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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%

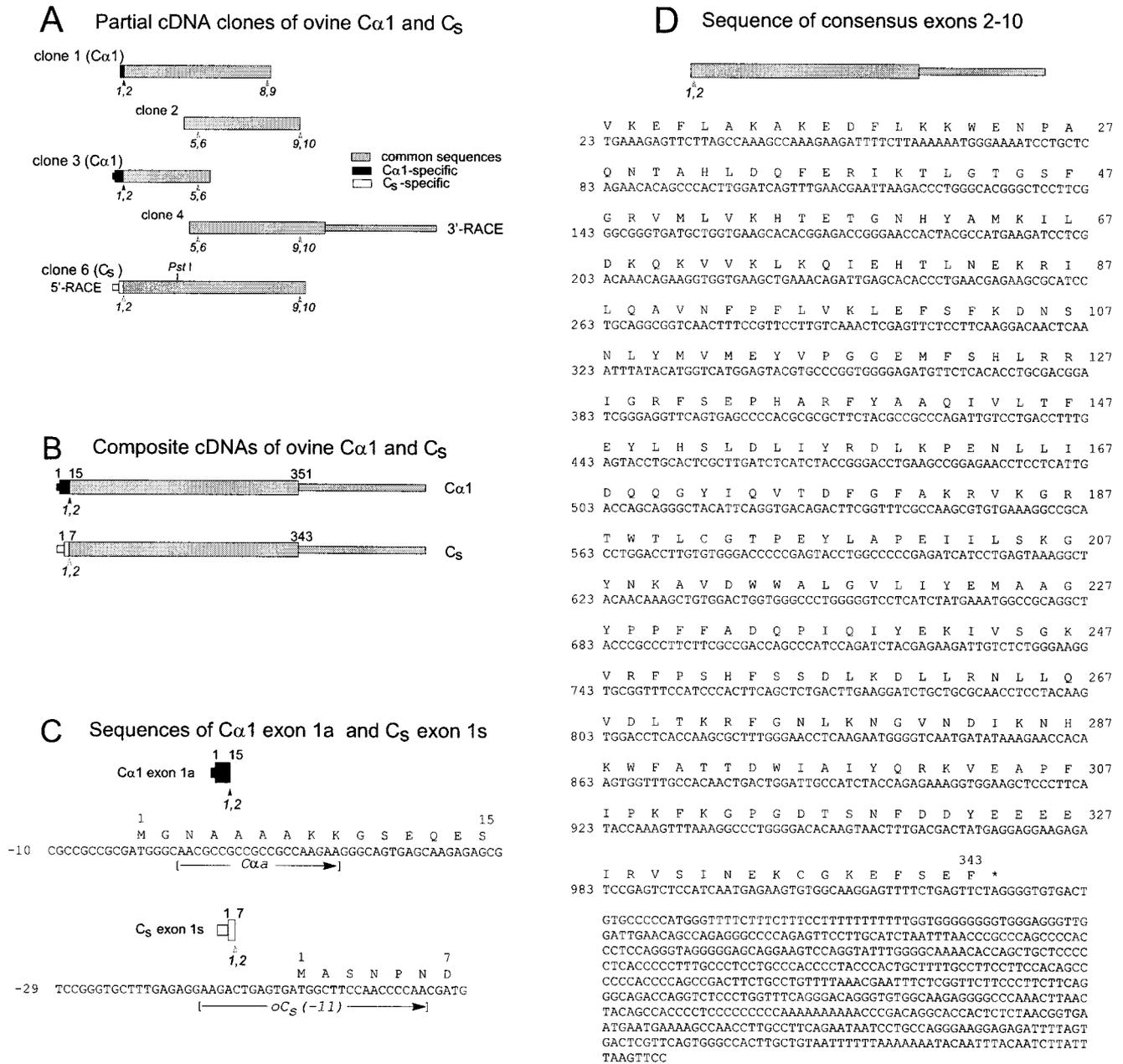


Figure 1. Cloning and sequences of ovine $C\alpha 1$ and C_s cDNAs. The initiating methionine is designated as amino acid residue 1, and the first base of the initiation codon ATG is designated as nucleotide 1. (A) Bars represent the five cDNA clones used to obtain the composite cDNAs and nucleotide 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°C . Murine oocyte total RNA was prepared from ~ 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 μg of poly(A)⁺ RNA. About 350 μg of murine testis poly(A)⁺ 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⁻ 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)₁₂₋₁₈ 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 ³²P-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 ³²P-labeled clone 1. Clone 7 was verified to be a murine C clone by high-stringency hybridization to ³²P-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 ³²P-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 alanyl 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 μ 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 μ 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 μ 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 μ 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 μ 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 (Glycergel, 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_s(-11)*, based on sequences located in exons 1a and 1s, respectively, with the reverse primer *oC_s1402R*, 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-

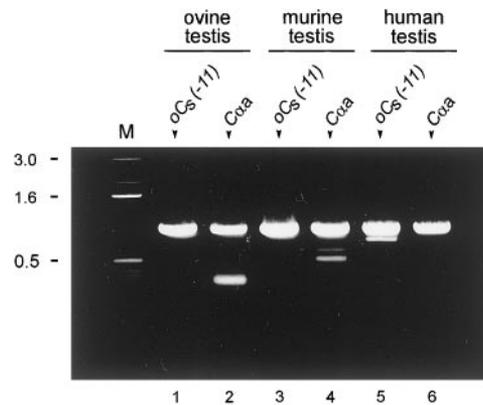


Figure 2. Detection of $C\alpha 1$ and C_s mRNA in various species. RT-PCR of total RNA from ovine and murine testes and PCR of cDNA from human testis. The forward primers used to generate the PCR products are shown at the top of the lanes: *oC_s(-11)* to amplify C_s and *Caa* to amplify $C\alpha 1$. 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_s(-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_s482R*). 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

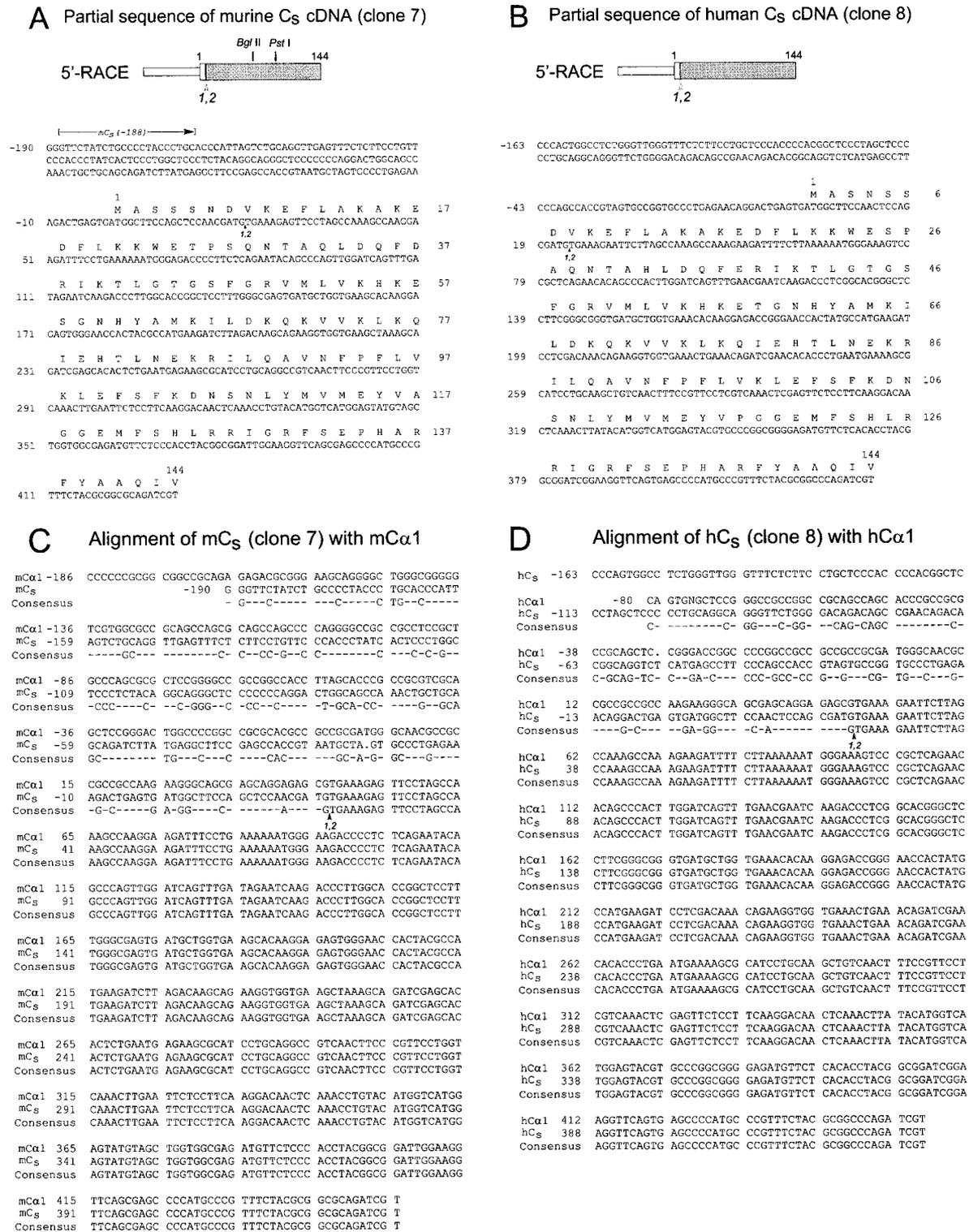


Figure 3. Partial nucleotide and amino acid sequences of murine C_s and human C_s cDNA. (A and B) Murine C_s cDNA (clone 7) and human C_s cDNA (clone 8) were obtained by 5'-RACE with the use of murine and human testis cDNAs as template. The shading and numbering are as in Figure 1. The *Bgl*III and *Pst*I 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_s exon 1s

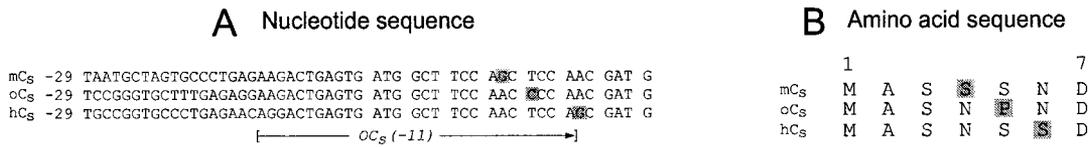


Figure 4. Comparison of exon 1s–encoded regions of ovine, murine, and human C_s. Partial cDNA nucleotide (A) and predicted amino acid (B) sequences of the murine (*mCs*), ovine (*oCs*), and human (*hCs*) versions of C_s exon 1s are aligned. The nonconsensus bases of the ORFs are highlighted, as are the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

human Ca1 with human C_s. As in the sheep, exon 1s of the murine C_s cDNA and exon 1s of the human C_s cDNA showed very little identity with their Ca1 counterparts, whereas C_s 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_s and exon 1s of human C_s were very similar to the ovine C_s 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_s 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_s mRNA Is Found Exclusively in the Testis

To investigate the tissue distribution of C_s, we carried out RT-PCR with the use of murine total RNA from various tissues as template. *mCs*- 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_s mRNA was detected only in testis (Figure 5, lane 19). It is important to note that C_s mRNA was not detected in ciliated tissues such as brain, lung, and trachea, indicating that C_s is not a component of cilia. Moreover, C_s mRNA was not detected in ovarian tissue or oocytes, indicating that C_s is not expressed in the female germ line. These results strongly suggest that C_s is expressed only in the testis, where the translated protein becomes integrated into the sperm tail.

C_s Is Expressed Only in Germ Cells and First Appears in Mid Pachytene Spermatocytes

To determine the pattern of expression of C_s in the testis, a rabbit anti-peptide antibody was made against the unique amino-terminal sequence of murine C_s. 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_s and ovine Ca1 in SDS-polyacrylamide gels (Figure 6, lanes 1–4). These proteins are presumed to represent C_s 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_s 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_s. 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_s 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-

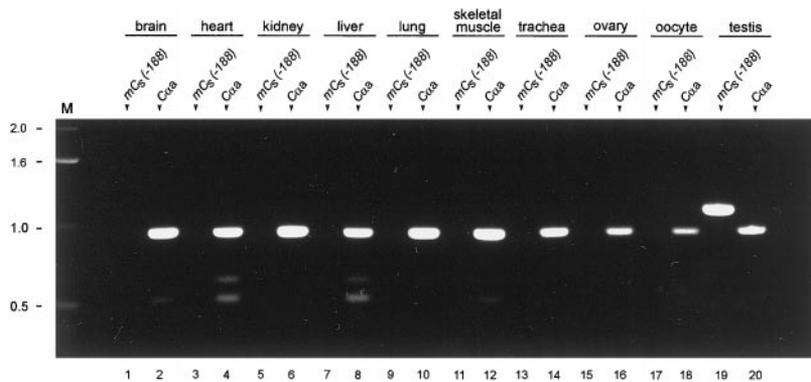
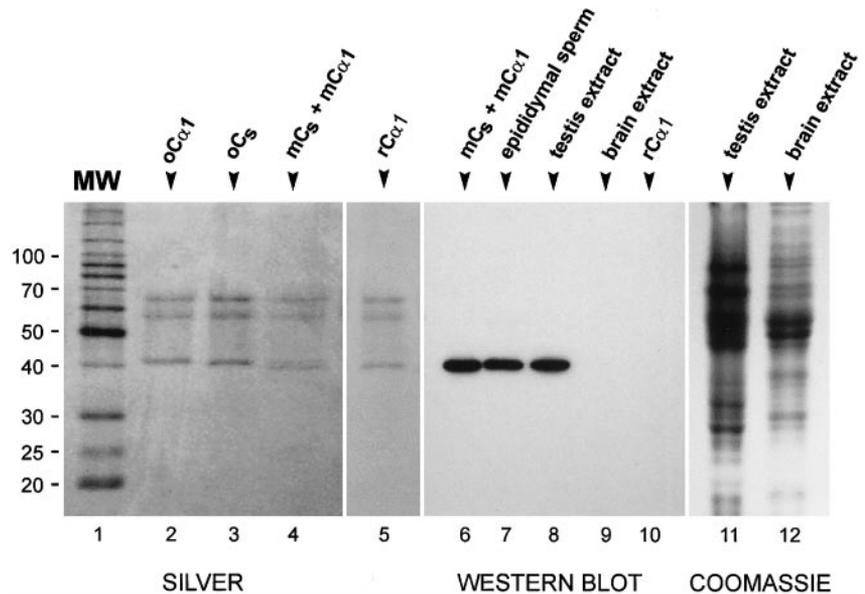


Figure 5. Detection of Ca1 and C_s mRNA in murine tissues. RT-PCR of total RNA from various murine tissues. The forward primers used to generate the PCR products are shown at the top of the lanes: *mCs*(-188) to amplify C_s and *Caa* to amplify Ca1. The reverse primer in all cases was *CaeR*. These primer sets are predicted to yield PCR products of 949 bases for murine Ca1 and 1119 bases for murine C_s. 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_s 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 (1×10^6 sperm), and in murine testis extract (50 μ g of total protein) but does not react with any band in murine brain extract (30 μ g of total protein) or with recombinant $C\alpha 1$ (37 ng). (Right) SDS-polyacrylamide gel of murine testis extract (50 μ g) and murine brain extract (30 μ 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.

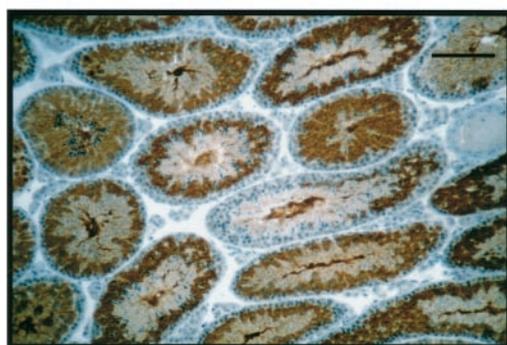
C_s is the third $C\alpha$ isoform to be reported. Thomis *et al.* (1992) described a partial human cDNA that was identical with human $C\alpha 1$ cDNA sequence at its 5' end but that contained sequences derived from introns flanking both sides of exon 8. This cDNA predicts a $C\alpha$ isoform, termed $C\alpha 2$, 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

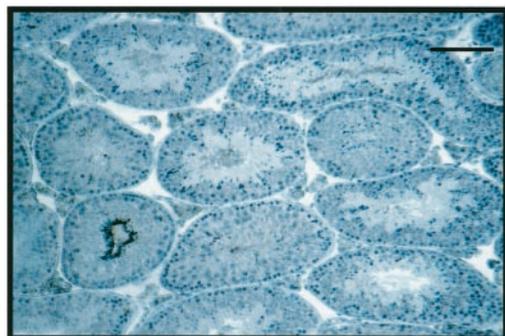
PCR with the use of a primer based on the nucleotide sequence of exon 1s of ovine C_s indicated that C_s is expressed in the testes of mouse and human as well as sheep. The nucleotide sequences of partial cDNAs encoding the murine and human C_s isoforms revealed that C_s exon 1s is very similar in all 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 μm .

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

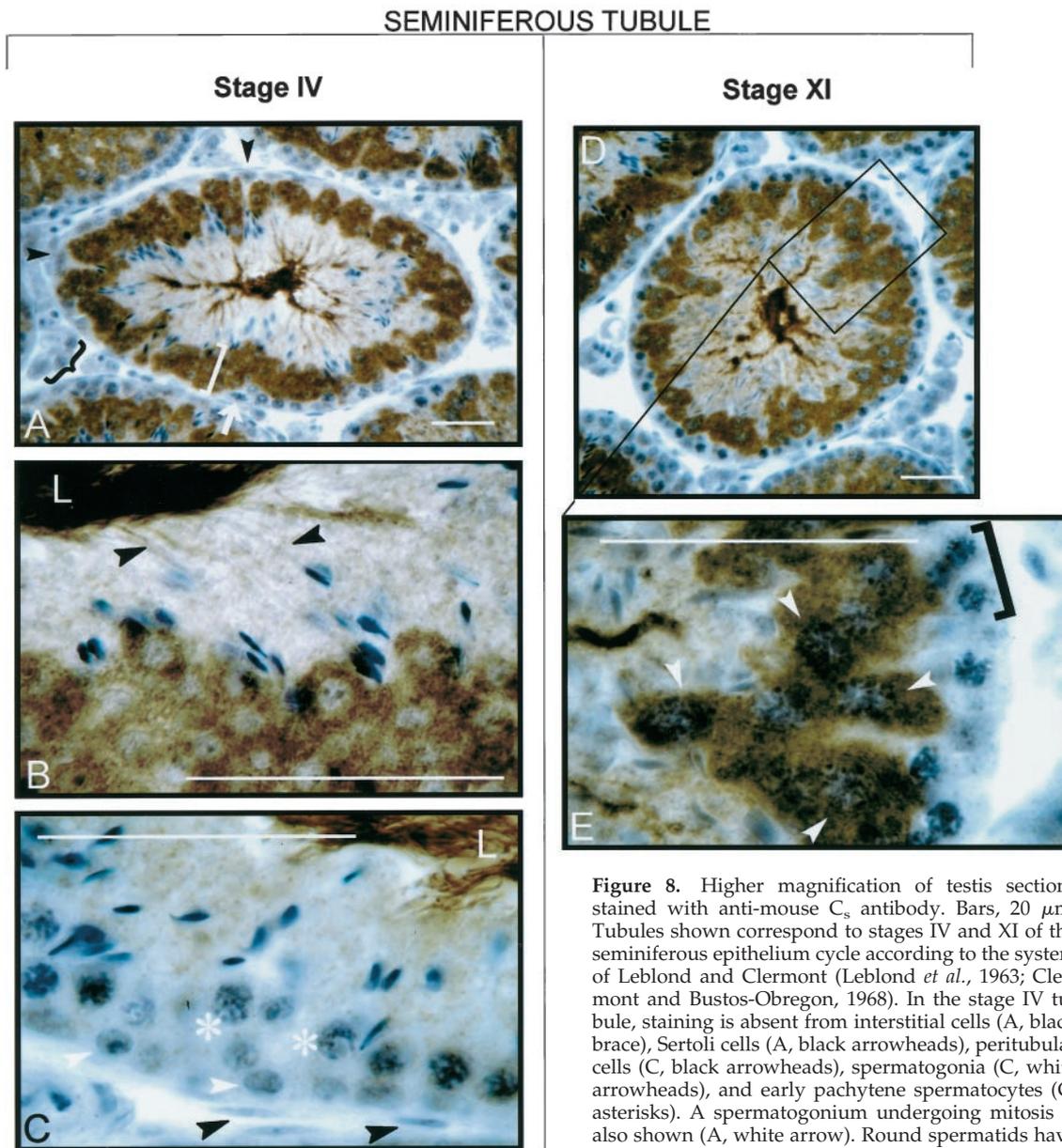


Figure 8. Higher magnification of testis sections stained with anti-mouse C_s antibody. Bars, 20 μm . Tubules shown correspond to stages IV and XI of the seminiferous epithelium cycle according to the system of Leblond and Clermont (Leblond *et al.*, 1963; Clermont and Bustos-Obregon, 1968). In the stage 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 $C\alpha 1$, the exon 1a-encoded residues form the first two turns of a long α -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 $C\alpha 1$ exon 1a residues are unstructured (Knighton *et al.*, 1991). In contrast to the situation in $C\alpha 1$, the residues encoded by exon 1s of C_s form a shorter domain,

are not predicted to form an α -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_s 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 CTACCCCTGCA CCCATTAGTC TGCAGGTTGA GTTCTCTCTC
Cx -186 TCTCAAACCT TGCGTAGATA 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 ATTCAAAATC
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

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

insensitive bonds would explain the inability of cAMP to release C_s from demembrated sperm.

Such anchoring of activated C_s 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_s would be ensured. Conversely, if cAMP levels decreased, C_s 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_s 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_α was mislocalized in sperm of a knockout mouse lacking RII_α, the predominant PKA regulatory subunit in sperm (Burton *et al.*, 1999). If the C_α isoform monitored in that study was indeed C_s, this result suggests that the unique structure of C_s is insufficient to properly localize the subunit in the absence of RII_α. However, it is quite possible that correct localization of C_s requires interactions with both R and another protein that interacts with C_s via an exposed hydrophobic site.

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