The unique catalytic subunit of sperm cAMP-dependent protein kinase is the product of an alternative Calpha mRNA expressed specifically in spermatogenic cells

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The Unique Catalytic Subunit of Sperm cAMP-dependent Protein Kinase Is the Product of an Alternative Ca 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 Cs, whose amino terminus differs from those of published C isoforms of other species. Isolation and sequencing of cDNA clones encoding ovine Cs and Ca1 (the predominant somatic isoform) now reveal that Cs is the product of an alternative transcript of the Ca gene. Cs cDNA clones from murine and human testes also were isolated and sequenced, indicating that Cs is of ancient origin and widespread in mammals. In the mouse, Cs 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 Cs first appears in pachytene spermatocytes. This is the first demonstration of a cell type–specific expression for any C isoform. The conservation of Cs throughout mammalian evolution suggests that the unique structure of Cs 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; Atiken 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 (R2C2). There are three known genes encoding mammalian C. The Ca gene is expressed in most tissues (Showers and Maurer, 1986; Uhler et al., 1986a,b). The CB gene also is expressed in multiple tissues but generally at lower levels than Ca (Showers and Maurer, 1986; Uhler et al., 1986b). Cy 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 (Cs) differs from that of bovine, murine, or human Ca1 (the predominant somatic isoform) in its amino terminus (San Agustin et al., 1998). A combination of tandem mass spectrometry and Edman degradation of Cs peptides indicated that the amino-terminal myristate and first 14 amino acids of the published Ca1 subunits are replaced by an amino-terminal acetate and 6 different amino acids in ovine Cs. However, short peptide sequences from more carboxyl-terminal portions of ovine Cs were identical to the published sequence of bovine Ca1. Although the complete...
sequence of neither the sperm nor the somatic form of ovine C was determined, the results indicated that ovine C is a novel isoform more closely related to Ca1 than to CB or Cj.

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 Ca1? 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 evolve relatively recently in the sheep or its immediate ancestors? Third, in what tissues is C 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 is expressed in both male and female reproductive tissues, it may be specific to the germ line. Immunohistochemistry with the use of this antibody indicates that Cs 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 Cs is a sperm-specific isoform of C that has been conserved throughout mammalian evolution. The unique structure of C 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. Coa, Cab, CaR, CbR, and CaR were derived from consensus sequences of bovine, murine, rat, and human Ca1 mRNAs (Uhler et al., 1986a; Chrivia et al., 1988; Maldonado and Hanks, 1988; Wiemann et al., 1991, 1992). Oc376 and Oc482R were derived from the composite ovine C and Ca1 cDNA sequences reported in this paper (Figure 1). The C-specific primers Oc376 (–11) and mC1 (–187) were derived from the sequences of ovine C exon 1s and murine C exon 1s, respectively (Figure 1C; see also Figure 3A). OvC791R was from the murine Ca1 cDNA sequence (Uhler et al., 1986a; Chrivia et al., 1988), and hCa60 was from the human Ca1 cDNA sequence (Maldonado and Hanks, 1988). API and nested API (Marathon cDNA amplification kit, Clontech Laboratories, Palo Alto, CA) were adapter-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%...
ethanol, and stored at 
-80°C. Murine oocyte total RNA was pre-
pared from [30 oocytes kindly provided by Dr. Joyce Tay (Univer-
]]. In more recent RNA prepa-
rations, tissues from mice were immersed immediately after

Figure 1. Cloning and sequences of ovine Ca1 and Cs 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 Ca1 and Cs. The ORFs are depicted as the wider portions of the bars. Sequences common to both Ca1 and
Cs are gray, sequences specific for Ca1 are black, and sequences specific for Cs are white. For orientation, selected presumptive exon junctions
based on the murine Ca genomic sequence (Chrivia et al., 1988) are marked below the bars (arrowheads). (B) Bars represent the composite
cDNAs of ovine testis Ca1 and Cs. 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 Ca1 and Cs, respectively. Shading is as in A. (C) Partial nucleotide
and predicted amino acid sequences of ovine Ca1 exon 1a and ovine Cs exon 1s. The positions of the forward primers Ca1 and oCs (211)
also are shown. (D) The nucleotide sequence of exons 2–10, which are identical for ovine Ca1 and Cs cDNAs, and their predicted amino acid
sequence. The amino acid and nucleotide positions indicated (right and left margins, respectively) are those for Cs. Sequence data for ovine
Ca1 and ovine Cs have been deposited in GenBank/EMBL/DDBJ under accession numbers AF238979 and AF238980, respectively.

Vol. 11, September 2000 3033
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 Cα1 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 primer (Life Technologies). Table 2 summarizes the amplification schemes used. PCR was carried out on human testis cDNA (Marathon-Ready human testis cDNA, Clontech). Two sets of gene-specific primers were used: oCα376, oCα376; and ApI, ApI to detect Cα1 mRNA in Murine and Human Tissues

### Detection of Cα and Cα1 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: oCα376, oCα376; and ApI, ApI to detect the presence of Cα mRNA, and Cα1 mRNA in Murine and Human Tissues

### 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 Cα or Cα1 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 I, B and C).

### Table 2. Generation of ovine Cα1 clones by RT-PCR and 3’-RACE

<table>
<thead>
<tr>
<th>Amplification</th>
<th>Primers</th>
<th>Thermocycler conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone 1</td>
<td>RT-PCR</td>
<td>Cαα, CααR</td>
</tr>
<tr>
<td>Clone 2</td>
<td>RT-PCR</td>
<td>Cαα, Cααβ</td>
</tr>
<tr>
<td>Clone 3</td>
<td>RT-touchdown PCR</td>
<td>hCαα(−60), oCαα82R</td>
</tr>
<tr>
<td>Clone 4</td>
<td>Touchdown PCR</td>
<td>oCα376, API</td>
</tr>
</tbody>
</table>

### Table 3. Generation of ovine, murine, and human Cα subclones by 5’-RACE

<table>
<thead>
<tr>
<th>First round (5’-RACE)</th>
<th>Thermocycler conditions</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Clone 6 (ovine)</td>
<td>API, Cαα</td>
</tr>
<tr>
<td>Clone 7 (murine)</td>
<td>API, mCα791R</td>
</tr>
<tr>
<td>Clone 8 (human)</td>
<td>API, Cαα</td>
</tr>
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</table>

### Second round | Thermocycler conditions

|                       |                         |
| Clone 6 (ovine)       | Nested API, oCαα82R     | Annealing at 59°C, extension at 68°C, 35 cycles, final 10-min extension at 68°C |

3034 Molecular Biology of the Cell
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 Ca1 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 CaR to detect C_s mRNA, and Caa and CaR to detect Ca1 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 Ca1. 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-m 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 piperase-N,N,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 × g for 15 min at 4°C. The supernatants were further clarified by centrifugation at 96,000 × g for 75 min at 4°C.

**Isolation of mC_s and mCa1 from Murine Testis**

Because both murine C_s and Ca1 are expressed in testis (see RESULTS), both isoforms were present in the clarified testes extract. The two isoforms were copurified with the use of the protocol for the purification of ovine Ca1 from ram skeletal muscle as described previously (San Agustin et al., 1998). Fractions containing murine C_s and Ca1 eluted from the CM Fast Flow column (0.5 × 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 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 orbital 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 μm 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 cover slips for 20 min in 10 mM citrate, pH 6 (Polak and Van Noorden, 1997). The cover slips 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 cover slips (Sanders and Salisbury, 1995). The testis sections were incubated with 250 μl 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 μl 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 μl 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 μl 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 cover slips 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 Ca1 and C_s cDNAs**

To determine the relationship of ovine C_s to ovine Ca1, 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 Ca1 and C_s.

Clone 1, corresponding to a portion of the Ca1 mRNA extending from exon 1 to exon 9, was obtained with the use of the Ca1-specific primer Ca1 and the reverse primer CaR. Sequencing confirmed that this clone encoded amino acids specific to the amino terminus of Ca1 (Figure 1C). Clone 2, obtained with the use of consensus primers based on published mammalian Ca1 sequences, was 100% identical with clone 1 in the region of overlap.

The remaining sequence of the 5’ end of the ORF of ovine Ca1 mRNA was obtained from clone 3, which was generated with the use of hCa1(−60) as the forward primer and Ca1cR as the reverse primer. A forward primer based on the 5’-UTR of human Ca1 mRNA was used because the 5’-UTR of bovine Ca1 mRNA is not known, and we reasoned that the 5’-UTR of human Ca1 was likely to be similar to that of ovine Ca1. Clone 3 encoded amino acids specific to
the amino terminus of Ca1 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 Cs 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 Cs were obtained by 5′-RACE with the use of CaeR as gene-specific primer and ovine testis 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 Cs and Ca1 cDNAs, all subclones contained sequences coding for the unique amino terminus of Cs contiguous to sequences identical to exons 2–10 of the Ca1 clones. The finding that the cDNA sequences of exons 2–10 of Ca1 and Cs are identical at the nucleotide level provided strong evidence that Cs is the product of an alternatively spliced gene.

Further proof that exon 1 (hereafter referred to as exon 1a) of the Ca1 mRNA and exon 1s of the Cs mRNA are spliced to the same downstream sequence was obtained by carrying out RT-PCR of ovine testis mRNA using the forward primers Cao and Cc(−11), based on sequences located in exons 1a and 1s, respectively, with the reverse primer Cc4102R, which is complementary to sequence located in the 3′ noncoding region of exon 10. Both primer pairs yielded products of the expected size (unpublished results), confirming that the 3′-UTR of exon 10 is common to both Ca1 and Cs mRNAs.

**Nucleotide and Predicted Amino Acid Sequences of Ovine Ca1 and Cs cDNAs**

Figure 1C shows the partial sequences of Ca1 exon 1a and Cs exon 1s obtained from the ovine cDNA clones. The ORF of Ca1 exon 1a codes for 15 amino acids, whereas that of Cs exon 1s codes for 7 different amino acids. The amino acid residues encoded by ovine Ca1 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; Chrivia et al., 1988), rat (Wiemann et al., 1991), hamster (Howard et al., 1991), and human (Maldonado and Hanks, 1988) Ca1, whereas the amino acid sequence predicted from Cs exon 1s (minus the initiator methionine) exactly matches the amino-terminal sequence for ovine Cs, obtained through protein biochemistry (San Agustin et al., 1998). The nucleotide sequence of exons 2–10, which are identical for both the Ca1 and Cs 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 Cs obtained from Edman analysis of its cyanogen bromide and tryptic fragments (San Agustin et al., 1998).

The ovine Cs cDNA predicts a protein of 343 amino acids (including the initiating methionine) with a mass of 39,858 Da, whereas the ovine Ca1 cDNA predicts a protein of 351 amino acids with a mass of 40,589 Da. Because the amino terminus of Ca1 is myristylated and that of Cs is acetylated (San Agustin et al., 1998), the mass of the modified Ca1 is predicted to be 899 Da greater than the mass of modified Cs, in excellent agreement with the difference of 890 Da determined empirically by mass spectrometry (San Agustin et al., 1998).

**Similar Cs mRNAs Are Present in Murine and Human Testes**

To determine if Cs 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 Cs [Cc(−11)] or Ca1 [Cao]. In all cases, the reverse primer was Cc4102R. In all three species, the Cs-specific primer yielded PCR products of the expected size (Figure 2, lanes 1, 3 and 5). Therefore, Cs is widespread in mammals. Ca1 transcripts also were found in the testes of all three species (Figure 2, lanes 2, 4, and 6), confirming that both Cs isoforms occur in the testis. The Ca1 and Cs PCR products had very similar sizes (slightly less than 1 kilobase), which agrees with the calculated sizes of 949 bases for the Ca1 PCR product and 942 bases for the Cs PCR product.

**Nucleotide Sequences of cDNAs Encoding the Amino Termini of Murine and Human Cs**

To confirm that murine and human testes have Cs, and to determine the degree of similarity between the amino termini of these proteins and that of ovine Cs, cDNAs of murine and human Cs were amplified from testis cDNA by 5′-RACE with the use of identical sets of primers (nested API and Cc482R). 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 Cs. Figure 3, A and B, show the partial nucleotide and predicted amino acid sequences of murine Cs cDNA (clone 7) and human Cs cDNA (clone 8), respectively. Figure 3, C and D, show the alignment of murine Ca1 with murine Cs and...
Figure 3. Partial nucleotide and amino acid sequences of murine Cs and human Cs cDNA. (A and B) Murine Cs cDNA (clone 7) and human Cs 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 II and Pst I sites that are present in clone 7 but not in clone 8 are indicated. The murine and human Cs sequences are available from GenBank/EMBL/DDBJ under the accession numbers AF239743 and AF239744, respectively. (C and D) The cDNA sequences of clones 7 (mCs) and 8 (hCs) are compared with the corresponding regions of the murine Ca (mCa) (Uhler et al., 1986a) and human Ca (hCa) (Maldonado and Hanks, 1988) sequences. Dashes indicate nonconsensus nucleotides.
human Ca1 with human Cs. As in the sheep, exon 1s of the
murine Cs cDNA and exon 1s of the human Cs cDNA
showed very little identity with their Ca1 counterparts,
whereas Cs 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 Ca1 and exon 1s
of human Cs were very similar to the ovine Cs 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 Cs 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.

Cs mRNA Is Found Exclusively in the Testis

To investigate the tissue distribution of Cs, 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
caseR as the reverse primer. Ca1 mRNA was detected in all
tissues assayed (Figure 5), whereas Cs mRNA was detected
only in testis (Figure 5, lane 19). It is important to note that
Cs mRNA was not detected in ciliated tissues such as brain,
lung, and trachea, indicating that Cs is not a component of
cilia. Moreover, Cs mRNA was not detected in ovarian tissue
or oocytes, indicating that Cs is not expressed in the female
germ line. These results strongly suggest that Cs is expressed
only in the testis, where the translated protein becomes
integrated into the sperm tail.

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

To determine the pattern of expression of Cs in the testis, a
rabbit anti-peptide antibody was made against the unique
amino-terminal sequence of murine Cs. The specificity of the
antibody was demonstrated in Western blots. Fractions of
purified Cs from murine testes contain two proteins that
migrate with mobilities very similar to those of pure ovine
Cs and ovine Ca1 in SDS-polyacrylamide gels (Figure 6,
lanes 1–4). These proteins are presumed to represent Cs 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 Cs 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
Cb but not Cs. The antibody also did not recognize purified
ovine Ca1, which has the same amino-terminal sequence as
murine Cs (our unpublished results), nor murine recombi-
nant 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 Cs 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 4. Comparison of exon 1s–encoded regions of ovine, murine, and human Cs. Partial cDNA nucleotide (A) and predicted amino acid
(B) sequences of the murine (mCs), ovine (oCs), and human (hCs) versions of Cs 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) is also shown.

Figure 5. Detection of Ca1 and Cs 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 Cs and Ca1 to amplify Ca1. The reverse primer in all cases was Ca1R.
These primer sets are predicted to yield PCR products of 949 bases for murine Ca1 and 1119 bases for murine Cs. 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 Cs transcripts are detected only in the testis. Lane M, DNA molecular mass markers (in kilo-
base pairs).
Figure 6. Specificity of the anti-mC\textsubscript{s} antibody. (Left) Silver-stained SDS-polyacrylamide gels of purified ovine C\textsubscript{s} (mC\textsubscript{s}), purified ovine C\textsubscript{s} (oC\textsubscript{s}), a mixture of murine C\textsubscript{s} and C\textsubscript{a} (mC\textsubscript{s} + mC\textsubscript{a}), isolated from murine testis, and mouse recombinant C\textsubscript{a} (rC\textsubscript{a}). Molecular mass markers (MW) are in kilodaltons. As reported previously (San Agustin et al., 1998), ovine C\textsubscript{s} migrates slightly faster than ovine C\textsubscript{a}. The partially purified murine C\textsubscript{s} and C\textsubscript{a} 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\textsubscript{s}. The antibody reacts with a single protein in the mixture of murine C\textsubscript{s} and C\textsubscript{a} (mC\textsubscript{s} + mC\textsubscript{a}), in murine epididymal sperm (1 x 10\textsuperscript{8} 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\textsubscript{a} (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 (our unpublished results), and stained late pachytene spermatocytes of stage VIII tubules slightly more strongly (Figure 8). Therefore, C\textsubscript{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\textsubscript{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\textsubscript{s} Is the Product of an Alternative Transcript of the C\textsubscript{a} Gene

C\textsubscript{s} originally was characterized by protein biochemistry as an ovine sperm PKA catalytic subunit differing from ovine somatic C\textsubscript{a}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\textsubscript{s} is the product of an alternative transcript of the C\textsubscript{a} gene. First, the nucleotide sequences of C\textsubscript{s} and C\textsubscript{a}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\textsubscript{s} and exon 1a of C\textsubscript{a}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\textsubscript{a}. Therefore, the C\textsubscript{s} mRNA must result from alternative splicing of a C\textsubscript{a} transcript. Production of the C\textsubscript{s} transcript also may depend on an alternative initiation site within the C\textsubscript{a} gene.

C\textsubscript{s} is the third C\textsubscript{a} isoform to be reported. Thomis et al. (1992) described a partial human cDNA that was identical with human C\textsubscript{a}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\textsubscript{a} isoform, termed C\textsubscript{a}2, that would be substantially truncated at its carboxy-terminal end. The C\textsubscript{a}2 cDNA appeared to be expressed in at least two human cell lines.

Similar C\textsubscript{s} Isoforms Are Widespread in Mammals

PCR with the use of a primer based on the nucleotide sequence of exon 1s of ovine C\textsubscript{s} indicated that C\textsubscript{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\textsubscript{s} isoforms revealed that C\textsubscript{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\textsubscript{a}1 exon 1a are replaced by 7 different amino acids in C\textsubscript{s}. In all three species, an alanine replaces the glycine that follows the first methionine in C\textsubscript{a}1. In C\textsubscript{s}, 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\textsubscript{s}, they probably are not myristylated but rather are acetylated, as is ovine C\textsubscript{s} (San Agustin et al., 1998). The presence of C\textsubscript{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).
The Murine Cx Pseudogene Likely Arose from a Cs 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 Ca gene, but it lacks introns and, relative to Cs, contains frameshift mutations, premature termination codons, and missense mutations. It is not transcribed. Therefore, it appears to be a pseudogene of the retroponon class (Weiner et al., 1986). Cx is closely related to Ca downstream of the Ca exon 1/exon 2 junction but does not resemble the Ca 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 Cs exon 1s nucleotide sequence with the Cx 5′ sequence reveals near identity from Cs nucleotide –20 to the Cx exon 1/exon 2 junction (Figure 9). Therefore, Cx probably arose by reverse transcription of a Cs mRNA followed by nonhomologous recombination of the cDNA into the genome of a male germ cell.

Function of Unique Cs Structure

The fact that Cs 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. Cs is not released from demembranated 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.
even when activated. The unique structure of C\textsubscript{s} may be responsible for this behavior. In C\textsubscript{a1}, 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 \textit{et al.}, 1993). In the absence of this myristate, the C\textsubscript{a1} exon 1a residues are unstructured (Knighton \textit{et al.}, 1991). In contrast to the situation in C\textsubscript{a1}, the residues encoded by exon 1s of C\textsubscript{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 \textit{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\textsubscript{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\textsubscript{s} to that structure. In either case, the attachment of C\textsubscript{s} to the sperm tail by cAMP-

\[\text{Sperm PKA Catalytic Subunit} \]

\[\text{Vol. 11, September 2000 3041}\]
calized in sperm of a knockout mouse lacking RII α nearly identical to that extending from C s nucleotide recombinant C. We are grateful to Dr. Susan Taylor for her generous gift of murine ACKNOWLEDGMENTS of RII is insufficient to properly localize the subunit in the absence release Cs from demembranated sperm. insensitive bonds would explain the inability of cAMP to Comparison of the 5 sequence of murine Cx pseudogene with that of exon 1s of murine Cx. The Cx nucleotide sequence is nearly identical to that extending from C s, nucleotide −20 downstream to the end of Cx exon 1s. An asterisk indicates the translation start site of C s. Dashes indicate nonconsensus nucleotides.

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 pathways, 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 Cα was mislocalized in sperm of a knockout mouse lacking RIα, the predominant PKA regulatory subunit in sperm (Burton et al., 1999). If the Cα isoform monitored in that study was indeed Cα, this result suggests that the unique structure of Cα is insufficient to properly localize the subunit in the absence of RIα. However, it is quite possible that correct localization of Cα requires interactions with both R and another protein that interacts with Cα via an exposed hydrophobic site.

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