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# The Unique Catalytic Subunit of Sperm cAMP-dependent Protein Kinase Is the Product of an Alternative $C\alpha$ mRNA Expressed Specifically in Spermatogenic Cells

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cAMP-dependent protein kinase has a central role in the control of mammalian sperm capacitation and motility. Previous protein biochemical studies indicated that the only cAMP-dependent protein kinase catalytic subunit (C) in ovine sperm is an unusual isoform, termed  $C_s$ , whose amino terminus differs from those of published C isoforms of other species. Isolation and sequencing of cDNA clones encoding ovine  $C_s$  and  $C\alpha 1$  (the predominant somatic isoform) now reveal that  $C_s$  is the product of an alternative transcript of the  $C\alpha$  gene.  $C_s$  cDNA clones from murine and human testes also were isolated and sequenced, indicating that  $C_s$  is of ancient origin and widespread in mammals. In the mouse,  $C_s$  transcripts were detected only in testis and not in any other tissue examined, including ciliated tissues and ovaries. Finally, immunohistochemistry of the testis shows that  $C_s$  first appears in pachytene spermatocytes. This is the first demonstration of a cell type-specific expression for any C isoform. The conservation of  $C_s$  throughout mammalian evolution suggests that the unique structure of  $C_s$  is important in the subunit's localization or function within the sperm.

## INTRODUCTION

cAMP-dependent protein kinase (PKA) (for review, see Taylor *et al.*, 1990) is a key enzyme in the control of mammalian sperm function (Garbers and Kopf, 1980). PKA-dependent protein phosphorylation is essential for rendering mammalian sperm capable of movement during epididymal maturation (Pariset *et al.*, 1985; Jaiswal and Majumder, 1996; Yeung *et al.*, 1999) and is critical for the maintenance of motility in mature sperm (Garbers *et al.*, 1971; Lindemann, 1978; Tash and Means, 1982; Brokaw, 1987; San Agustin and Witman, 1994; Chaudhry *et al.*, 1995). PKA also is important in the signaling events leading to capacitation and the acrosome reaction in sperm (Duncan and Fraser, 1993; Visconti *et al.*, 1995, 1997, 1999a,b; Galantino-Homer *et al.*, 1997; Aitken *et al.*, 1998; Osheroff *et al.*, 1999). Thus, an understanding of

the proteins involved in sperm cAMP-dependent control pathways is a major goal of current research in reproductive biology (Cummings *et al.*, 1994; Burton *et al.*, 1999; Osheroff *et al.*, 1999).

The PKA holoenzyme consists of two catalytic subunits (C) bound to two regulatory subunits (R) in a tetrameric complex ( $R_2C_2$ ). There are three known genes encoding mammalian C. The  $C\alpha$  gene is expressed in most tissues (Showers and Maurer, 1986; Uhler *et al.*, 1986a,b). The  $C\beta$  gene also is expressed in multiple tissues but generally at lower levels than  $C\alpha$  (Showers and Maurer, 1986; Uhler *et al.*, 1986b).  $C\gamma$  is a transcribed retroposon found only in primates and expressed only in testis (Beebe *et al.*, 1990; Reinton *et al.*, 1998).

We recently determined that the PKA catalytic subunit of ovine sperm ( $C_s$ ) differs from that of bovine, murine, or human  $C\alpha 1$  (the predominant somatic isoform) in its amino terminus (San Agustin *et al.*, 1998). A combination of tandem mass spectrometry and Edman degradation of  $C_s$  peptides indicated that the amino-terminal myristate and first 14 amino acids of the published  $C\alpha 1$  subunits are replaced by an amino-terminal acetate and 6 different amino acids in ovine  $C_s$ . However, short peptide sequences from more carboxyl-terminal portions of ovine  $C_s$  were identical to the published sequence of bovine  $C\alpha 1$ . Although the complete

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Abbreviations used: C, catalytic subunit of PKA; PKA, cAMP-dependent protein kinase; R, regulatory subunit of PKA; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; TBS, Tris-buffered saline, pH 7.5; UTR, untranslated region.

**Table 1.** Oligonucleotide primers used in amplifying  $C\alpha 1$  and  $C_s$  cDNAs

Primer	Description	5' to 3' nucleotide sequence
<i>Caa</i>	Bovine $C\alpha 1$ 7–26	AACGCCGCCGCCGCCAAGAA
<i>Cab</i>	Bovine $C\alpha 1$ 328–347	TCCTTCAAGGACAACCTCAA
<i>CacR</i>	Complement of bovine $C\alpha 1$ 782–800	TTCAAGTCAGAGCTGAAGT
<i>CadR</i>	Complement of bovine $C\alpha 1$ 928–947	ATGAAGGGAGCTTCCACCTT
<i>CaeR</i>	Complement of bovine $C\alpha 1$ 929–955	ACTTTGGTATGAAGGGAGCTTCCACCT
<i>oCa376</i>	Ovine $C\alpha 1$ 376–402 (equivalent to ovine $C_s$ 352–378)	GGTGGGGAGATGTTCTCACACCTGCGA
<i>oCa482R</i>	Complement of ovine $C\alpha 1$ 456–482 (equivalent to ovine $C_s$ 432–458)	AGCGAGTGCAGTACTCTAAGGTCAGG
<i>oCs(-11)</i>	Ovine $C_s$ -11 to 16	AAGACTGAGTGATGGCTTCCAACCCCA
<i>mCa791R</i>	Complement of murine $C\alpha 1$ 771–791	GAGCTGAAGTGGGATGGGAAC
<i>mCs(-188)</i>	Murine $C_s$ -188 to -167	GTTCTATCTGCCCTACCCTGC
<i>hCa(-60)</i>	Human $C\alpha 1$ -60 to -43	GCCGCAGCCAGCACCCGC
<i>AP1</i>	Adaptor primer	CCATCCTAATACGACTCACTATAGGGC
<i>Nested AP1</i>	Adaptor primer	ACTCACTATAGGGCTCGAGCGGC

Numbers represent nucleotide positions in  $C\alpha 1$  or  $C_s$  mRNA. Nucleotides upstream of a translation start site are numbered 3' to 5' beginning with -1; those downstream are numbered 5' to 3' beginning with +1.

sequence of neither the sperm nor the somatic form of ovine C was determined, the results indicated that ovine  $C_s$  is a novel isoform more closely related to  $C\alpha 1$  than to  $C\beta$  or  $C\gamma$ .

The discovery that ovine sperm contain a novel isoform of C raised a number of important questions. First, how is the sperm isoform generated? Is it the product of a unique gene or of an alternative transcript derived from the same gene as  $C\alpha 1$ ? Second, how widely distributed is it phylogenetically? The unique isoform was not identified in previous biochemical, immunological, and molecular genetic analyses of sperm PKA or C RNAs and cDNAs from testis of rodents and primates (Beebe *et al.*, 1990; Øyen *et al.*, 1990; Reinton *et al.*, 1998; Burton *et al.*, 1999); was it simply overlooked, or did  $C_s$  evolve relatively recently in the sheep or its immediate ancestors? Third, in what tissues is  $C_s$  expressed? If it is expressed in a range of ciliated tissues, it may have been selected for assembly into ciliary and flagellar axonemes in general. If  $C_s$  is expressed in both male and female reproductive tissues, it may be specific to the germ line. If it is expressed only in testis, is it present in all testicular cells, only in the germ cells, or only in those germ cells producing protein for incorporation into the sperm? If the latter,  $C_s$  may have evolved for assembly or function in the unusual intracellular environment of the sperm.

We have now isolated cDNA clones encoding ovine testis  $C_s$  and  $C\alpha 1$  and determined their nucleotide sequences. In agreement with our previous amino acid sequence data, the cDNAs predict different amino-terminal sequences for  $C_s$  and  $C\alpha 1$ . The differences extend from the subunits' amino termini to their presumptive exon 1/exon 2 boundaries. (Presumptive exon junctions for the ovine  $C\alpha 1$  and  $C_s$  cDNAs and the human  $C_s$  cDNA are based on the mouse  $C\alpha$  genomic sequence [Chrivia *et al.*, 1988]). However, the nucleotide sequences of the  $C_s$  and  $C\alpha 1$  cDNAs downstream of these boundaries are identical. Moreover, the first exon for  $C_s$  (termed exon 1s) and the first exon for  $C\alpha 1$  (termed exon 1a) are spliced to the same 3'-untranslated region (UTR) in mature transcripts. These results provide conclusive evidence that  $C_s$  is the product of an alternative transcript of the  $C\alpha$  gene. We found that  $C_s$  also is present in murine and human testis, and we cloned and sequenced cDNAs encod-

ing the  $C_s$  from these species. Thus,  $C_s$  is of ancient origin and widespread in mammals. We used reverse transcriptase (RT)-PCR to probe for  $C_s$  transcripts in a wide variety of murine tissues, including ciliated tissues and ovarian tissue, and found that  $C_s$  transcripts are present only in the testis. Finally, we generated an antibody specific for the amino terminus of murine  $C_s$ . Immunohistochemistry with the use of this antibody indicates that  $C_s$  is present only in spermatogenic cells and appears first in pachytene spermatocytes when many other proteins destined for assembly into the developing sperm are first synthesized. This is the first demonstration of a cell type-specific expression of any C isoform. Together, these findings indicate that  $C_s$  is a sperm-specific isoform of  $C\alpha$  that has been conserved throughout mammalian evolution. The unique structure of  $C_s$  may be important in the assembly, localization, or function of this key regulatory subunit in the sperm.

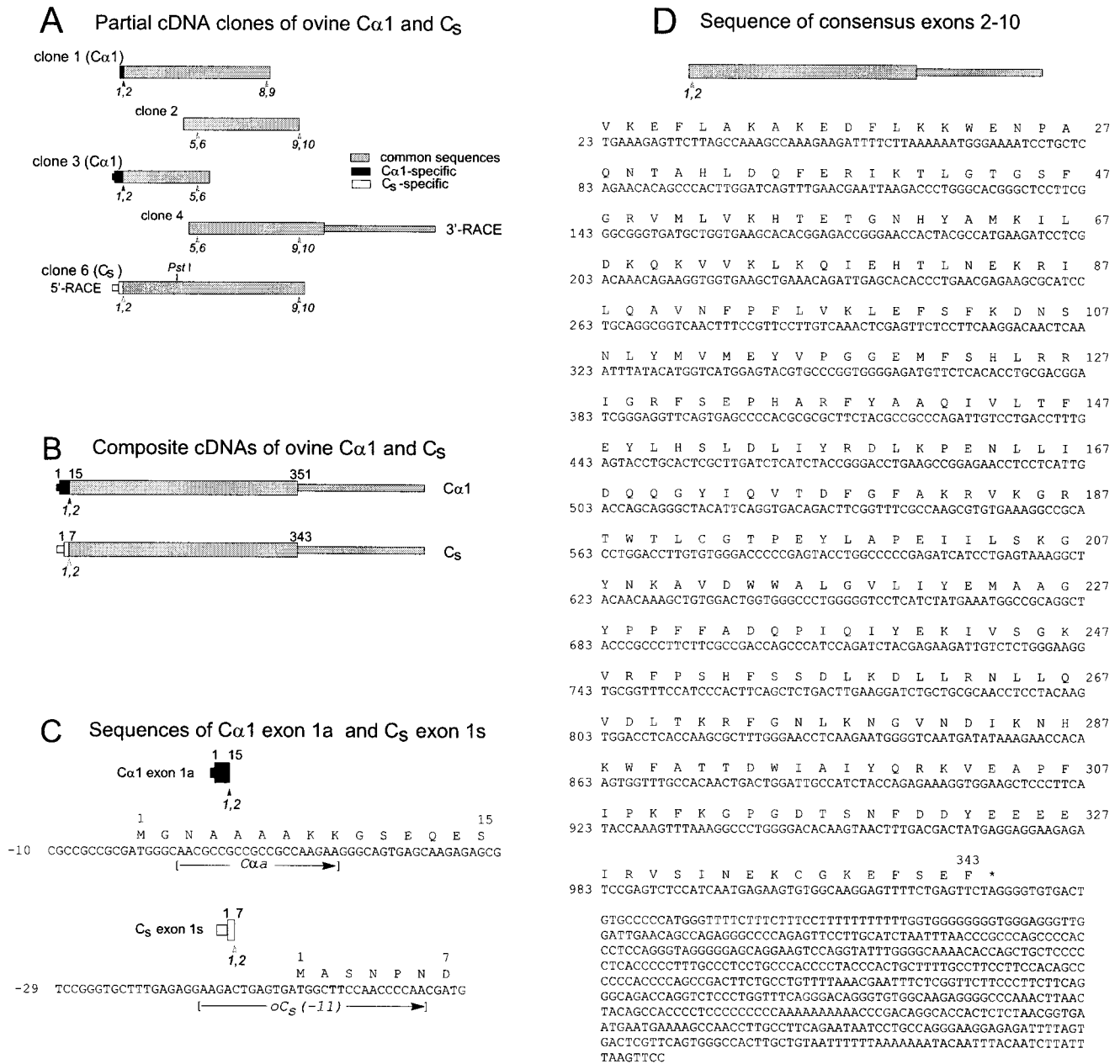
## MATERIALS AND METHODS

### PCR Primers

Oligonucleotide primers used in this work are listed in Table 1. *Caa*, *Cab*, *CacR*, *CadR*, and *CaeR* were derived from consensus sequences of bovine, murine, rat, and human  $C\alpha 1$  mRNAs (Uhler *et al.*, 1986a; Chrivia *et al.*, 1988; Maldonado and Hanks, 1988; Wiemann *et al.*, 1991, 1992). *oCa376* and *oCa482R* were derived from the composite ovine  $C_s$  and  $C\alpha 1$  cDNA sequences reported in this paper (Figure 1). The  $C_s$ -specific primers *oCs(-11)* and *mCs(-188)* were derived from the sequences of ovine  $C_s$  exon 1s and murine  $C_s$  exon 1s, respectively (Figure 1C; see also Figure 3A). *mCa791R* was from the murine  $C\alpha 1$  cDNA sequence (Uhler *et al.*, 1986a; Chrivia *et al.*, 1988), and *hCa(-60)* was from the human  $C\alpha 1$  cDNA sequence (Maldonado and Hanks, 1988). *AP1* and *nested AP1* (Marathon cDNA amplification kit, Clontech Laboratories, Palo Alto, CA) were adaptor-specific primers used in rapid amplification of cDNA ends (RACE) reactions.

### Preparation of RNA and Synthesis of cDNA for RACE

Total RNA was prepared as described by Ausubel *et al.* (1989). The final preparation was suspended in 300 mM sodium acetate, 70%



**Figure 1.** Cloning and sequences of ovine  $C\alpha 1$  and  $C_s$  cDNAs. The initiating methionine is designated as amino acid residue 1, and the first base of the initiation codon ATG is designated as nucleotide 1. (A) Bars represent the five cDNA clones used to obtain the composite cDNAs and nucleotide sequences of ovine  $C\alpha 1$  and  $C_s$ . The ORFs are depicted as the wider portions of the bars. Sequences common to both  $C\alpha 1$  and  $C_s$  are gray, sequences specific for  $C\alpha 1$  are black, and sequences specific for  $C_s$  are white. For orientation, selected presumptive exon junctions based on the murine  $C\alpha$  genomic sequence (Chrivia et al., 1988) are marked below the bars (arrowheads). (B) Bars represent the composite cDNAs of ovine testis  $C\alpha 1$  and  $C_s$ . The numbers on top of the bars indicate the positions of amino acid residues encoded at the start (1) and ends (351 and 343) of the ORFs and the ends (15 and 7) of exon 1 of ovine  $C\alpha 1$  and  $C_s$ , respectively. Shading is as in A. (C) Partial nucleotide and predicted amino acid sequences of ovine  $C\alpha 1$  exon 1a and ovine  $C_s$  exon 1s. The positions of the forward primers  $C\alpha a$  and  $oC_s(-11)$  also are shown. (D) The nucleotide sequence of exons 2-10, which are identical for ovine  $C\alpha 1$  and  $C_s$  cDNAs, and their predicted amino acid sequence. The amino acid and nucleotide positions indicated (right and left margins, respectively) are those for  $C_s$ . Sequence data for ovine  $C\alpha 1$  and ovine  $C_s$  have been deposited in GenBank/EMBL/DBJ under accession numbers AF238979 and AF238980, respectively.

ethanol, and stored at  $-80^\circ\text{C}$ . Murine oocyte total RNA was prepared from  $\sim 30$  oocytes kindly provided by Dr. Joyce Tay (Univer-

sity of Massachusetts Medical School). In more recent RNA preparations, tissues from mice were immersed immediately after

**Table 2.** Generation of ovine  $C\alpha 1$  clones by RT-PCR and 3'-RACE

	Amplification	Primers	Thermocycler conditions
Clone 1	RT-PCR	<i>Caa</i> , <i>CacR</i>	Annealing at 50°C, extension at 68°C, 35 cycles, final 10-min extension at 68°C
Clone 2	RT-PCR	<i>Cab</i> , <i>CadR</i>	
Clone 3	RT-touchdown PCR (Don <i>et al.</i> , 1991)	<i>hCa(-60)</i> , <i>oCa482R</i>	94°C, 72°C, 5 cycles; 94°C, 70°C, 5 cycles; 94°C, 68°C, 30 cycles
Clone 4	Touchdown PCR (3'-RACE)	<i>oCa376</i> , <i>AP1</i>	94°C, 70°C, 5 cycles; 94°C, 68°C, 5 cycles; 94°C, 65°C, 30 cycles

excision in RNA Later (Ambion, Austin, TX), eliminating the need for immediate storage in liquid nitrogen. Ovine testis mRNA was prepared from 1.9 mg of total RNA (Clontech PT1353-1), yielding ~100  $\mu\text{g}$  of poly(A)<sup>+</sup> RNA. About 350  $\mu\text{g}$  of murine testis poly(A)<sup>+</sup> RNA was obtained from 1 mg of murine testis total RNA. Marathon adaptor-ligated ovine and murine testis cDNAs for RACE were prepared as recommended (Clontech protocol PT 1115-1, with SuperScript II Rnase H<sup>-</sup> RT [Life Technologies, Grand Island, NY] used instead of avian myeloblastosis virus RT). Marathon-ready human testis cDNA was purchased from Clontech.

#### Cloning of Ovine Testis $C\alpha 1$ cDNA (Clones 1, 2, 3, and 4)

PCR was carried out with the use of the Elongase enzyme mix (Life Technologies). Table 2 summarizes the amplification schemes used. In the RT reactions, first-strand cDNA was synthesized from ovine testis total RNA with the use of SuperScript II RT and oligo(dT)<sub>12-18</sub> as primer (Life Technologies).

The PCR products were cloned by ligation to pBluescript II KS(-) phagemid (Stratagene, La Jolla, CA) followed by electroporation into Epicurean Coli XL1-Blue cells (Stratagene). Clones 1, 2, and 3 were identified by restriction mapping. Clone 4 was identified by hybridization to a <sup>32</sup>P-labeled clone 2.

#### Cloning of Ovine, Murine, and Human $C_s$ cDNAs (Clones 6, 7, and 8)

5'-RACE was performed on Marathon adaptor-ligated ovine, murine, and human testis cDNAs (Table 3). The 5'-RACE products were then subcloned as described above. Ovine  $C_s$  subclones (clone

6) were identified by hybridization to a <sup>32</sup>P-labeled clone 1. Clone 7 was verified to be a murine  $C$  clone by high-stringency hybridization to <sup>32</sup>P-labeled clone 1 and by its characteristic digestion patterns by specific restriction enzymes. Murine  $C\alpha$  cDNA is cut by *Bgl*III at position 218 of  $C\alpha 1$ , whereas ovine  $C\alpha$  is not; both are cut by *Pst*I at position 290. Clone 8 was verified to be a human  $C_s$  clone by high-stringency hybridization to <sup>32</sup>P-labeled clone 1 and by its resistance to digestion by *Pst*I.

#### Sequencing of Clones 1 to 8

Sequencing of the cDNA clones was done at the Iowa State University DNA Sequencing Facility (Ames, IA). Analysis of sequences was carried out with the use of version 10.0-UNIX of the Wisconsin Package (Genetics Computer Group, Madison, WI). Nucleotides upstream of a translation start site are numbered 3' to 5' beginning with -1; those downstream are numbered 5' to 3' beginning with +1. Translation of  $C_s$  or  $C\alpha 1$  is presumed to begin with the methionine immediately upstream of the amino-terminal glycine or alanine, respectively (Uhler *et al.*, 1986a; San Agustin *et al.*, 1998) (Figure 1, B and C).

#### Detection of $C_s$ and $C\alpha 1$ mRNA in Murine and Human Tissues

RT-PCR was carried out on total RNA from murine and ovine testes. PCR was carried out on human testis cDNA (Marathon-Ready human testis cDNA, Clontech). Two sets of gene-specific primers were used: *oCs(-11)* and *CaeR* to detect the presence of  $C_s$  mRNA, and *Caa* and *CaeR* to detect  $C\alpha 1$  mRNA. The thermocycler program

**Table 3.** Generation of ovine, murine, and human  $C_s$  clones by 5'-RACE

	First round (5'-RACE)		Second round	
	Primers	Thermocycler conditions	Primers	Thermocycler conditions
Clone 6 (ovine)	<i>AP1</i> , <i>CaeR</i>	Annealing at 59°C, extension at 68°C, 40 cycles, final 10-min extension at 68°C	–	–
Clone 7 (murine)	<i>AP1</i> , <i>mCa791R</i>	Annealing at 56°C, extension at 68°C, 40 cycles, final 10-min extension at 68°C	<i>Nested AP1</i> , <i>oCa482R</i>	Annealing at 59°C, extension at 68°C, 35 cycles, final 10-min extension at 68°C
Clone 8 (human)	<i>AP1</i> , <i>CaeR</i>	Annealing at 59°C, extension at 68°C, 40 cycles, final 10-min extension at 68°C		

was similar to that used for clone 1 except that the reaction was carried out for 35 cycles with annealing at 59°C.

To determine the presence of  $C_s$  and  $C\alpha 1$  transcripts in various murine tissues, RT-PCR was performed on total RNA from murine brain, heart, kidney, liver, lung, ovary, oocytes, skeletal muscle, testis, and trachea with the use of two sets of primers:  $mC_s(-188)$  and  $CaeR$  to detect  $C_s$  mRNA, and  $Caa$  and  $CaeR$  to detect  $C\alpha 1$  mRNA. Thermocycler conditions were 30 cycles (35 cycles for oocytes) and annealing at 61°C.

### Polyclonal Antibody against Murine $C_s$

The peptide Ac-ASSNDVK was synthesized and injected into rabbits (Research Genetics, Huntsville, AL). The first six residues of the peptide correspond to the predicted unique  $mC_s$  amino terminus without the initiator methionine (see Figure 3A); the seventh residue, K, is shared by both murine  $C_s$  and  $C\alpha 1$ . It was assumed that the amino-terminal alanine residue of murine  $C_s$  is acetylated, as is the case with ovine  $C_s$  (San Agustin *et al.*, 1998). The antibodies were affinity purified by a two-step procedure. The antisera first were applied to a column containing the synthetic acetylated peptide coupled to Sepharose 4B, and the bound antibodies were eluted by low pH. The released antibodies then were applied to a second column containing the unacetylated synthetic peptide coupled to Sepharose 4B, and the antibodies that did not bind were collected and retained. The concentration of the affinity-purified antibody was 0.83 mg/ml.

### Preparation of Murine Testis and Brain Extracts

Testes (~1.6 g) from six adult mice were excised, minced in 4-ml of cold testis homogenization buffer (10 mM potassium phosphate, pH 6.8, 1 mM EDTA, 0.1 mM DTT), and ground in a glass homogenizer. Brain tissue (~1.3 g) from three mice was mixed with 1 ml of cold brain homogenization buffer (100 mM piperazine- $N,N'$ -bis[2-ethanesulfonic acid], pH 6.8, 2 mM EGTA, 1 mM  $MgSO_4$ , 2 mM DTT, 4 M glycerol) and ground in a glass homogenizer. The homogenates were centrifuged at  $6500 \times g$  for 15 min at 4°C. The supernatants were further clarified by centrifugation at  $96,000 \times g$  for 75 min at 4°C.

### Isolation of $mC_s$ and $mC\alpha 1$ from Murine Testis

Because both murine  $C_s$  and  $C\alpha 1$  are expressed in testis (see RESULTS), both isoforms were present in the clarified testis extract. The two isoforms were copurified with the use of the protocol for the purification of ovine  $C\alpha 1$  from ram skeletal muscle as described previously (San Agustin *et al.*, 1998). Fractions containing murine  $C_s$  and  $C\alpha 1$  eluted from the CM Fast Flow column (0.5  $\times$  5 cm, Amersham Pharmacia Biotech, Piscataway, NJ) between 180 and 230 mM NaCl (see Figure 6). No other polypeptide was detected in the fractions containing these two proteins.

### Western Blotting

Protein samples were subjected to electrophoresis in a 10% polyacrylamide gel and blotted to polyvinylidene difluoride membrane (San Agustin *et al.*, 1998). The blot was then treated with blocking solution (Tris-buffered saline [TBS] with 0.1% Tween-20, 1% cold fish scale gelatin [Sigma Chemical, St. Louis, MO], 5% nonfat dry milk) for 1 h at room temperature and incubated overnight at 4°C with the anti-murine  $C_s$  antibody diluted 1:4000 with the blocking solution. The blot was brought to room temperature, washed three times with blocking solution, and then incubated for 1 h with secondary antibody (HRP-conjugated goat anti-rabbit immunoglobulin G diluted 1:2000 with blocking solution). It was then washed twice with blocking solution and once with TBST (TBS with 0.1% Tween-20). Cross-reacting proteins were detected with the use of the ECL detection reagent (hydrogen peroxide/luminol; Amersham

Life Science, Boston, MA). Exposure of the blot to film (AR X-Omat, Kodak, Rochester, NY) was usually between 10 and 50 s.

### Immunohistochemistry

Mouse testes were excised from freshly killed adult mice and placed in 40 ml of chilled Bouin's fixative. Testes were punctured at several places with a needle (26 gauge) to allow quicker penetration of the fixative and agitated gently in an orbit shaker at 4°C. After 2 h of shaking, the testes were cut in half. Fixation was continued for an additional 24 h at 4°C. The fixed testes were washed five times with TBS, passed through a series of graded ethanol solutions followed by xylene, and then embedded in paraffin. Thin sections, typically 5  $\mu$ m thick, were cut from the paraffin block, transferred to silanized coverslips, and dried overnight in an oven at 37°C. The testis sections were deparaffinized with xylene and then rehydrated by immersion in a graded series of aqueous isopropanol solutions.

Antigens were retrieved by boiling the coverslips for 20 min in 10 mM citrate, pH 6 (Polak and Van Noorden, 1997). The coverslips were rinsed in water and then transferred to individualized humidors, i.e., a Petri dish with moistened filter paper and Parafilm on top to hold the coverslip (Sanders and Salisbury, 1995). The testis sections were incubated with 250  $\mu$ l of blocker solution (TBS containing 5% BSA, 20% normal swine serum) for 1 h at room temperature. The blocker solution was removed by blotting and replaced with 250  $\mu$ l of anti-murine  $C_s$  antibody diluted 1:1000 to 1:2000 with one-fifth blocker solution (TBS, 1% BSA, 4% normal swine serum). The sections were incubated with the antibody overnight at 4°C, returned to room temperature, washed with TBST, and treated for 40 min with 250  $\mu$ l of biotinylated swine anti-rabbit immunoglobulin G (DAKO, Carpinteria, CA) diluted 1:200 with TBS, 1% BSA, 10% normal mouse serum. After washing with TBST, the sections were incubated for 40 min with 250  $\mu$ l of alkaline phosphatase-conjugated streptavidin (DAKO) diluted 1:300 with TBS, 0.5% BSA. The sections were washed with TBST and then exposed to the BCIP/NBT/INT (5-bromo-4-chloro-3-indoxyl phosphate/nitroblue tetrazolium chloride/iodonitrotetrazolium violet) substrate system (DAKO). Color was allowed to develop for 30 min, after which the coverslips were rinsed with water. The sections were then counterstained with Harris' hematoxylin (5 min) and finally mounted on glass slides with an aqueous-based mountant (Glycerol, DAKO).

## RESULTS

### Cloning of Ovine Testis $C\alpha 1$ and $C_s$ cDNAs

To determine the relationship of ovine  $C_s$  to ovine  $C\alpha 1$ , we cloned and sequenced the complete ORFs of their cDNAs. Figure 1A illustrates the overlapping cDNA clones, arranged to scale and position, that were used to assemble the composite cDNAs (Figure 1B) of ovine testis  $C\alpha 1$  and  $C_s$ .

Clone 1, corresponding to a portion of the  $C\alpha 1$  mRNA extending from exon 1 to exon 9, was obtained with the use of the  $C\alpha 1$ -specific primer  $Caa$  and the reverse primer  $CacR$ . Sequencing confirmed that this clone encoded amino acids specific to the amino terminus of  $C\alpha 1$  (Figure 1C). Clone 2, obtained with the use of consensus primers based on published mammalian  $C\alpha 1$  sequences, was 100% identical with clone 1 in the region of overlap.

The remaining sequence of the 5' end of the ORF of ovine  $C\alpha 1$  mRNA was obtained from clone 3, which was generated with the use of  $hCa(-60)$  as the forward primer and  $oCa482R$  as the reverse primer. A forward primer based on the 5'-UTR of human  $C\alpha 1$  mRNA was used because the 5'-UTR of bovine  $C\alpha 1$  mRNA is not known, and we reasoned that the 5'-UTR of human  $C\alpha 1$  was likely to be similar to that of ovine  $C\alpha 1$ . Clone 3 encoded amino acids specific to

the amino terminus of  $C\alpha 1$  and was identical to clones 1 and 2 in the regions of overlap.

Clone 4, containing the 3' end of the ORF and the 3'-UTR of ovine  $C\alpha$  mRNA, was obtained as a 3'-RACE product of ovine testis cDNA. Clone 4 was 100% identical to clones 1, 2, and 3 in their regions of overlap.

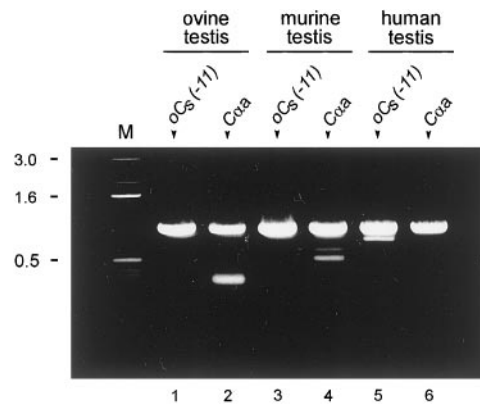
The 5' end of the ORF and the 5'-UTR of ovine  $C_s$  were obtained by 5'-RACE with the use of *CaeR* as gene-specific primer and ovine testes cDNA as template. A single band of product was observed in agarose gels, and a number of subclones of this PCR band were isolated for nucleotide sequencing. Although the *CaeR* primer could have amplified both  $C_s$  and  $C\alpha 1$  cDNAs, all subclones contained sequences coding for the unique amino terminus of  $C_s$  contiguous to sequences identical to exons 2–10 of the  $C\alpha 1$  clones. The finding that the cDNA sequences of exons 2–10 of  $C\alpha 1$  and  $C_s$  are identical at the nucleotide level provided strong evidence that  $C_s$  is the product of an alternatively spliced mRNA in which a unique  $C_s$  exon (hereafter referred to as exon 1s) is spliced to exon 2 of the  $C\alpha$  gene.

Further proof that exon 1 (hereafter referred to as exon 1a) of the  $C\alpha 1$  mRNA and exon 1s of the  $C_s$  mRNA are spliced to the same downstream sequence was obtained by carrying out RT-PCR of ovine testis mRNA with the use of the forward primers *Caa* and *oC<sub>s</sub>(-11)*, based on sequences located in exons 1a and 1s, respectively, with the reverse primer *oC<sub>s</sub>1402R*, which is complementary to sequence located in the 3' noncoding region of exon 10. Both primer pairs yielded products of the expected size (our unpublished results), confirming that the 3'-UTR of exon 10 is common to both  $C_s$  and  $C\alpha 1$  mRNAs.

### Nucleotide and Predicted Amino Acid Sequences of Ovine $C\alpha 1$ and $C_s$ cDNAs

Figure 1C shows the partial sequences of  $C\alpha 1$  exon 1a and  $C_s$  exon 1s obtained from the ovine cDNA clones. The ORF of  $C\alpha 1$  exon 1a codes for 15 amino acids, whereas that of  $C_s$  exon 1s codes for 7 different amino acids. The amino acid residues encoded by ovine  $C\alpha 1$  exon 1a (minus the initiator methionine) are identical to those reported for bovine (Shoji *et al.*, 1983; Wiemann *et al.*, 1992), murine (Uhler *et al.*, 1986a; Chrvia *et al.*, 1988), rat (Wiemann *et al.*, 1991), hamster (Howard *et al.*, 1991), and human (Maldonado and Hanks, 1988)  $C\alpha 1$ , whereas the amino acid sequence predicted from  $C_s$  exon 1s (minus the initiator methionine) exactly matches the amino-terminal sequence for ovine  $C_s$  obtained through protein biochemistry (San Agustin *et al.*, 1998). The nucleotide sequence of exons 2–10, which are identical for both the  $C\alpha 1$  and  $C_s$  cDNAs, is presented in Figure 1D; the predicted amino acid sequence is 100% identical (78 of 78 residues) with the partial amino acid sequence of this portion of ovine  $C_s$  obtained from Edman analysis of its cyanogen bromide and tryptic fragments (San Agustin *et al.*, 1998).

The ovine  $C_s$  cDNA predicts a protein of 343 amino acids (including the initiating methionine) with a mass of 39,858 Da, whereas the ovine  $C\alpha 1$  cDNA predicts a protein of 351 amino acids with a mass of 40,589 Da. Because the amino terminus of  $C\alpha 1$  is myristylated and that of  $C_s$  is acetylated (San Agustin *et al.*, 1998), the mass of the modified  $C\alpha 1$  is predicted to be 899 Da greater than the mass of modified  $C_s$ , in excellent agreement with the difference of 890 Da deter-



**Figure 2.** Detection of  $C\alpha 1$  and  $C_s$  mRNA in various species. RT-PCR of total RNA from ovine and murine testes and PCR of cDNA from human testis. The forward primers used to generate the PCR products are shown at the top of the lanes: *oC<sub>s</sub>(-11)* to amplify  $C_s$  and *Caa* to amplify  $C\alpha 1$ . The reverse primer in all cases was *CaeR*. The PCR products were subjected to electrophoresis in an 0.8% agarose gel and stained with ethidium bromide.  $C\alpha 1$  and  $C_s$  products were obtained from all three species. Controls in which the RT was omitted yielded no bands. Lane M, DNA molecular mass markers (in kilobases).

mined empirically by mass spectrometry (San Agustin *et al.*, 1998).

### Similar $C_s$ mRNAs Are Present in Murine and Human Testis

To determine if  $C_s$  mRNAs are present in the testes of other mammalian species, we carried out PCR with the use of ovine, murine, and human testicular cDNA as template and forward primers (Figure 1C) specific for either  $C_s$  [*oC<sub>s</sub>(-11)*] or  $C\alpha 1$  [*Caa*]. In all cases, the reverse primer was *CaeR*. In all three species, the  $C_s$ -specific primer yielded PCR product of the expected size (Figure 2, lanes 1, 3 and 5). Therefore,  $C_s$  is widespread in mammals.  $C\alpha 1$  transcripts also were found in the testes of all three species (Figure 2, lanes 2, 4, and 6), confirming that both C isoforms occur in the testis. The  $C\alpha 1$  and  $C_s$  PCR products had very similar sizes (slightly less than 1 kilobase), which agrees with the calculated sizes of 949 bases for the  $C\alpha 1$  PCR product and 942 bases for the  $C_s$  PCR product.

### Nucleotide Sequences of cDNAs Encoding the Amino Termini of Murine and Human $C_s$

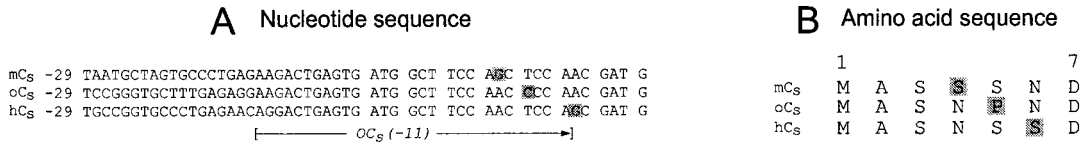
To confirm that murine and human testes have  $C_s$ , and to determine the degree of similarity between the amino termini of these proteins and that of ovine  $C_s$ , cDNAs of murine and human  $C_s$  were amplified from testis cDNA by 5'-RACE with the use of identical sets of primers (*nested AP1* and *oC<sub>s</sub>482R*). Only one PCR band was observed in each case. These products were cloned and sequenced. As with the ovine 5'-RACE cDNA, all of the clones encoded  $C_s$ . Figure 3, A and B, show the partial nucleotide and predicted amino acid sequences of murine  $C_s$  cDNA (clone 7) and human  $C_s$  cDNA (clone 8), respectively. Figure 3, C and D, show the alignment of murine  $C\alpha 1$  with murine  $C_s$  and



**Figure 3.** Partial nucleotide and amino acid sequences of murine  $C_s$  and human  $C_s$  cDNA. (A and B) Murine  $C_s$  cDNA (clone 7) and human  $C_s$  cDNA (clone 8) were obtained by 5'-RACE with the use of murine and human testis cDNAs as template. The shading and numbering are as in Figure 1. The *Bgl*III and *Pst*I sites that are present in clone 7 but not in clone 8 are indicated. The murine and human  $C_s$  sequences are available from GenBank/EMBL/DBJ under the accession numbers AF239743 and AF239744, respectively. (C and D) The cDNA sequences of clones 7 ( $mC_s$ ) and 8 ( $hC_s$ ) are compared with the corresponding regions of the murine  $Ca1$  ( $mCa1$ ) (Uehler *et al.*, 1986a) and human  $Ca1$  ( $hCa1$ ) (Maldonado and Hanks, 1988) sequences. Dashes indicate nonconsensus nucleotides.



Comparison of murine, ovine, and human C<sub>s</sub> exon 1s



**Figure 4.** Comparison of exon 1s–encoded regions of ovine, murine, and human C<sub>s</sub>. Partial cDNA nucleotide (A) and predicted amino acid (B) sequences of the murine (*mC<sub>s</sub>*), ovine (*oC<sub>s</sub>*), and human (*hC<sub>s</sub>*) versions of C<sub>s</sub> exon 1s are aligned. The nonconsensus bases of the ORFs are highlighted, as are the amino acid residues that will result from these substitutions. The position of the primer *oC<sub>s</sub>*(-11) also is shown.

human Ca1 with human C<sub>s</sub>. As in the sheep, exon 1s of the murine C<sub>s</sub> cDNA and exon 1s of the human C<sub>s</sub> cDNA showed very little identity with their Ca1 counterparts, whereas C<sub>s</sub> nucleotides downstream of the exon 1/exon 2 junction were 100% identical to the published sequences for the Ca1 cDNAs. However, exon 1s of murine C<sub>s</sub> and exon 1s of human C<sub>s</sub> were very similar to the ovine C<sub>s</sub> exon 1s (Figure 4A). The coding region of exon 1s of each of the three cDNAs differs from the others at only 2 of 22 positions. Each of these substitutions would result in the incorporation of a different amino acid residue into the C<sub>s</sub> molecule (Figure 4B). The first three amino acid residues are predicted to be identical for all three species, but the next three residues are S or N at positions 4 and 6 and P or S at position 5.

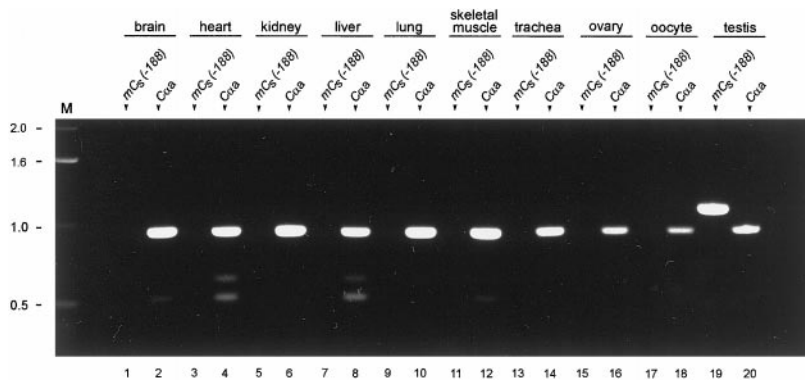
**C<sub>s</sub> mRNA Is Found Exclusively in the Testis**

To investigate the tissue distribution of C<sub>s</sub>, we carried out RT-PCR with the use of murine total RNA from various tissues as template. *mC<sub>s</sub>*- and *Ca1*-specific forward primers were chosen to yield different-sized PCR products with *CaeR* as the reverse primer. *Ca1* mRNA was detected in all tissues assayed (Figure 5), whereas C<sub>s</sub> mRNA was detected only in testis (Figure 5, lane 19). It is important to note that C<sub>s</sub> mRNA was not detected in ciliated tissues such as brain, lung, and trachea, indicating that C<sub>s</sub> is not a component of cilia. Moreover, C<sub>s</sub> mRNA was not detected in ovarian tissue or oocytes, indicating that C<sub>s</sub> is not expressed in the female germ line. These results strongly suggest that C<sub>s</sub> is expressed only in the testis, where the translated protein becomes integrated into the sperm tail.

**C<sub>s</sub> Is Expressed Only in Germ Cells and First Appears in Mid Pachytene Spermatocytes**

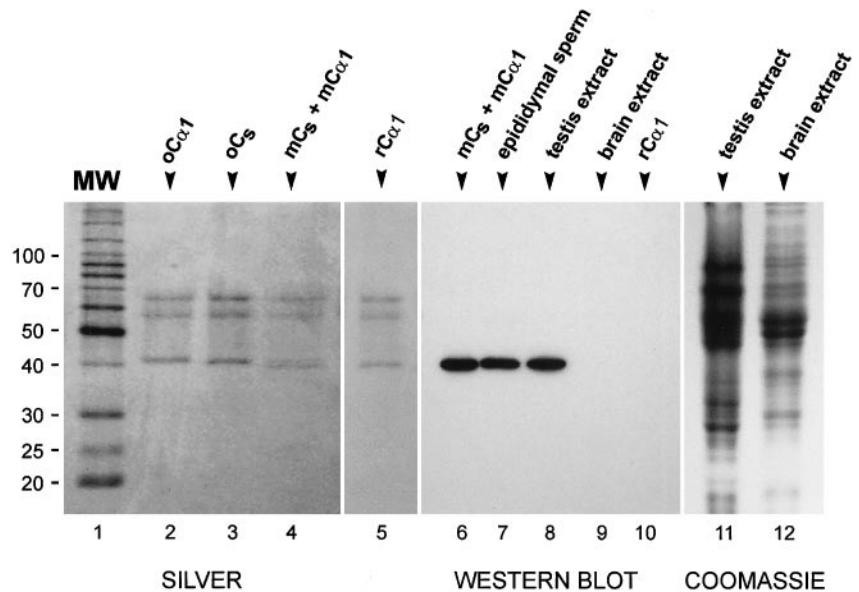
To determine the pattern of expression of C<sub>s</sub> in the testis, a rabbit anti-peptide antibody was made against the unique amino-terminal sequence of murine C<sub>s</sub>. The specificity of the antibody was demonstrated in Western blots. Fractions of purified C from murine testes contain two proteins that migrate with mobilities very similar to those of pure ovine C<sub>s</sub> and ovine Ca1 in SDS-polyacrylamide gels (Figure 6, lanes 1–4). These proteins are presumed to represent C<sub>s</sub> and Ca1, both of which are expressed in the testis (Figures 2 and 5). When Western blots of this mixture were probed with the antibody, a single protein of ~40 kDa was detected (Figure 6, lane 6). The antibody reacted strongly with a single band of the same size in murine epididymal sperm, which are presumed to contain C<sub>s</sub> but not Ca1 (San Agustin *et al.*, 1998), and in murine testis extract, but it did not recognize any protein in murine brain extract, which contains Ca1 and Cβ but not C<sub>s</sub>. The antibody also did not recognize purified ovine Ca1, which has the same amino-terminal sequence as murine Ca1 (our unpublished results), nor murine recombinant Ca1 (kindly provided by Dr. S. Taylor, University of California, San Diego) (Figure 6, lanes 5 and 10). Therefore, the antibody is highly specific for C<sub>s</sub> and does not appear to recognize any other protein in the testis.

In sections of murine testes (Figures 7 and 8), the antibody stained only germ cells and did not react with Sertoli cells, Leydig cells, or any other non-germ cells. It also did not stain spermatogonia, zygotene spermatocytes, or early pachytene spermatocytes. The antibody stained mid pachytene sper-



**Figure 5.** Detection of Ca1 and C<sub>s</sub> mRNA in murine tissues. RT-PCR of total RNA from various murine tissues. The forward primers used to generate the PCR products are shown at the top of the lanes: *mC<sub>s</sub>*(-188) to amplify C<sub>s</sub> and *Cae* to amplify Ca1. The reverse primer in all cases was *CaeR*. These primer sets are predicted to yield PCR products of 949 bases for murine Ca1 and 1119 bases for murine C<sub>s</sub>. The PCR products were subjected to electrophoresis in an 0.8% agarose gel and stained with ethidium bromide. Transcripts encoding the Ca1 isoform are present in all the tissues analyzed, whereas C<sub>s</sub> transcripts are detected only in the testis. Lane M, DNA molecular mass markers (in kilobases).

**Figure 6.** Specificity of the anti- $mC_s$  antibody. (Left) Silver-stained SDS-polyacrylamide gels of purified ovine  $C\alpha 1$  ( $oC\alpha 1$ ), purified ovine  $C_s$  ( $oC_s$ ), a mixture of murine  $C_s$  and  $C\alpha 1$  ( $mC_s + mC\alpha 1$ ) isolated from murine testis, and mouse recombinant  $C\alpha 1$  ( $rC\alpha 1$ ). Molecular mass markers (MW) are in kilodaltons. As reported previously (San Agustin *et al.*, 1998), ovine  $C_s$  migrates slightly faster than ovine  $C\alpha 1$ . The partially purified murine  $C\alpha 1$  and  $C_s$ , which are resolved as two bands at ~40 kDa, appear to migrate slightly faster than their ovine homologues. The bands in the 60- to 70-kDa range are human keratin contaminants (San Agustin *et al.*, 1998). (Center) Western blot probed with an affinity-purified antibody generated against an acetylated peptide corresponding to the unique amino terminus of murine  $C_s$ . The antibody reacts with a single protein in the mixture of murine  $C_s$  and  $C\alpha 1$  ( $mC_s + mC\alpha 1$ ), in murine epididymal sperm ( $1 \times 10^6$  sperm), and in murine testis extract (50  $\mu$ g of total protein) but does not react with any band in murine brain extract (30  $\mu$ g of total protein) or with recombinant  $C\alpha 1$  (37 ng). (Right) SDS-polyacrylamide gel of murine testis extract (50  $\mu$ g) and murine brain extract (30  $\mu$ g) stained with Coomassie blue as loading control for lanes 8 and 9 of the Western blot.



matocytes of stage VI tubules very weakly, stained mid pachytene spermatocytes of stage VIII tubules slightly more strongly (our unpublished results), and stained late pachytene spermatocytes of stage XI tubules very strongly (Figure 8). Therefore,  $C_s$  appears to be synthesized first in mid pachytene and is highly expressed by late pachytene. The antibody also stained round spermatids, elongating spermatids, and mature sperm present in the lumen of the seminiferous tubules (Figure 8).  $C_s$  was present in the cytosol of round spermatids and appeared to move from the cytosol into the developing flagella as the spermatids matured. Controls in which the primary antibody was omitted did not exhibit any staining.

## DISCUSSION

### $C_s$ Is the Product of an Alternative Transcript of the $C\alpha$ Gene

$C_s$  originally was characterized by protein biochemistry as an ovine sperm PKA catalytic subunit differing from ovine somatic  $C\alpha 1$  in its electrophoretic mobility, mass, and amino-terminal sequence up to the presumptive exon 1/exon 2 junction (San Agustin *et al.*, 1998). The current study provides definitive molecular genetic evidence that ovine  $C_s$  is the product of an alternative transcript of the  $C\alpha$  gene. First, the nucleotide sequences of  $C_s$  and  $C\alpha 1$  cDNAs downstream of the exon 1/exon 2 junction are absolutely identical. If the proteins were the products of different genes, at least some substitutions would have occurred at the nucleotide level since the divergence of the two genes at least 65 million years ago (see below). Second, exon 1s of  $C_s$  and exon 1a of  $C\alpha 1$  are both spliced to the same 3'-UTR.

Examination of the mouse genome sequence (GenBank accession number M18241) indicates that the mouse exon 1s sequence (see below) is not contiguous with the 5' sequence

of exon 2 of  $C\alpha$ . Therefore, the  $C_s$  mRNA must result from alternative splicing of a  $C\alpha$  transcript. Production of the  $C_s$  transcript also may depend on an alternative initiation site within the  $C\alpha$  gene.

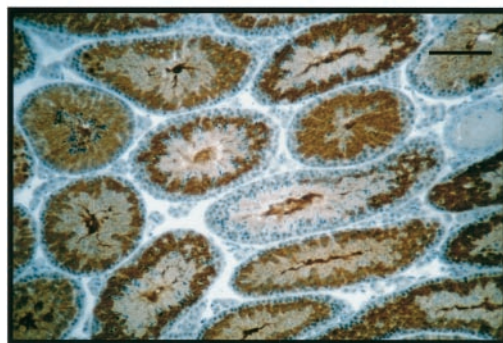
$C_s$  is the third  $C\alpha$  isoform to be reported. Thomis *et al.* (1992) described a partial human cDNA that was identical with human  $C\alpha 1$  cDNA sequence at its 5' end but that contained sequences derived from introns flanking both sides of exon 8. This cDNA predicts a  $C\alpha$  isoform, termed  $C\alpha 2$ , that would be substantially truncated at its carboxyl-terminal end. The  $C\alpha 2$  cDNA appeared to be expressed in at least two human cell lines.

### Similar $C_s$ Isoforms Are Widespread in Mammals

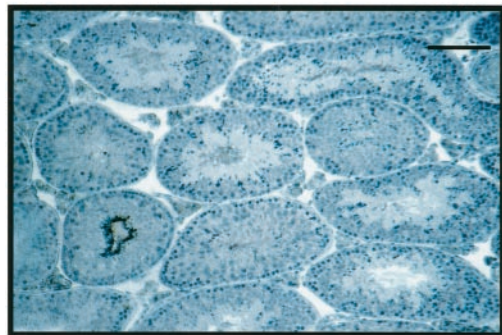
PCR with the use of a primer based on the nucleotide sequence of exon 1s of ovine  $C_s$  indicated that  $C_s$  is expressed in the testes of mouse and human as well as sheep. The nucleotide sequences of partial cDNAs encoding the murine and human  $C_s$  isoforms revealed that  $C_s$  exon 1s is very similar in all three species, each differing from the other at only two positions. In the mouse and human, as in the sheep, the sequences indicate that the 15 amino acids encoded by  $C\alpha 1$  exon 1a are replaced by 7 different amino acids in  $C_s$ . In all three species, an alanine replaces the glycine that follows the first methionine in  $C\alpha 1$ . In  $C\alpha 1$ , this methionine is cleaved off posttranslationally, and the newly exposed amino-terminal glycine is myristylated (Shoji *et al.*, 1983). Because the glycine is replaced with alanine in murine and human  $C_s$ , they probably are not myristylated but rather are acetylated, as is ovine  $C_s$  (San Agustin *et al.*, 1998).

The presence of  $C_s$  in primates, rodents, and ungulates indicates that this isoform arose early in evolution, at least before the divergence of these mammalian orders more than 65 million years ago (Young, 1962).

## MOUSE TESTIS SECTIONS



+ mCs antibody



- mCs antibody

**Figure 7.** Immunohistochemical staining of murine testis sections with the use of the anti-mouse  $C_s$  antibody. Cells stained brown are positive for  $C_s$ . Only germ cells at later stages of spermatogenesis stain with the antibody (top); because the cell associations seen in cross-sections of the seminiferous tubules vary depending on their stage in the spermatogenic cycle, the different tubules display different staining patterns. No staining is detected in the absence of the primary antibody (bottom). Bars, 100  $\mu\text{m}$ .

### The Murine *Cx* Pseudogene Likely Arose from a $C_s$ mRNA

A PKA catalytic subunit-related sequence, *Cx*, is present in the murine genome (Cummings *et al.*, 1994). This sequence was reported to be most closely related to that of the  $C\alpha$  gene, but it lacks introns and, relative to  $C\alpha$ , contains frameshift mutations, premature termination codons, and mis-sense mutations. It is not transcribed. Therefore, it appears to be a pseudogene of the retroposon class (Weiner *et al.*, 1986). *Cx* is closely related to  $C\alpha$  downstream of the  $C\alpha$  exon 1/exon 2 junction but does not resemble the  $C\alpha$  sequence upstream of this site, leading to speculation that the mRNA intermediate that gave rise to *Cx* may have been incompletely spliced (Cummings *et al.*, 1994). However, a comparison of the murine  $C_s$  exon 1s nucleotide sequence with the *Cx* 5' sequence reveals near identity from  $C_s$  nucleotide -20 to the  $C_s$  exon 1/exon 2 junction (Figure 9). Therefore, *Cx* probably arose by reverse transcription of a  $C_s$  mRNA followed by nonhomologous recombination of the cDNA into the genome of a male germ cell.

### Tissue and Cell Distribution of $C_s$

Using a RT-PCR assay and primers specific for  $C_s$  or  $C\alpha 1$ , we detected  $C_s$  transcripts in murine testis but not in murine brain, heart, kidney, liver, lung, ovary, oocytes, trachea, or skeletal muscle. In contrast,  $C\alpha 1$  transcripts were present in all tissues tested. Therefore,  $C_s$  appears to be expressed only in the testis.

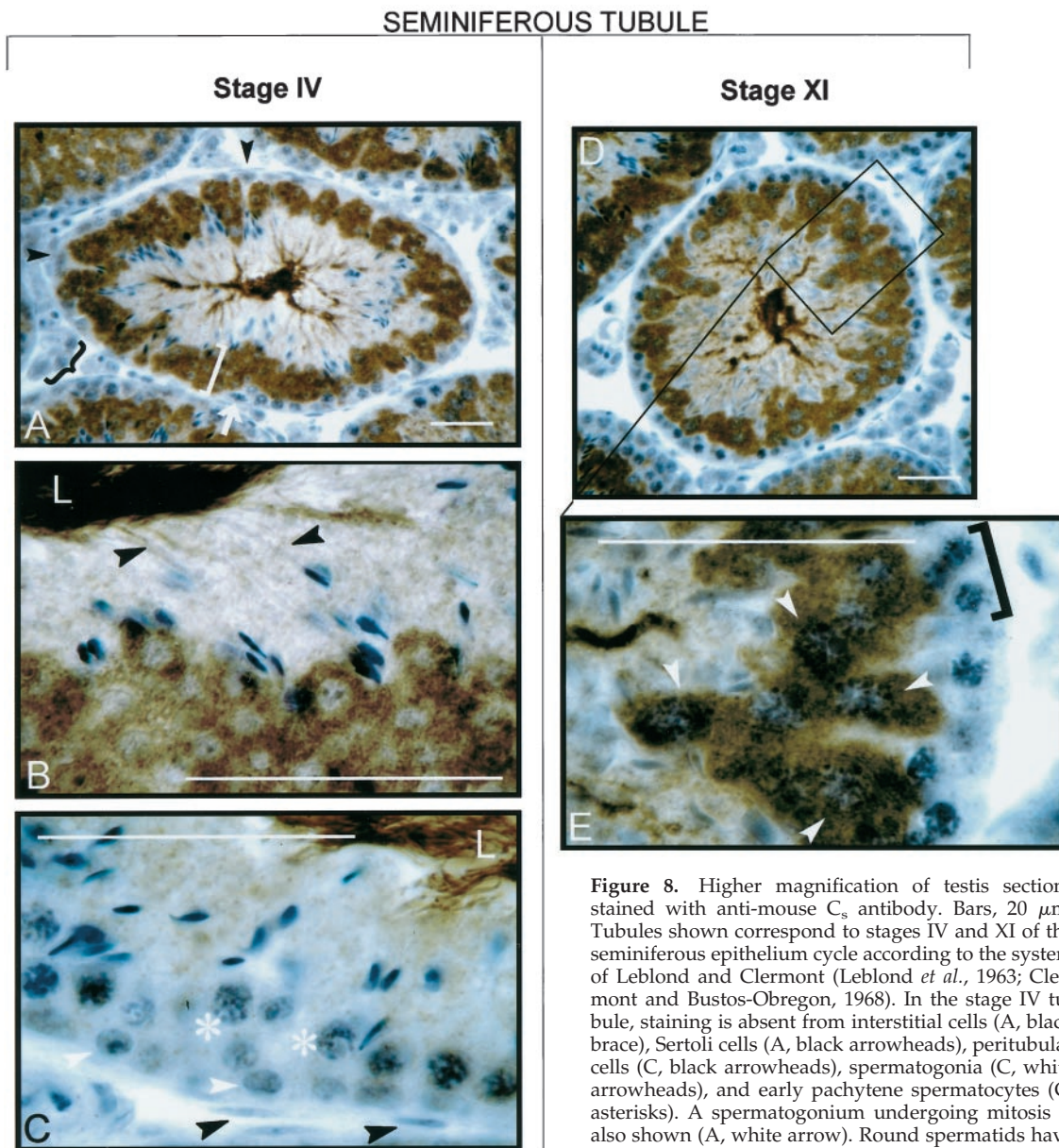
It is significant that  $C_s$  is not expressed in highly ciliated tissues such as the lung, trachea, and brain. PKA is important in the control of somatic cilia (for review, see Witman, 1990), and it was possible that  $C_s$  is an isoform specific for cilia and flagella in general. However, the current results indicate that this is not the case. Similarly, the absence of  $C_s$  expression in ovaries and oocytes rules out the possibility that  $C_s$  is expressed in all germ cells. Rather, it appears to be present only in the male. In oocytes, PKA is believed to play a major role in the maintenance of meiotic arrest (Schultz, 1988; Rose-Hellekant and Bavister, 1996). This important function probably is performed by  $C\alpha 1$ , which our results indicate is present in ovaries and oocytes.

Immunohistochemistry of murine testis sections with the use of an anti-peptide antibody against the unique amino terminus of murine  $C_s$  indicated that  $C_s$  is present only in germ cells. Synthesis of  $C_s$  appears to be initiated during mid pachytene. Therefore, transcription of  $C_s$  must be directed, at least initially, by the diploid nucleus. This finding is consistent with previous studies showing that synthesis of SDS-soluble sperm proteins is highest during meiosis (O'Brien and Bellvé, 1980) and that transcription and translation during spermatogenesis both peak in mid pachytene (Monesi, 1965). Subsequently,  $C_s$  is localized to the developing flagellum of the elongating spermatids. It should be noted that this is the first demonstration of a cell type-specific expression of any C isoform.

The fact that  $C_s$  does not appear to be present in spermatogonia and prepachytene spermatocytes suggests that any cAMP-dependent functions in these cells are mediated by  $C\alpha 1$  or some other isoform of C. It will be of interest to determine if  $C\alpha 1$  is present together with  $C_s$  in meiotic and postmeiotic cells or if  $C_s$  mediates all cAMP-dependent functions (Amat *et al.*, 1990; Delmas *et al.*, 1993) during spermiogenesis. It was reported that  $C\alpha$  mRNA is present in pachytene spermatocytes (Øyen *et al.*, 1990; Landmark *et al.*, 1993), but the probes used would not have distinguished between  $C\alpha 1$  and  $C_s$  mRNAs, so this should be reexamined. In any case,  $C_s$  was the only C isoform detected in Western blots of ovine ejaculated, epididymal, and rete testis sperm (San Agustin *et al.*, 1998), and it was the only isoform isolated from ovine sperm flagella (San Agustin *et al.*, 1998), despite the fact that  $C\alpha 1$  would have copurified with  $C_s$  had it been present in the flagella. Therefore, if  $C_s$  and  $C\alpha 1$  occur together in spermatids,  $C_s$  must be specifically targeted to the developing sperm structures.

### Function of Unique $C_s$ Structure

The fact that  $C_s$  is present in a wide range of mammals raises the possibility that its unique structure has an important role in the assembly or function of the subunit.  $C_s$  is not released from demembrated ovine sperm in the presence of cAMP (San Agustin and Witman, 1994; San Agustin *et al.*, 1998), indicating that it is attached to structures within the sperm



**Figure 8.** Higher magnification of testis sections stained with anti-mouse  $C_s$  antibody. Bars, 20  $\mu$ m. Tubules shown correspond to stages IV and XI of the seminiferous epithelium cycle according to the system of Leblond and Clermont (Leblond *et al.*, 1963; Clermont and Bustos-Obregon, 1968). In the stage IV tubule, staining is absent from interstitial cells (A, black brace), Sertoli cells (A, black arrowheads), peritubular cells (C, black arrowheads), spermatogonia (C, white arrowheads), and early pachytene spermatocytes (C, asterisks). A spermatogonium undergoing mitosis is also shown (A, white arrow). Round spermatids have intensely stained cytosol (A, white bracket). In the previous generation of elongated spermatids that have

moved farther toward the lumen (L), the cytoplasm now stains less intensely but the developing flagella (B, black arrowheads) are darkly stained. Darkly stained tails of mature sperm are visible in the lumens (L) of the stage IV tubules (B and C). In the stage XI tubule, staining of the cytosol of the spermatids occupying the inner portion of the tubule diminishes as they elongate (D). Staining is absent from zygotene spermatocytes (E, black bracket) but is prominent in the cytoplasm of late pachytene spermatocytes (E, white arrowheads).

even when activated. The unique structure of  $C_s$  may be responsible for this behavior. In  $Ca1$ , the exon 1a-encoded residues form the first two turns of a long  $\alpha$ -helix that extends across the surface of the catalytic core of the enzyme. This helix is anchored to the hydrophobic core by the amino-terminal myristate (Zheng *et al.*, 1993). In the absence of this myristate, the  $Ca1$  exon 1a residues are unstructured (Knighton *et al.*, 1991). In contrast to the situation in  $Ca1$ , the residues encoded by exon 1s of  $C_s$  form a shorter domain,

are not predicted to form an  $\alpha$ -helix (Chou and Fasman, 1978), and lack a terminal myristate to serve as an anchor (San Agustin *et al.*, 1998). Such a short, probably unstructured amino-terminal domain is likely to leave the catalytic subunit's hydrophobic core exposed, possibly allowing  $C_s$  to bind to hydrophobic sites within the sperm. Alternatively, a flexible amino-terminal tail might itself bind to a structure within the sperm and tether  $C_s$  to that structure. In either case, the attachment of  $C_s$  to the sperm tail by cAMP-

Alignment of Cx with mC<sub>s</sub> exon 1s

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Cx -286 AAGAGGCTCT TTTCTCCCGG GGAATCCCTT TCCTGTTATC TAACTACTCT
mCs -190 GGGTT
Cx -236 CTCTGGGACC ATTGTATCTT GATATTTTGG AGGATTAAC TCTCCCTCTT
Consensus -----C--- --C-----A ----T--G-C T---G-TT-- GT-T-----C

mCs -185 CTATCTGCCC CTACCCTGCA CCCATTAGTC TGCAGGTTGA GTTCTCTCTC
Cx -186 TCTCAAACCT TGGCTAGATA GIGCTCTGCC TCTTGATTTT GTATAATAAC
Consensus -----C--- --C-----A ----T--G-C T---G-TT-- GT-T-----C

mCs -135 CTGTTCCAC CCTATCACTC COTGGCTCCC TCTACAGGGA GGGCTCCCCC
Cx -136 TTCTAAACCG CTTTCCACCG ATTTTCACCA TCATATAGGA ATTCAAATC
Consensus -T-T--C-C C-T--CAC-- --T--C-CC- TC-----G-A --C-----C

mCs -85 CCAGGACTGG CAGCCAAACT GCTGCAGCAG ATCTTATGAG GCTTCCGAGC
Cx -86 CAGCCCCATC ACCAATTGCA GGGACCACAA TTCACAGTGA GCTATAAAAA
Consensus C-----C--- -----C- G--C--CA- -TC--A----- GCT----A--

mCs -35 CACCGTAATG CTAGTGCCCT GAGAAAGAC. TGAGTGATGG* CTTCCAGCTC
Cx -36 AGTGTCTGT AAGAGGCCCT GAGAAAGACT TGAGTGATGC CTTCCAGCTC
Consensus -----GCCCT GAGAAAGAC- TGAGTGATG- CTTCCAGCTC

mCs 15 CAACGATG
Cx 15 CAATGATG
Consensus CAA-GATG

```

**Figure 9.** Comparison of the 5' sequence of murine Cx pseudogene with that of exon 1s of murine C<sub>s</sub>. The Cx nucleotide sequence is nearly identical to that extending from C<sub>s</sub> nucleotide -20 downstream to the end of C<sub>s</sub> exon 1s. An asterisk indicates the translation start site of C<sub>s</sub>. Dashes indicate nonconsensus nucleotides.

insensitive bonds would explain the inability of cAMP to release C<sub>s</sub> from demembrated sperm.

Such anchoring of activated C<sub>s</sub> in the sperm could be advantageous. First, the phosphorylation of its substrates could be accomplished more efficiently. By maintaining the activated catalytic subunit in close proximity to its target substrates, rapid phosphorylation of these proteins upon activation of C<sub>s</sub> would be ensured. Conversely, if cAMP levels decreased, C<sub>s</sub> would be able to rapidly rebind to R, which itself would be anchored in the same general vicinity by A-kinase-anchoring proteins. Second, by limiting the distance that activated C<sub>s</sub> can travel, promiscuous phosphorylation of other flagellar proteins and its potentially deleterious effects would be avoided. This type of spatial arrangement has been observed in other signal transduction complexes, in which the components of the signaling pathway are assembled on scaffold proteins for more effective physical interaction between enzyme and substrate and for enhanced specificity (Faux and Scott, 1996; Whitmarsh *et al.*, 1998).

Recently, it was found that the majority of C<sub>α</sub> was mislocalized in sperm of a knockout mouse lacking RII<sub>α</sub>, the predominant PKA regulatory subunit in sperm (Burton *et al.*, 1999). If the C<sub>α</sub> isoform monitored in that study was indeed C<sub>s</sub>, this result suggests that the unique structure of C<sub>s</sub> is insufficient to properly localize the subunit in the absence of RII<sub>α</sub>. However, it is quite possible that correct localization of C<sub>s</sub> requires interactions with both R and another protein that interacts with C<sub>s</sub> via an exposed hydrophobic site.

## ACKNOWLEDGMENTS

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