

Recapitulation of Germ Cell- and Pituitary-Specific Expression With 1.6 kb of the Cystatin-Related Epididymal Spermatogenic (*Cres*) Gene Promoter in Transgenic Mice

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ABSTRACT: The *Cres* (cystatin-related epididymal spermatogenic) gene encodes the defining member of a new subgroup within the family 2 cystatins of cysteine protease inhibitors. *Cres* expression is highly tissue- and cell-specific, with messenger RNA (mRNA) present in the testicular round/elongating spermatids, proximal caput epididymal epithelium, gonadotroph cells in the anterior pituitary gland, and corpus luteum of the ovary. To begin to elucidate the molecular mechanisms controlling the tissue- and cell-specific expression of the *Cres* gene, transgenic mice were generated containing 1.6 kilobases (kb) of the mouse *Cres* promoter linked to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene. A CAT enzyme-linked immunosorbent assay detected CAT protein in the testis, epididymis, isolated cauda epididymal spermatozoa, and anterior pituitary gland from mice heterozygous and homozygous for the

transgene. However, reverse transcription (RT)-PCR did not detect CAT mRNA in any regions of the epididymis, suggesting that the CAT protein detected in the epididymis was from spermatozoa. RT-PCR also did not detect CAT mRNA in the ovary. RT-PCR analysis of the testes from mice of different postnatal ages showed CAT mRNA first detected at day 22, which correlated with the first appearance of *Cres* mRNA and with the presence of round spermatids. These studies demonstrate that 1.6 kb of *Cres* promoter contains the DNA elements necessary for germ cell and pituitary gland-specific expression but lacks critical sequences necessary for expression in the epididymis and ovary.

Key words: Epididymis, testis, spermatogenesis, gene regulation.
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The cystatin-related epididymal spermatogenic (CRES) protein defines a new subgroup in the cystatin family 2 of cysteine protease inhibitors by virtue of its conserved gene and protein structure and the cosegregation of the *Cres* gene with cystatin C on mouse chromosome 2 (Cornwall et al, 1999). Although CRES has the 4 highly conserved cysteine residues and thus is predicted to adopt the conformation of a prototypical family 2 cystatin, it lacks 2 of the 3 consensus sequences necessary for inhibition of C1 cysteine proteases and therefore might have a different function. Indeed, in recent studies, we demonstrated that, unlike cystatin C, CRES showed no inhibitory activity against the cysteine proteases papain or cathepsin B but rather inhibited the serine protease prohormone convertase 2 (Cornwall et al, 2003).

Cres also differs from the cystatins by exhibiting tis-

sue-specific expression. In contrast to the ubiquitous expression of cystatin C, *Cres* messenger RNA (mRNA) is present in the principal cells of the proximal caput epididymis, round spermatids, and anterior pituitary gonadotropes and was found recently in the ovary (Cornwall et al, 1992; Cornwall and Hann, 1995; Sutton et al, 1999; Hsia and Cornwall, 2003). Furthermore, *Cres* expression in the epididymis is dependent on testicular factors (Cornwall et al, 1992). To identify transcriptional elements necessary for the cell- and tissue-specific expression of the *Cres* gene, we initiated studies to examine the *Cres* gene promoter. Previously, an analysis of 1.6 kilobases (kb) of the mouse *Cres* promoter revealed transcription factor binding motifs present in highly regulated genes (Cornwall et al, 1999). These predicted DNA-binding elements included GATA, SF-1 (steroidogenic factor 1), SRY (sex-determining region Y), ERE (estrogen responsive element), Ptx1 (pituitary homeobox 1), and C/EBP (CCAAT/enhancer binding protein). We recently demonstrated that the transcription factor C/EBP β is the predominant C/EBP family member in the mouse epididymis and gonadotroph cells and is required for maximal expression of the *Cres* gene in these tissues (Hsia and Cornwall, 2001).

The purpose of this study was to determine whether 1.6 kb of *Cres* 5'-flanking sequences contain the neces-

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sary elements for cell- and tissue-specific expression. Toward this end, transgenic mice were generated that expressed the chloramphenicol acetyltransferase (CAT) gene under the control of a mouse *Cres* promoter fragment.

Materials and Methods

Animals

All experiments were conducted in accordance with the principles and procedures outlined in the NIH Guidelines for Care and Use of Experimental Animals.

Chimeric Construct

A DNA fragment containing approximately 1.6 kb of *Cres* promoter was generated by PCR with *Cres* 5'-flanking pGEM-T plasmid as template (Cornwall et al, 1999). The DNA fragment was purified on a 1% agarose gel and ligated into the promoterless pBLCAT3 plasmid (containing the bacterial CAT sequence) by standard methods such that the *Cres* promoter DNA fragment would drive expression of the CAT reporter gene. The 1.6-kb *Cres*-pBLCAT3 plasmid was digested with appropriate enzymes to remove vector sequences. The digested DNA was electrophoresed on a 1% low-melting point agarose gel in 1× Tris-acetate EDTA (TAE) buffer (Invitrogen, Carlsbad, Calif) for several hours, and the *Cres*-CAT cassette was excised and purified with GELase (Epicentre, Madison, Wis). The DNA pellet was washed several times in 70% ethanol before resuspending in Tris-EDTA (TE) buffer, pH 7.5. The A_{260}/A_{280} was determined, and a small aliquot was run on a 1% agarose/TAE gel to determine the DNA quality before pronuclear DNA microinjection.

Transgenic Mice and DNA Isolation

Transgenic mice (strain B6D2, Harlan Sprague-Dawley) were generated by microinjection of the linearized 1.6-kb *Cres*-CAT DNA into the male pronucleus of fertilized oocytes with the use of standard techniques (Palmiter and Brinster, 1985). Transgenic animals were identified by genomic Southern blot analysis. Approximately 3 to 4 mm of the tails from weaned mice was digested overnight at 55°C in 210 μ L of digestion solution (50 mM Tris-Cl, pH 8, 100 mM NaCl, 100 mM EDTA, 1% sodium dodecyl sulfate (SDS), 1.0 mg/mL of proteinase K). The DNA was extracted with 1 volume of Tris-saturated phenol, followed by 1:1 (vol/vol) phenol/chloroform, and then twice with chloroform. The DNA was subsequently precipitated at room temperature with 2.5 volumes of 100% ethanol. Samples were centrifuged at $10000 \times g$ at 4°C for 15 minutes, washed with 70% ethanol, and dried briefly at room temperature. The pellets were gently resuspended in 100 μ L of 0.1× saline-sodium citrate (SSC) and allowed to dissolve overnight at 4°C.

Southern Blot Analysis

Genomic DNA (10 μ g) prepared from tail snips was digested overnight with 40 U of *Pst*I restriction enzyme (Invitrogen) and electrophoresed overnight on a 0.8% agarose/1× TAE gel. The gel was incubated in 0.25 N HCl for 20 minutes, denatured in 0.5 M NaOH and 1.5 M NaCl for 45 minutes, and washed in

0.5 M Tris, pH 8, and 1.5 M NaCl for 45 minutes. The DNA was then transferred by vacuum blotting (Appligene, Illkirch, France) to nylon membrane (Nytran Supercharge, Schleicher and Schuell, Keene, NH). The membrane was ultraviolet (UV) cross-linked (Stratalinker; Stratagene, La Jolla, Calif) and prehybridized in Church buffer (0.5 M NaPO₄, pH 7.5, 1 mM EDTA, 7% SDS) at 65°C for 2 hours. Random-primed ³²P-labeled probes were synthesized from a 500-bp *Cres* promoter insert or the CAT insert (Prime It II kit; Stratagene) and were incubated overnight with the membrane (10⁶ cpm/mL) in Church buffer at 65°C. The membrane was washed twice for 20 minutes at room temperature in 0.2× SSC and 0.1% SDS before exposure to autoradiographic film. Transgene copy number was determined from the ratio of the intensity of the DNA fragment corresponding to the transgene and the 7-kb *Pst*I fragment corresponding to the endogenous *Cres* gene multiplied by 2. Mice homozygous for the CAT transgene were identified from those heterozygous for the transgene by comparing the relative band intensities following Southern blot analysis.

CAT Enzyme-Linked Immunosorbent Assay

The amount of CAT protein present in different tissues from transgenic mice heterozygous and homozygous for the *Cres*-CAT transgene was measured with a CAT enzyme-linked immunosorbent assay (ELISA; Roche Molecular Biochemicals, Indianapolis, Ind). Briefly, small tissue fragments from several mice were pooled and homogenized in 1× lysis buffer provided by the kit and protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, Ill). CAT protein standards and 150 μ g of each tissue lysate were added to the microplate and incubated for 2 hours at 37°C. The wells were washed 5 times with washing buffer provided in the kit and then incubated for 1 hour at 37°C with anti-CAT antibody conjugated to digoxigenin (anti-CAT DIG). The wells were washed again with buffer and incubated with anti-DIG antibody conjugated to peroxidase (anti-DIG POD) for 1 hour at 37°C. Sample wells were washed with washing buffer and incubated with peroxidase substrate and enhancer and the absorbance at 405 nm was measured with a Bio-Tek EL 312e microplate reader (Bio-Tek Instruments Inc, Winooski, Vt). Data (pg CAT protein/100 μ g of tissue) are presented as the mean \pm SEM of 3 independent experiments.

Reverse Transcription-PCR

Total RNA was isolated from mouse tissues with Trizol reagent (Invitrogen) following the manufacturer's protocol. The RNA was quantitated by A_{260}/A_{280} and visualized by gel electrophoresis in 1% agarose gel containing 1× borate buffer, pH 8.2, and 0.66 M formaldehyde with ethidium bromide in the RNA samples. For reverse transcription (RT)-PCR, 2.5 μ g total RNA was incubated in RT reaction buffer containing 5 mM MgCl₂, 50 mM KCl, 10 mM Tris, pH 8.3, 0.5 mM desoxynucleotide triphosphates (dNTPs), 20 U RNasin (RNase inhibitor, Promega, Madison, Wis), and 2.5 μ M oligo-dT (Promega) in a final volume of 25 μ L for 30 minutes at 37°C in the presence of 2.5 U RNase-free DNase I (Roche Molecular Biochemicals, Indianapolis, Ind). After heat inactivation of DNase I at 75°C for 5 minutes, an aliquot was reserved for PCR amplification as a no-RT control

to confirm the absence of contaminating DNA. Fifty units of MuLV reverse transcriptase (Perkin-Elmer Biosystems, Foster City, Calif) was added to the remainder and reverse transcription was carried out at 42°C for 30 minutes, 99°C for 5 minutes, and 4°C for 5 minutes.

Three microliters each of RT and no-RT reaction was amplified by PCR in separate reactions with primers recognizing CAT, *Cres*, and S16 complementary DNAs (cDNAs). S16 was amplified as a constitutive control to measure the relative efficiency of each RT reaction. The identity of the PCR products generated with each primer pair was confirmed by sequence analysis. PCR master mixes containing 10 mM Tris, pH 8.3, 50 mM KCl, 0.5 μM each of forward and reverse primers, and 1.25 U of *Taq* DNA polymerase (Sigma Chemical Co, St Louis, Mo) were prepared so that RNA samples within a particular experiment were amplified from a single master mix. MgCl₂ and dNTP concentrations, as well as cycle number, were optimized for each set of primers. *Cres* PCR reactions were carried out in 2.5 mM MgCl₂ and 0.25 mM dNTPs for 25 (reproductive tract samples) or 40 (pituitary samples) cycles. CAT and S16 reactions were amplified with 2 mM MgCl₂ and 0.2 mM dNTPs for 40 and 26 cycles, respectively. The cycling parameters consisted of 45 seconds at 95°C for denaturation, 25 seconds at annealing temperature (T_a) for each primer set, and 1 minute at 72°C for extension, after which the reactions were incubated at 72°C for 7 minutes with a minicycler (MJ Research Inc, Watertown, Mass). RT-PCR products were analyzed by gel electrophoresis in 1.5% agarose/1× TAE gels followed by ethidium bromide staining and examination under UV light. Each animal experiment was repeated, and in each case, the results of a representative experiment are shown.

Oligonucleotide Primer Pairs

PCR primers (Invitrogen) were designed from the sequences for mouse CAT, *Cres*, and S16 cDNAs with the use of PrimerSelect (Lasergene Suite; DNA Star, Madison, Wis) as follows: *Cres* sense: 5'-CAAGGAAAGTGAAGACAAATATGTC-3' and antisense: 5'-GTGACAGACTTGAACCACAGGTT-3', $T_a = 64^\circ\text{C}$, 25 or 40 cycles; CAT sense: 5'-TCTTGCCCGCCTGATGAATGCTC-3' and antisense: 5'-TACGCCCGCCCTGCCACTC-3', $T_a = 56^\circ\text{C}$, 40 cycles; and S16 sense: 5'-CGTGTCAGTCCGTGCAGGTCTT-3' and antisense: 5'-TCCAACTTTTTGGATTGCAGCG-3', $T_a = 56^\circ\text{C}$, 25 cycles.

Results

Identification of Mice Expressing the Cres-CAT Transgene

A DNA fragment containing 1.6 kb of the *Cres* gene promoter was subcloned into the promoterless pBLCAT3 plasmid to generate a construct in which the *Cres* sequence would drive expression of the CAT reporter gene (Figure 1A). Three founder animals (numbers 4, 20, and 30) were identified by genomic Southern blot analysis with a *Cres* promoter probe (Figure 1B, top panel). Unfortunately, female founder 20 died before a breeding line

could be established, whereas the transgene in male founder 30 appeared to have integrated into a silenced region of the Y chromosome because no F1 female offspring possessed the transgene and F1 males, although containing the transgene, showed no CAT expression (data not shown). For subsequent studies, male founder 4 was mated with wild-type females and passed the *Cres-CAT* transgene to its offspring in a Mendelian manner. As shown in Figure 1B (bottom panel), Southern blot analysis was carried out with the use of genomic DNA isolated from the tails of F1 mice and probes representing either the first 500 bp of the *Cres* promoter or the CAT cDNA. The *Cres* probe hybridized to a 7-kb *Pst*I fragment that was present in all mice and represented the endogenous *Cres* gene. In addition, a larger DNA fragment was detected in some mice, indicating the presence of the transgene in the genomes of these animals. This was confirmed by Southern analysis with the CAT probe, which only detected the larger *Pst*I DNA fragment and not the endogenous *Cres* gene. A comparison of the hybridization intensities for the transgene and endogenous *Cres* gene indicated a transgene copy number of 1 for line 4 heterozygous mice.

CAT Protein Expression in Transgenic Mouse Tissues

We previously showed that the *Cres* gene is expressed in the principal cells of the proximal caput epididymis, round spermatids in the testis, anterior pituitary gonadotropes, and, most recently, corpora lutea of the ovary (Cornwall et al, 1992; Cornwall and Hann, 1995; Sutton et al, 1999; Hsia and Cornwall, 2003). To determine whether the CAT transgene was expressed in mouse tissues in a similar tissue-specific manner, a CAT ELISA was used to measure CAT protein in tissue extracts from different regions of the mouse epididymis, as well as in 16 other tissues from male and female mice heterozygous and homozygous for the transgene. As shown in Figure 2, CAT protein exhibited tissue-specific expression similar to that of endogenous CRES and was detected in mouse testis and the distal regions of the epididymis; however, the highest amount of CAT protein was present in spermatozoa isolated from the cauda epididymis. Because of variations within the CAT assay, however, CAT protein levels in spermatozoa were not statistically different from that in the testis. Lower levels of CAT protein were present in the proximal regions of the epididymis, vas deferens, female reproductive tract, and pituitary gland. CAT protein was not detected in the seminal vesicle, prostate, brain, heart, liver, kidney, spleen, adrenal, and lung of transgenic mice. In general, mice homozygous for the transgene had approximately twice the levels of CAT protein present in mice heterozygous for the transgene, suggesting a proportional increase as the result of an increase in copy number. One exception to this was in the female anterior pituitary gland, in which there ap-

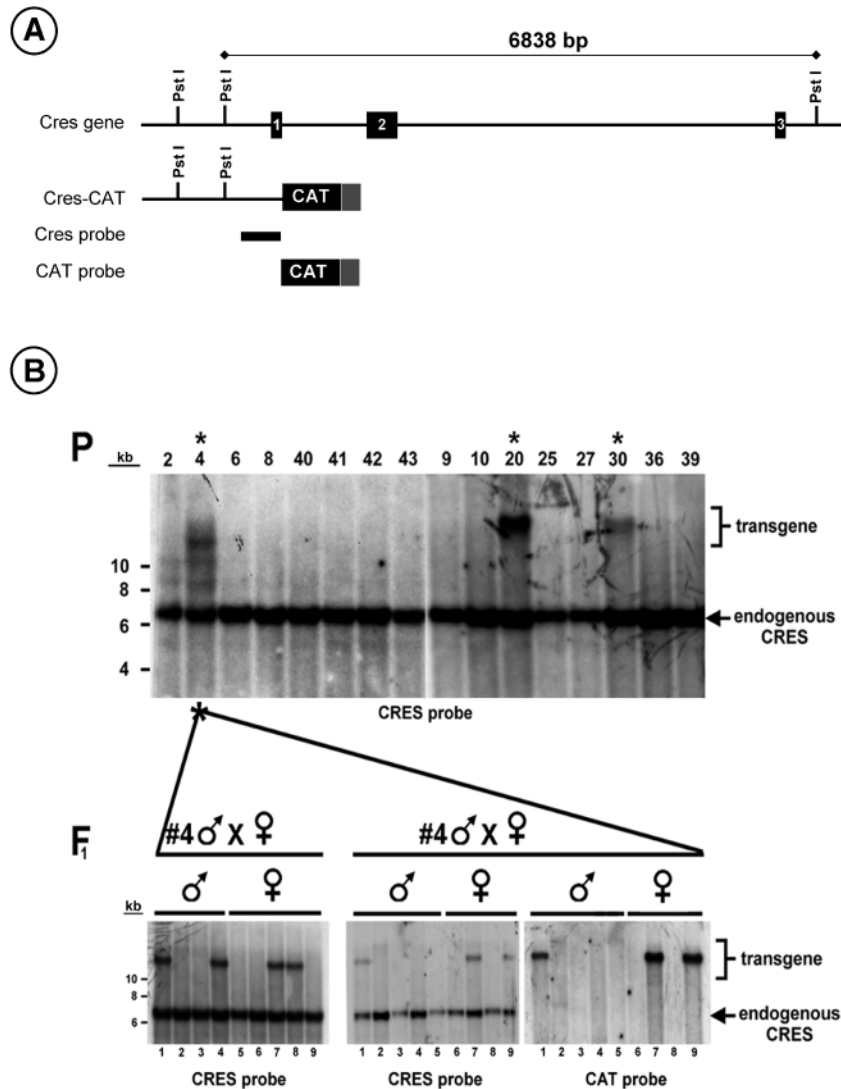


Figure 1. Genomic Southern blot analysis. **(A)** Schematic diagram of the endogenous *Cres* (cystatin-related epididymal spermatogenic) gene and the *Cres*-chloramphenicol acetyltransferase (CAT) transgene with *Pst*I sites indicated. The primary transcription start site is located at the first residue of exon 1, with a minor start site located 15 basepairs (bp) upstream (Cornwall et al, 1999). The regions of DNA from which probes were generated for Southern blot analysis are noted. The *Cres* probe recognized the first 500 bp of the *Cres* promoter, whereas the CAT cDNA probe contained the full coding sequence for CAT. **(B, top panel)** Genomic DNA isolated from tail snips was digested with *Pst*I and examined by Southern blot analysis with the *Cres* probe to identify founder mice expressing the transgene. The endogenous 7-kb *Cres* gene was present in all animals, whereas larger *Pst*I fragments representing the *Cres*-CAT transgene were only present in 3 animals. **(B, bottom panel)** Founder male 4 was mated with wild-type females to generate F1 offspring that were examined for transgene expression with the use of either *Cres* or both *Cres* and CAT probes in Southern blot analysis.

peared to be higher levels of CAT protein in the heterozygous animals compared with the homozygous animals. Also, CAT protein appeared to be higher in female anterior pituitary glands compared with male glands. Because in separate studies we have determined that *Cres* mRNA and protein in the anterior pituitary gland are differentially regulated by gonadotropin-releasing hormone (GnRH) and steroid hormones (Sutton-Walsh and Cornwall, unpublished observations), the differences we see in CAT protein levels likely reflect the hormonal state of the animals rather than true sex differences or differences between hetero-

zygous and homozygous mice. As expected, tissues isolated from control, nontransgenic mice had no detectable CAT protein (data not shown). The high amount of CAT protein in the testis and in isolated cauda spermatozoa, as well as the increasing amounts of CAT protein along the epididymal tubule, strongly suggested that the 1.6-kb *Cres* promoter possessed the necessary elements to confer expression of the CAT reporter in the testis and, specifically, in the germ cells. However, at this time, we could not rule out that the epididymal epithelium also expressed CAT protein.

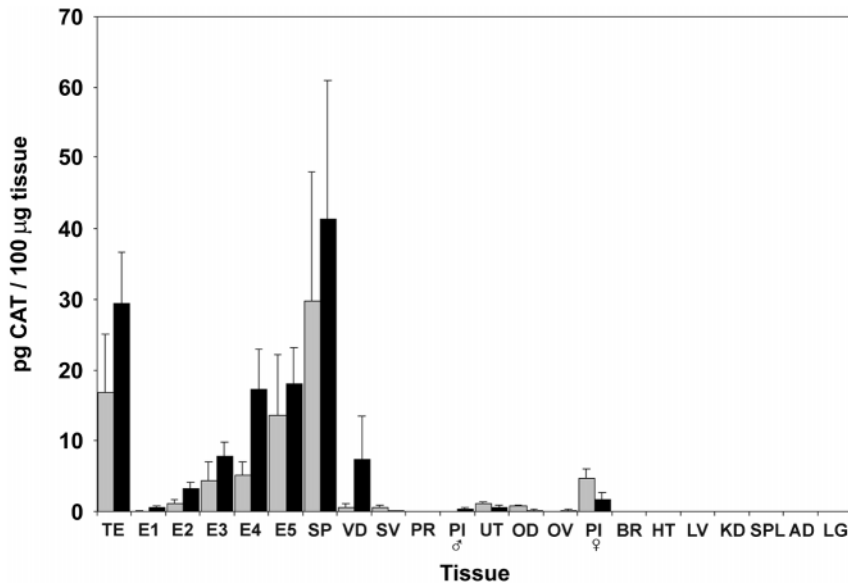


Figure 2. Chloramphenicol acetyltransferase (CAT) enzyme-linked immunosorbent assay (ELISA) of transgenic mouse tissues. Protein extracts prepared from tissues pooled from 3 mice heterozygous (gray bars) and homozygous (black bars) for the transgene were examined for CAT protein with a colorimetric CAT ELISA. TE indicates testis; E1, proximal caput; E2, midcaput; E3, distal caput; E4, corpus; E5, cauda epididymis; SP, cauda spermatozoa; VD, vas deferens; SV, seminal vesicle; PR, prostate; PI, anterior pituitary gland; UT, uterus; OD, oviduct; OV, ovary; BR, brain; HT, heart; LV, liver; KD, kidney; SPL, spleen; AD, adrenal gland; and LG, lung. Data represent the mean \pm SEM of 3 experiments, each using protein extracts prepared from 3 mice.

RT-PCR Analysis of CAT mRNA in Transgenic Mouse Tissues

RT-PCR was carried out to examine *Cres* and CAT mRNA expression in the testis and 5 regions of the mouse epididymis from mice homozygous for the *Cres-CAT*

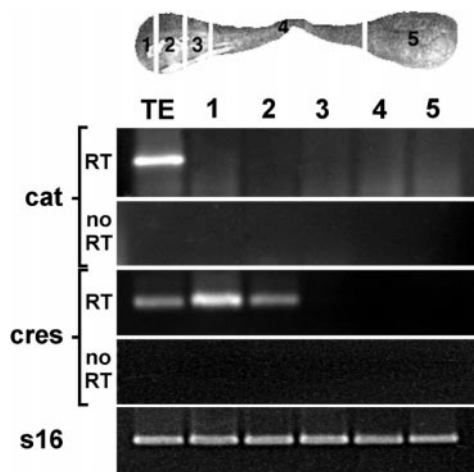


Figure 3. Reverse transcription (RT)-PCR analysis of chloramphenicol acetyltransferase (CAT) and *Cres* (cystatin-related epididymal spermatogenic) messenger RNAs (mRNAs) in the testis and different regions of the epididymis. Total RNA was isolated from the testis and the 5 epididymal regions from mice homozygous for the transgene. S16, an mRNA encoding a ribosomal protein, was used as a loading control. No RT-PCR was carried out on template that was not reverse transcribed, to confirm the absence of contaminating DNA. Data show a representative RT-PCR experiment from 3 replicate experiments, each using different RNA preparations.

transgene. Previously, we demonstrated by Northern blot analysis that expression of the *Cres* gene was highest in the proximal caput (region 1), with lower expression in the midcaput (region 2) and testis (Cornwall et al, 1992). RT-PCR confirmed our initial studies and showed *Cres* mRNA in the testis and epididymal regions 1 and 2 (Figure 3). In contrast, CAT mRNA was only detected in the testis and not the epididymis, suggesting that the CAT protein detected along the epididymal tubule and in the vas deferens of CAT transgenic mice was from spermatozoa. We cannot eliminate the possibility, however, that CAT mRNA is present in these tissues, but at very low levels.

We also used RT-PCR to examine *Cres* and CAT mRNA expression in the female reproductive tract and pituitary gland from male and female transgenic mice (Figure 4). As expected, *Cres* mRNA was present in the ovary, with lower levels in both male and female pituitary glands. Because *Cres* mRNA in the pituitary gland is hormonally regulated (Sutton-Walsh and Cornwall, unpublished observations), the differences in *Cres* mRNA levels between the pituitaries from males and females likely reflect the hormonal state of the animals. Interestingly, low levels of *Cres* mRNA were also detected in the oviduct and uterus, revealing new sites of *Cres* expression not previously detected by our Northern blot studies (Cornwall et al, 1992). CAT mRNA was detected in the male and female pituitary gland but not in the female reproductive tissues, suggesting that, in addition to driving

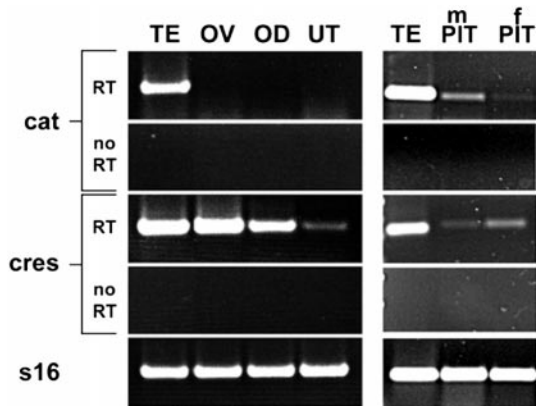


Figure 4. Reverse transcription (RT)–PCR analysis of chloramphenicol acetyltransferase (CAT) and *Cres* (cystatin-related epididymal spermatogenic) messenger RNAs (mRNAs) in testis, anterior pituitary gland, and female reproductive tissues. Total RNA was isolated from various tissues. TE indicates testis; OV, ovary; OD, oviduct; UT, uterus; and male (m) and female (f)PIT, anterior pituitary glands from mice homozygous for the transgene. The S16 and no-RT controls are described in the legend to Figure 3. Data show a representative RT-PCR experiment from 6 replicate experiments, each using different RNA preparations.

transgene expression in the testis, the 1.6-kb *Cres* promoter fragment can also drive pituitary gland expression. Whether the 1.6-kb promoter fragment also contains all the necessary elements for the same response to hormonal stimuli as the endogenous *Cres* gene in the pituitary gland is not known. The higher levels of CAT mRNA in the male compared with the female pituitary, which is opposite that of *Cres* mRNA levels, might suggest subtle differences between the transgene and the endogenous *Cres* gene in their response to hormones. Similar to our observations in the epididymis and vas deferens, we can-

not rule out that CAT mRNA is present, but at very low levels, in female reproductive tissues.

Temporal Expression of CAT mRNA in the Testes of Postnatal Mice

The mRNA and protein analyses thus far suggested that the CAT transgene was expressed by the testicular germ cells. We previously established by in situ hybridization that *Cres* mRNA is primarily expressed by round and elongating spermatids (Cornwall and Hann, 1995). However, because CAT mRNA and protein levels were low in the transgenic mice, we were unable to use in situ hybridization or immunohistochemistry to determine whether the CAT transgene was expressed in the same germ cell populations as the endogenous *Cres* gene. An indirect approach to look at expression during different stages of male germ cell development was to examine CAT mRNA levels in testes from mice at different postnatal ages, which reflected expression at different points along the first wave of spermatogenesis. The first round of spermatogenesis in mouse starts after birth and is characterized by the sequential appearance of cells corresponding to each stage of spermatogenesis (Bellve et al, 1977). Spermatogonia typically appear at postnatal days 6 through 7 and round spermatids at postnatal days 22 through 25. As shown in Figure 5, CAT mRNA was first detected in the testes from day 22 mice, which corresponded with the appearance of *Cres* mRNA and with approximately the first appearance of round spermatids. CAT and *Cres* mRNA were also detected in the testes from mice at days 26 and 31, which likely reflected accumulation of round spermatids and the appearance of

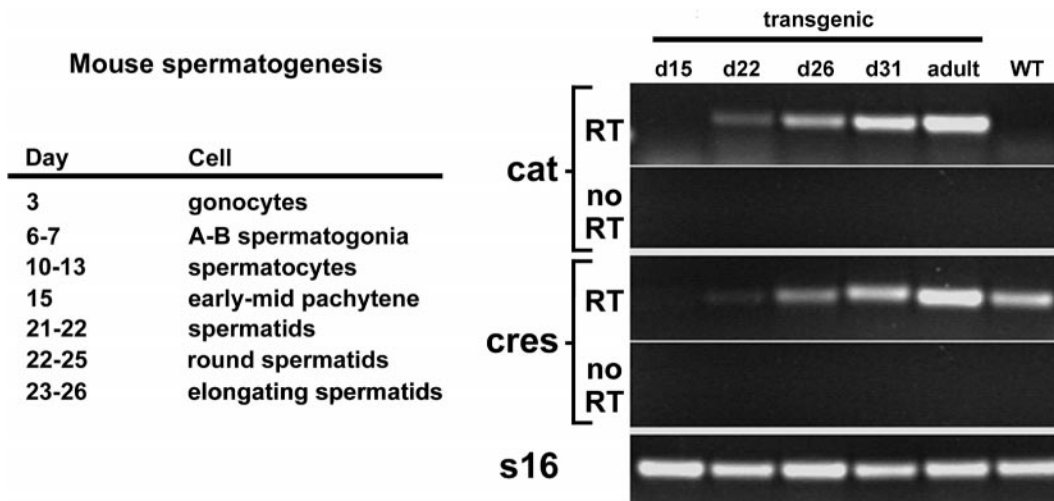


Figure 5. Reverse transcription (RT)–PCR analysis of chloramphenicol acetyltransferase (CAT) and *Cres* (cystatin-related epididymal spermatogenic) messenger RNAs (mRNAs) in the testes from mice from different postnatal ages. Total RNA was isolated from testes at postnatal days 15, 22, 26, and 31 and from adult (>12 weeks) testes from mice homozygous for the transgene. Total RNA was also prepared from the testes of adult wild-type, nontransgenic mice (WT). The S16 and no-RT controls are described in the legend to Figure 3. Data show a representative RT-PCR experiment from 5 replicate experiments, 3 of which were performed with different RNA preparations. The approximate postnatal ages when specific germ cell populations are present during the first wave of spermatogenesis are indicated.

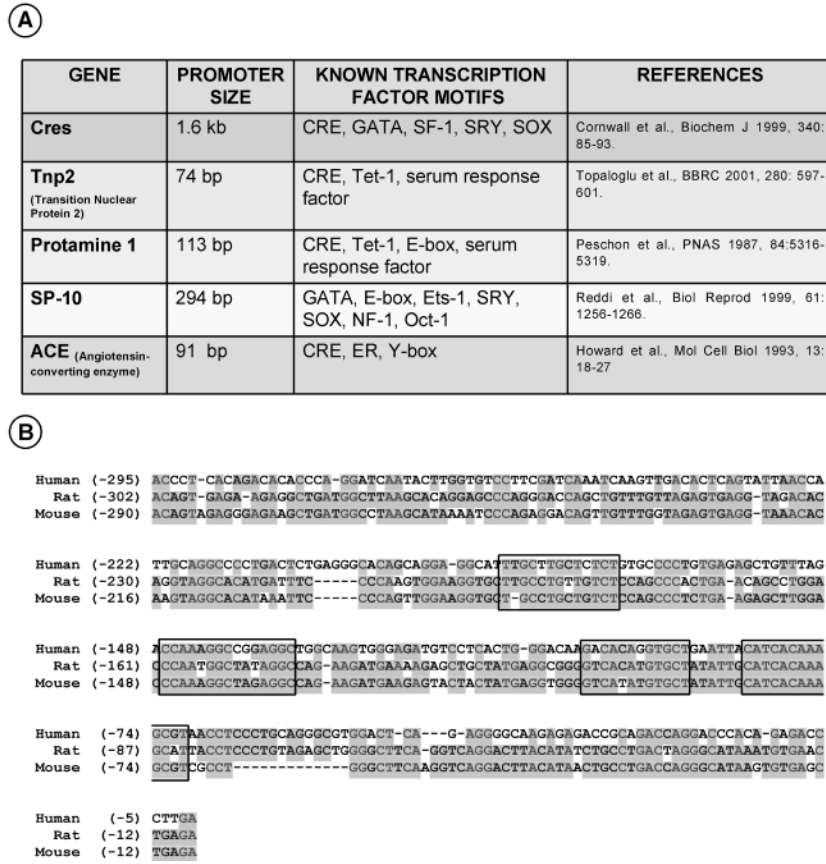


Figure 6. Potential DNA regulatory elements for spermatid-specific expression. **(A)** Gene promoters that target spermatid-specific expression, including size of the promoter fragment studied and transcription factor motifs present within the promoter fragment. **(B)** Alignment of 5'-flanking sequences of mouse, rat, and human *Cres* (cystatin-related epididymal spermatogenic) genes. Sequence similarity between the proximal promoters of mouse and rat, 83%; mouse and human, 50%; and rat and human, 53%. Boxes indicate regions of high identity between the promoters of all 3 species, which could be important for germ cell-specific expression.

elongating spermatids. Although we cannot completely eliminate the possibility that CAT mRNA is expressed at specific stages by Sertoli, Leydig, or other somatic cells in the testis, the parallel temporal expression of *Cres* and CAT mRNAs in the postnatal mice suggests that, like the endogenous *Cres* gene, CAT is present in the round and elongating spermatids. These data also indicate that the 1.6-kb *Cres* promoter fragment has sufficient regulatory sequences to recapitulate the developmentally regulated expression of the endogenous *Cres* gene in the testis.

Discussion

Our studies show that 1.6 kb of *Cres* gene promoter was able to direct CAT transgene expression in the testis and pituitary gland, but not the epididymis or ovary, thereby mimicking, in part, the tissue-specific expression of the endogenous *Cres* gene. Although the low levels of CAT mRNA and protein expression in the transgenic mice precluded a definitive identification of the cell populations

expressing CAT in the testis and pituitary gland, the presence of CAT protein in isolated cauda epididymal spermatozoa and the parallel temporal expression of *Cres* and CAT mRNAs in the testes of postnatal mice support that CAT, like the endogenous *Cres* gene, is expressed in the round and elongating spermatids.

In addition to our studies presented here, several studies have identified spermatid-specific gene promoters. It is of interest that many of these promoters have been localized to short proximal promoter sequences, including protamine 1 (119 bp), t-ACE (91 bp), proenkephalin (119 bp), *Phda-2* (187 bp), and *Ldhc-4* (100 bp) (Howard et al, 1993; Zambrowicz et al, 1993; Iannello et al, 1997; Liu et al, 1997; Li et al, 1998). A comparison of several of these promoters with the *Cres* promoter revealed no similar stretches of sequence homology. Although a common motif is not present in all spermatid-specific promoters, there is a commonality of putative transcription factor binding motifs (Figure 6A), which could have important consequences for germ cell-specific expression.

Another approach to identifying regions of sequence

important for cell-specific expression is the comparison of gene promoters from different species. Previously, we sequenced portions of the rat and human *Cres* 5'-flanking sequences, and their alignment with the mouse *Cres* promoter sequence is shown in Figure 6B. We have also established that, like mouse and rat, human *Cres* mRNA localizes to the round spermatids (Wassler et al, 2002); therefore, promoter elements that are common to mouse, rat, and human could be critical for round spermatid-specific expression. In Figure 6B, regions of the 5'-flanking sequence that have high identity between all 3 species, and thus might bind transcription factors important for germ cell expression, are indicated by boxes. Computer analyses for known transcription factor binding sites yielded no matches, suggesting that these regions of sequence could represent novel transcription factor binding sites involved in the germ cell-specific expression of *Cres*.

In contrast to the somewhat limited studies of germ cell promoters, gene promoters that target expression to the anterior pituitary gland, and specifically to gonadotroph cells, have been more fully characterized. The gonadotrope-specific element (GSE), which interacts with the SF-1 transcription factor (Keri and Nilson, 1996), is important for conferring gonadotrope-specific expression and has been shown to regulate the expression of several genes involved in steroidogenesis and reproductive function (Ingraham et al, 1994). In addition to SF-1, the transcription factor Egr-1 (early growth response protein-1) has been shown to be important for full LH β gene expression, possibly by a synergistic interaction with SF-1, as well as GnRH responsiveness (Halvorson et al, 1998; Tremblay and Drouin, 1999). Pitx1 (pituitary homeobox 1) transcription factor also plays a key role in normal gonadotrope function (Lanctot et al, 1999; Szeto et al, 1999). Studies of the proximal LH β promoter indicate that full activation of the LH β gene, including GnRH responsiveness, requires the functional interactions of SF-1, Egr-1, and Pitx1 (Tremblay and Drouin, 1999; Quirk et al, 2001). Our studies showed that the 1.6-kb *Cres* promoter fragment targeted transgene expression in the anterior pituitary gland, thereby recapitulating the expression of the endogenous *Cres* gene. Similar to the LH β gene promoter, the 1.6-kb *Cres* promoter fragment contains a GSE (SF-1) element as well as an Egr-1 and several Pitx1 elements, suggesting that these sites might also play necessary roles for *Cres* expression in gonadotroph cells. As a result of the low levels of CAT mRNA and protein expression in the pituitary glands of transgenic mice, however, we were unable to confirm transgene expression in the gonadotroph cells.

Compared with tissues such as the liver or kidney that showed no CAT protein, low levels of CAT protein were consistently detected in the proximal epididymis and fe-

male reproductive tissues. However, we did not detect the corresponding CAT mRNA. The lack of CAT mRNA could be because specific DNA elements necessary for the activation of epididymal and female reproductive tract transcription are not present in the 1.6-kb *Cres* promoter fragment. Alternatively, because some CAT protein was detected, perhaps the levels of CAT mRNA are too low to be detected, suggesting that tissue-specific enhancers are missing from the promoter fragment. In fact, CAT expression in the testis and pituitary gland from transgenic animals was much lower than that of the endogenous *Cres* gene, suggesting that additional *cis*-acting sequences, such as specific enhancer elements in the endogenous *Cres* promoter, might also be necessary for high levels of expression in these tissues. Finally, we cannot rule out the possibility that the lack of CAT expression in the epididymis and ovary is because the transgene inserted into a region of the genome that prevented expression in these tissues.

It is of interest that, of the few transgenic studies that have been performed to identify promoter sequences that direct epididymal-specific expression, several have yielded inappropriate patterns of reporter gene expression or a complete lack of epididymal expression. The endogenous cysteine-rich secretory protein 1 (*CRISP-1*) gene is expressed in the corpus and cauda regions of the mouse epididymis; however, 3.8 kb of the *CRISP-1* 5'-flanking sequence linked to EGFP (enhanced green fluorescent protein)-directed transgene expression to the testis rather than the epididymis (Lahti et al, 2001). In contrast to the expression of the endogenous glutathione peroxidase 5 (*GPX5*) gene in the caput region, a 5-kb promoter fragment of the gene directed expression of EGFP to a smaller region of the caput, as well as to the cauda epididymis and other mouse tissues (Lahti et al, 2001). Transgenic mice expressing 0.3-kb of the *Pem* homeobox gene promoter conferred transgene expression to the caput, where the endogenous *Pem* gene is expressed, but also an aberrant expression in the corpus. The aberrant expression was lost when 0.6 kb of *Pem* promoter was included in the transgene, suggesting that negative regulatory elements exist between 0.6 and 0.3 kb of the *Pem* promoter (Rao et al, 2002). To date, only 5 kb of the epididymal retinoic acid-binding protein (E-RABP) gene promoter and 5.3 kb of the related 17-kd murine epididymal protein (mEP17) gene promoter have been shown to drive reporter expression in an appropriate region- and epididymal-specific manner (Lareyre et al, 1999; Suzuki et al, 2003). Thus, it appears that the DNA elements that mediate region-specific gene expression in the epididymis are quite complex and will require further investigation to dissect out the critical components.

Taken together, our studies demonstrate that 1.6 kb of the mouse *Cres* promoter contains all the necessary in-

formation to drive the tissue-specific expression of the CAT transgene to the testicular germ cells and the anterior pituitary gland, thereby recapitulating, in part, the expression of the endogenous *Cres* gene. The 1.6-kb promoter fragment, however, lacks essential enhancer elements required for endogenous levels of expression of the reporter gene in these tissues. The *Cres* promoter fragment also lacks essential tissue-specific elements or enhancers that allow expression or normal levels of expression in the other *Cres*-expressing tissues, such as the epididymis and ovary. Further studies that use promoter deletion analyses are required to establish whether smaller *Cres* promoter fragments retain the ability to direct germ cell-specific expression of *Cres*. In addition, larger *Cres* promoter fragments are needed to identify regions that confer epididymal and ovarian expression.

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