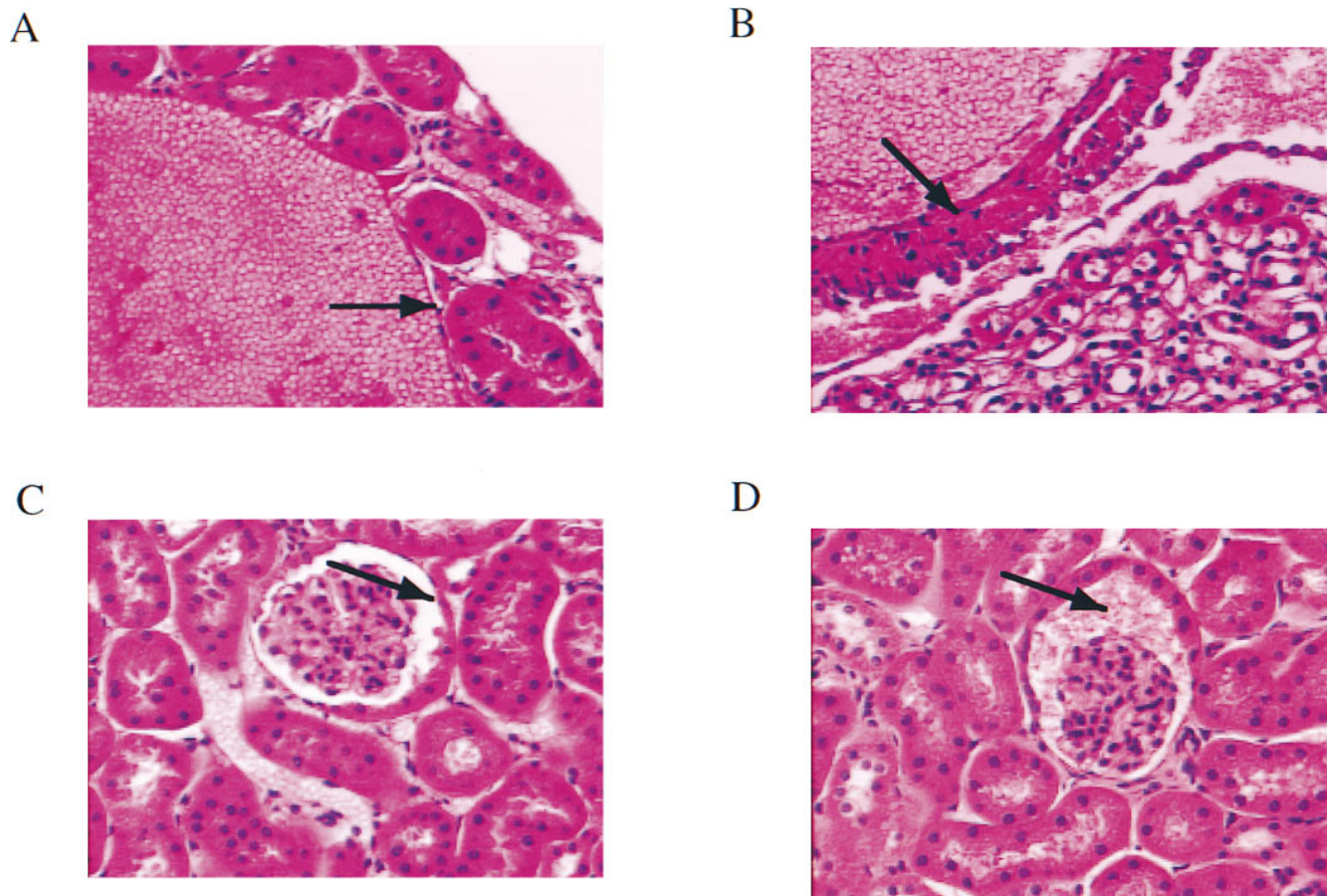


Figure 7. Abnormal kidney structure of the experimental mouse. Kidneys from age-matched wild-type (*A* and *C*) and experimental (*B* and *D*) mice were subjected to histological analysis. (*A*) The arrow shows an arteriole wall in the kidney of normal mouse. (*B*) The arrow shows a thickened arteriole wall in the kidney of the experimental mouse. (*C*) The arrow shows the Bowman's capsule. (*D*) The arrow shows cellular infiltration in the Bowman's capsule in the experimental mouse.

litters, the wild-type mice sired 85 pups, while in six matings, the null mice sired one pup, and in six matings, the experimental mice sired 47 pups. Thus, the fertility-defect of the null mice had been repaired in the experimental mice.

Discussion

In this study, we have used a combination of gene ablation and transgene insertion techniques to achieve expression of ACE in only one cell type in the experimental mouse, in contrast to its expression in many tissues in normal mice. Our experimental system has allowed us to make the unequivocal statement that gACE expression in sperm is necessary and sufficient for fulfilling the role of ACE in male fertility. In previous studies with ACE gene knockout mice, the requirement of ACE-expression for male fertility has been established (29, 30). These studies, however, could not determine the particular source of ACE-expression that is responsible for maintaining male fertility. Because ACE is known to be expressed in the testis, it is the most relevant tissue in the context of male fertility. The structure of the testes of the ACE^{-/-} mice, however, is normal as are the sperm count, shape, and motility (29, 30, 35). Moreover, ACE is expressed in many cell types in the tes-

tes: sperm, Leydig cells, epididymis, and the vasculature. Since ACE was missing in all these cell types in ACE^{-/-} mice, it was impossible to ascertain the requirement of ACE expression in specific cells in the testes as it relates to its role in fertility. Our studies have circumvented that problem and clearly established that expression of gACE in sperm was sufficient in this context.

gACE expression in the sperm of our experimental mice cured their deficiency in fertility but the kidney defects remained. The typical kidney defects described for the ACE^{-/-} mice, arterial thickening and infiltration of inflammatory cells (29–31), were observed in our experimental mice. We also observed, in experimental mice, additional defects previously reported for the ACE^{-/-} mice, namely, cortical and medullary thinning, crowding of glomeruli, focal atrophy, and shrinkage of tubules. From the observed structural defects, we anticipate that the kidney functions of these mice are also defective. Similarly, since no sACE was expressed in the vasculature of these mice, their blood pressure should be as low as that of the ACE^{-/-} mice. Such blood pressure measurements need to be done, in the future, in mice of a homogeneous genetic background. Mean blood pressure varies considerably among different strains of mice. Thus, such measurements are meaning-

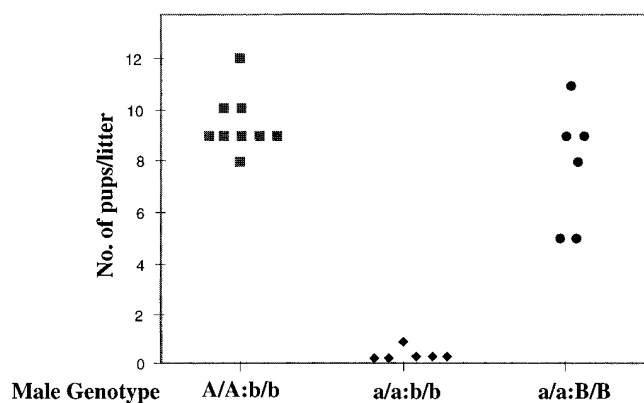


Figure 8. Fertility analysis of male experimental mice. Age-matched male mice of different genotypes were mated with normal female FVB mice and the numbers of pups born were counted. Two male mice of each group were mated with different females and the numbers of pups born from each mating are shown. Squares, A/A:b/b males; diamonds, a/a:b/b males; and circles, are for a/a:B/B males.

less in our experimental mice which inherited genes from both FVB and C57Bl/6 strains. Since blood pressure is regulated by a number of unknown genetic loci that would have segregated independently among the siblings, we could not compare the blood pressure of the experimental mice with that of a valid control group. Such measurements would require backcrossing of the transgene to the C57Bl/6 background or backcrossing of the ACE^{-/-} locus to the FVB strain. Experiments are currently in progress but their completion will require a substantial period of time.

A study, published recently by Hagaman et al. (35), provided further information about the function of ACE in male fertility. They observed that expression of ACE in sperm is important for egg fertilization. Sperm lacking ACE are deficient in transport within the oviduct and in binding to zonae pellucidae. By selective gene targeting, they also demonstrated that somatic ACE is not required for fertility, a conclusion supporting the results of our investigation although the experimental approaches used in the two studies were completely different. Our experimental strategy will be useful for addressing a number of issues that cannot be addressed adequately by the gene knockout technique alone. As illustrated in this study in the context of the testes, the transgenic knockout combination can assess the role of ACE expression specifically in one cell type of a given tissue. A relevant example is the kidney where ACE is expressed in the vasculature as well as in the epithelial cells of the proximal tubules. Which of the kidney defects of the ACE^{-/-} mouse is caused by the lack of ACE expression in which cells of the kidney? Such a question can be experimentally addressed now using our approach. We will be also able to assess the physiological functional equivalence of gACE and sACE isozymes. Whether sACE expression in sperm can substitute for gACE expression is a question that can now be answered. Similarly, we can determine if the expression of secreted ACE, as compared with tissue-bound ACE, is sufficient for the reproductive function of ACE in sperm. Thus, the molecular genetic approach used in this study can be effectively applied to a variety of physiological questions.

Acknowledgments

We are indebted to Oliver Smithies and John Krege for the donation of an ACE^{+/-} mouse and for helpful discussion. We thank Indira Sen for ACE antibody, Robert Erickson for the PGKII promoter, Theresa Rowe and Natasha Lerner for technical assistance, and Debbie Wilson for secretarial assistance.

This work was supported in part by the National Institutes of Health grant HL-48258.

References

- Soffer, R.L. 1981. Angiotensin-converting enzyme. In *Biochemical Regulation of Blood Pressure*. R.L. Soffer, editor. John Wiley & Sons, Inc., New York. 123-164.
- Soubrier, F., C. Hubert, P. Testut, S. Nadaud, F. Alhenc-Gelas, and P. Corvol. 1993. Molecular biology of the angiotensin I converting enzyme. I. Biochemistry and structure of the gene. *J. Hypertens.* 11:471-476.
- Erdo, E.G. 1990. Angiotensin I converting enzyme and the changes in our concepts through the years. *Hypertension (Dallas)*. 16:363-370.
- Roy, S.N., J. Kusari, R.L. Soffer, C.Y. Lai, and G.C. Sen. 1988. Isolation of cDNA clones of rabbit angiotensin converting enzyme: identification of two distinct mRNAs for the pulmonary and the testicular isozymes. *Biochem. Biophys. Res. Commun.* 155:678-684.
- Soubrier, F., F. Alhenc-Gelas, C. Hubert, J. Allegrini, M. John, G. Tregear, and P. Corvol. 1988. Two putative active centers in human angiotensin I-converting enzyme revealed by molecular cloning. *Proc. Natl. Acad. Sci. USA*. 85:9386-9390.
- Kumar, R.S., J. Kusari, S.N. Roy, R.L. Soffer, and G.C. Sen. 1989. Structure of testicular angiotensin converting enzyme: a segmental mosaic isozyme. *J. Biol. Chem.* 264:16754-16758.
- Bernstein, K.E., B.M. Martin, A.S. Edwards, and E.A. Bernstein. 1989. Mouse angiotensin-converting enzyme is a protein composed of two homologous domains. *J. Biol. Chem.* 264:11945-11951.
- Ehlers, M.R., E.A. Fox, D.J. Strydom, and J.F. Riordan. 1989. Molecular cloning of human testicular angiotensin-converting enzyme: the testis isozyme is identical to the C-terminal half of endothelial angiotensin-converting enzyme. *Proc. Natl. Acad. Sci. USA*. 79:4295-4297.
- Berg, T., J. Solner, C.Y. Lai, and R.L. Soffer. 1986. Immunohistochemical localization of two angiotensin I-converting isoenzymes in the reproductive tract of the male rabbit. *J. Histochem. Cytochem.* 34:753-760.
- Langford, K.G., Y. Zhou, L.D. Russell, J.N. Wilcox, and K.E. Bernstein. 1993. Regulated expression of testis angiotensin-converting enzyme during spermatogenesis. *Biol. Reprod.* 48:1210-1218.
- Erickson, R.P., S.P. Kessler, H. Kremling, and G.C. Sen. 1996. Species variation in the testicular angiotensin-converting enzyme promoter studied in transgenic mice. *Mol. Reprod. Dev.* 44: 324-331.
- Sibony, M., J.M. Gasc, F. Soubrier, F. Alhenc-Gelas, and P. Corvol. 1993. Gene expression and tissue localization of the two isoforms of angiotensin I converting enzyme. *Hypertension (Dallas)*. 21:827-835.
- Ehlers, M.R., and J.F. Riordan. 1991. Membrane proteins with soluble counterparts: role of proteolysis in the release of transmembrane proteins. *Biochemistry*. 30:10065-10074.
- Sen, I., H. Samanta, W. Livingston, and G.C. Sen. 1991. Establishment of transfected cell lines producing testicular angiotensin-converting enzyme. Structural relationship between its secreted and cellular forms. *J. Biol. Chem.* 266:21985-21990.
- Beldent, V., A. Michaud, L. Wei, M.T. Chauvet, and P. Corvol. 1993. Proteolytic release of human angiotensin-converting enzyme. Localization of the cleavage site. *J. Biol. Chem.* 268:26428-26434.
- Sadhukhan, R., G.C. Sen, R. Ramchandran, and I. Sen. 1998. The distal ectodomain of angiotensin-converting enzyme regulates its cleavage-secretion from the cell surface. *Proc. Natl. Acad. Sci. USA*. 95:138-143.
- Kumar, R.S., T.J. Thekkumkara, and G.C. Sen. 1991. The mRNAs encoding the two angiotensin-converting isozymes are transcribed from the same gene by a tissue-specific choice of alternative transcription initiation sites. *J. Biol. Chem.* 266:3854-3862.
- Thekkumkara, T.J., W. Livingston, R.S. Kumar, and G.C. Sen. 1992. Use of alternative polyadenylation sites for tissue-specific transcription of two angiotensin-converting enzyme mRNAs. *Nucl. Acids Res.* 20:683-687.
- Howard, T.E., S.Y. Shai, K.G. Langford, B.M. Martin, and K.E. Bernstein. 1990. Transcription of testicular angiotensin-converting enzyme (ACE) is initiated within the 12th intron of the somatic ACE gene. *Mol. Cell Biol.* 10: 4294-4302.
- Hubert, C., A.M. Houot, P. Corvol, and F. Soubrier. 1991. Structure of the angiotensin I-converting enzyme gene. Two alternate promoters correspond to evolutionary steps of a duplicated gene. *J. Biol. Chem.* 266:15377-15383.
- Goraya, T.Y., S.P. Kessler, P. Stanton, R.W. Hanson, and G.C. Sen.

1995. The cyclic AMP response elements of the genes for angiotensin converting enzyme and phosphoenolpyruvate carboxykinase (GTP) can mediate transcriptional activation by CREM tau and CREM alpha. *J. Biol. Chem.* 270:19078–19085.
22. Kessler, S.P., T.M. Rowe, J.A. Blendy, R.P. Erickson, and G.C. Sen. 1998. 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. *J. Biol. Chem.* 273:9971–9975.
23. Langford, K.G., S. Shai, T. Howard, M. Kovak, P. Overbeek, and K.E. Bernstein. 1991. Transgenic mice demonstrate a testis-specific promoter for angiotensin converting enzyme. *J. Biol. Chem.* 266:15559–15562.
24. Howard, T., R. Balogh, P. Overbeek, and K.E. Bernstein. 1993. Sperm-specific expression of angiotensin-converting enzyme (ACE) is mediated by a 91–base-pair promoter containing a CRE-like element. *Mol. Cell Biol.* 13:18–27.
25. Zho, Y., Z. Sun, A.R. Means, P. Sassone-Corsi, and K.E. Bernstein. 1996. CREM τ is a positive regulator of testis ACE transcription. *Proc. Natl. Acad. Sci. USA.* 93:12262–12266.
26. Esther, C.R., D. Semeniuk, E.M. Marino, Y. Zhou, P.A. Overbeek, and K.E. Bernstein. 1997. Expression of testis angiotensin-converting enzyme is mediated by a cyclic AMP responsive element. *Lab. Invest.* 77:483–488.
27. Goraya, T.Y., S.P. Kessler, R.S. Kumar, J. Douglas, and G.C. Sen. 1994. Identification of positive and negative transcriptional regulatory elements of the rabbit angiotensin-converting enzyme gene. *Nucl. Acids Res.* 22:1194–1201.
28. Kessler, S.P., T.Y. Goraya, and G.C. Sen. 1996. Analysis of the negative transcriptional regulatory element in the angiotensin-converting enzyme gene. *Gene Expr.* 6:73–85.
29. Kregge, J.H., S.W.M. John, L.L. Langenbach, J.B. Hodgins, J.R. Hagan, E.S. Bachman, J.C. Jennette, D.A. O'Brien, and O. Smithies. Male-female differences in fertility and blood pressure in ACE-deficient mice. *Nature.* 375:146–148.
30. Esther, C.R., T.E. Howard, E.M. Marino, J.M. Goddard, M.R. Capocchi, and K.E. Bernstein. 1996. Mice lacking angiotensin-converting enzyme have low blood pressure, renal pathology and reduced male fertility. *Lab. Invest.* 74:953–965.
31. Esther, C.R., E.M. Marino, T.E. Howard, A. Machaud, P. Corvol, M.R. Capocchi, and K.E. Bernstein. 1997. The critical role of tissue angiotensin-converting enzyme as revealed by gene targeting in mice. *J. Clin. Invest.* 99:2375–2385.
32. Tian, B., Q.C. Meng, Y.F. Chen, J.H. Kregge, O. Smithies, and S. Oparil. 1997. Blood pressures and cardiovascular homeostasis in mice having reduced or absent angiotensin-converting enzyme gene function. *Hypertension (Dallas).* 30:128–133.
33. Robinson, M.O., J.R. McCarrey, and M.I. Simon. 1989. Transcriptional regulatory regions of testis-specific PGK2 defined in transgenic mice. *Proc. Natl. Acad. Sci. USA.* 86:8437–8441.
34. Hogan, B., F. Costantini, and E. Lacy, 1986. *Manipulating the Mouse Embryo: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Plainview, NY. 91–203.
35. Hagan, J.R., J.S. Moyer, E.S. Bachman, M. Sibony, P.L. Magyar, J.E. Welch, O. Smithies, J.H. Kregge, and D.A. O'Brien. 1998. Angiotensin-converting enzyme and male fertility. *Proc. Natl. Acad. Sci. USA.* 95:2552–2557.