Identification and Functional Analysis of Splice Variants of the Germ Cell Soluble Adenylyl Cyclase*

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In mammalian germ cells, cAMP signaling is dependent on two forms of adenylyl cyclase, the conventional membrane-bound ACII and a soluble form of adenylyl cyclase (sAC). Recent elucidation of the sAC sequence indicates that this enzyme is phylogenetically distinct from the membrane-bound AC, does not interact with G proteins, and its activity is regulated by bicarbonate ions. Here we have investigated the properties and regulation of this enzyme during spermatogenesis. Two different transcripts encoding a full-length and truncated sAC were identified by reverse transcriptase-polymerase chain reaction and RNase protection analysis. The truncated sAC transcript lacks exon 11 with a premature termination of the open reading frame after the catalytic domain. Reverse transcriptase-polymerase chain reaction with testis RNA from adult mouse and rat of different ages, as well as RNase protection, showed that both transcripts are absent at 11 days of age, appear between 20 and 30 days of age, and are retained in the adult testis. The presence of corresponding proteins in testis, germ cells, and spermatozoa was demonstrated by fast protein liquid chromatography and differential immunoprecipitation with full-length sAC-specific antibodies. Bicarbonate ions activated both sAC forms and increased cAMP levels in germ cells isolated from 25- and 50-day-old rats and adult rats in a concentration-dependent manner. These findings provide evidence that full-length and truncated sAC are generated by alternate splicing. Both forms are active in spermatids, and the bicarbonate present in the seminiferous tubule may be a signal that regulates cAMP levels in these cells.

Cyclic AMP, the product of adenylyl cyclase, is an important second messenger controlling signaling in a wide variety of cells in almost all prokaryotic and eukaryotic organisms (1, 2). This also holds true for the male gonad, where cAMP signaling is essential for the hormonal regulation of somatic cell differentiation and function. Similarly, acquisition of motility, capacitation, and acrosome reaction, processes that render mature spermatozoa competent to fertilize an egg, are dependent on cAMP signaling (3–6). Conversely, little is known about the role of the cAMP pathway during the differentiation of germ cells. That cAMP signaling may be important for the spermatogenic cell is suggested by the genetic ablation of the CREB1-related protein CREM that is expressed at a high level in differentiating spermatids (7). Mice deficient in CREM are sterile, and spermatogenesis is arrested at the beginning of spermatid differentiation (8, 9). From this phenotype and the observation that several spermatid-specific genes activated during differentiation contain functional cAMP-response elements in their promoters (10, 11), it has been proposed that CREM functions as a master switch to activate the genetic program directing spermatid differentiation (12). Because CREM is phosphorylated and activated by the cAMP-regulated protein kinase A in a manner similar to the cAMP-response element-binding protein (7, 13), it has been inferred that cAMP signaling is crucial for spermatogenesis.

In male germ cells and mature spermatozoa, cAMP production is catalyzed by at least two cyclases. Spermatids express ACII, a membrane-bound, G protein-regulated adenyl cyclase, which is also expressed at high levels in the olfactory system and in brain (14, 15). In addition, it has long been known that a nonconventional, G protein- and forskolin-insensitive, adenylyl cyclase is present in maturing germ cells and spermatozoa (16–19).

The structure of the soluble form of adenylyl cyclase (sAC) was recently elucidated by protein purification and cloning (20). This adenylyl cyclase is an enzyme with no clearly identifiable transmembrane domain and is structurally related to cyclases found in prokaryotes (20). Forskolin and guanine nucleotides do not regulate sAC activity (20), a finding consistent with the properties reported for the germ cell soluble cyclase (18, 21, 22). Moreover, spermatozoa from all species studied express an adenylyl cyclase that is sensitive to bicarbonate anions (23, 24). Recently, it has been shown that sAC corresponds to the cyclase sensitive to bicarbonate found in testis, probably in spermatozoa, and possibly in other tissues, thus functioning as a bicarbonate sensor (25).

Initial biochemical characterization of the testis-soluble cyclase identified a protein with a molecular mass of 48 kDa (21, 22, 26). However, the cloning of the sAC gene revealed an open reading frame that codes for a 187-kDa protein (20). To reconcile these discrepancies, it has been proposed that the 187-kDa protein is an inactive precursor that is proteolytically cleaved to generate the active 48-kDa form (20). In

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1 The abbreviations used are: CREB, cAMP-response element-binding protein; CREM, cyclic AMP-response element modulator; sAC, soluble form of adenylyl cyclase; AC, adenylyl cyclase; FL-sAC, full-length sAC; T-sAC, truncated sAC; nsAC, rat sAC; RT-PCR, reverse transcriptase-polymerase chain reaction; FPLC, fast protein liquid chromatography; bp, base pair; kb, kilobase pair; DMEM, Dulbecco’s modified Eagle’s medium; RIA, radioimmunoassay; RPA, ribonuclease protection assay.

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support of this view, expression of a truncated sAC cDNA construct containing only the C1C2 catalytic domain yields a highly active protein of \(48 \text{kDa}\) (20).

In the present study, we have reinvestigated this issue and provide evidence for an additional mechanism for the generation of the short sAC form. By using independent approaches, we demonstrate that two alternate spliced transcripts are expressed in rodent germ cells, and both the full-length and truncated sACs contribute to the germ cell adenylyl cyclase activity. Given the high concentration of bicarbonate in the seminiferous tubule fluid environment in which germ cells differentiate, we propose that sAC is active early on during germ cell differentiation and its bicarbonate regulation may provide a means by which germ cell function is regulated by the surrounding environment.

**EXPERIMENTAL PROCEDURES**

**Culture Medium**—All culture media used were from Life Technologies, Inc. Restriction enzymes, polymerases, and ligases were from Roche Molecular Biochemicals or from Life Technologies, Inc. The \(\alpha^{32}\text{P}\)ATP, \(\alpha^{32}\text{P}\)CTP, and \(\text{[H]}\)cAMP were from PerkinElmer Life Sciences, and \(\text{[125]}\text{I}\)-cAMP was from Amersham Pharmacia Biotech. Collagenase (type I) is from Worthington. Unless otherwise specified, all other chemicals were the purest grade available from Sigma.

**RNA Preparations**—Total RNA was extracted from testis of male

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**TABLE I**

| Primer    | 5' position\(^a\) | Sequence | Restriction sites\(^b\) |
|-----------|-------------------|----------|------------------------|
| rsAC-1f   | 261               | 5'-GACACAGCTTCTGCACACTG-3' | EcoRI |
| rsAC-2f   | 283               | 5'-CTCGAGATTCAATCCCGACTGC-3' | EcoRI |
| rsAC-3f   | 1509              | 5'-CAACAGTACAGTCAGTTTG-3'  |         |
| rsAC-4f   | 1665              | 5'-GTCGGATCCGAGCGATGTG-3'  |         |
| rsAC-1r   | 5115              | 5'-GATGCGCAGAAAGGATGTGCGG-3' | ApaI |
| rsAC-2r   | 2393              | 5'-GATGCGCAGAAAGGATGTGCGG-3' | ApaI |
| rsAC-3r   | 1933              | 5'-GATGCGCAGAAAGGATGTGCGG-3' | ApaI |
| rsAC-4r   | 1698              | 5'-GATGCGCAGAAAGGATGTGCGG-3' | ApaI |

\(^a\) 5' position of the primer sequence on the cDNA.

\(^b\) Restriction sites are incorporated in the primers.

\(^c\) f, forward primer; r, reverse primer.
Harlan Sprague-Dawley rat and mouse using Trizol (Life Technologies, Inc.) following the manufacturer’s protocol and then precipitated with cold ethanol. The dried pellets for each preparation were dissolved in ribonuclease-free H$_2$O and stored at $-80$ °C for further use.

**Germ Cell Isolation**—Total germ cells were isolated from testis of different ages of male Harlan Sprague-Dawley rats by two subsequent collagenase digestions (0.33% collagenase type I, 220 units/mg) as described earlier (27).

**Spermatozoa Preparations**—Epithidal spermatozoa were isolated from rat cauda epididymis according to the methods described (28). After several washings with phosphate-buffered saline, the cells were suspended in homogenization buffer and sonicated 4 times, for 30 s each.

**RT-PCR Analysis**—For the amplification of FL-rsAC and C1C2-rsAC, rat testis total RNA (5 μg) was reverse-transcribed with Moloney murine leukemia virus RT, using oligo(dT) or random hexanucleotide primer for first-strand cDNA synthesis (First Strand Complementary DNA Synthesis kit, Amersham Pharmacia Biotech) following the manufacturer’s protocols. PCRs were performed directly on 3–5 μl of first-strand cDNA. PCR was carried out using 500 nmol each of rsAC-1f and rsAC-1r primers (for FL-rsAC) and rsAC-2f and rsAC-4r primers (C1C2-rsAC) (Table I) in a 100-μl reaction volume composed of 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl$_2$, 200 μM of each deoxynucleotide. PCR was carried out for initial denaturation of 1 cycle at 94 °C for 2 min, followed by 30 cycles of denaturation (94 °C for 45 s), annealing (55 °C for 45 s), and extension (72 °C for 10 min) with 5 units of TaqPlus Precision polymerase (Stratagene, La Jolla, CA) or 2.5 units of Takara Ex Taq polymerase (Takara Shuzo Co., Otsu, Shiga, Japan). This was followed by a final extension step of 72 °C for 10 min. At the end of the PCR amplification, products were analyzed on agarose gels stained with ethidium bromide and visualized with UV light. The amplified fragments of correct size were excised from the agarose gel and purified by a gel extraction kit (Qiagen, Inc., Chatsworth, CA) for subcloning and sequencing.

For identification of sAC splice variants, total RNA (5 μg) of rat and mouse testis was reverse-transcribed with Superscript II Reverse Transcriptase (SuperScript™ First-strand Synthesis system for RT-PCR, Life Technologies, Inc.) using 500 ng of oligo(dT) or gene-specific primer rsAC-2r (Table I) according to manufacturer’s directions. PCR was carried out using 250 nmol of rsAC-3f and rsAC-3r as well as rsAC-4f and rsAC-2r primers in a 50-μl reaction volume composed of 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl$_2$, 200 μM of each deoxynucleotide. PCR was carried out for initial denaturation of 1 cycle at 94 °C for 2 min, for 30 cycles at denaturation (94 °C for 45 s), annealing (55 °C for 45 s), and extension (72 °C for 10 min) with 5 units of TaqPlus Precision polymerase (Stratagene, La Jolla, CA) or 2.5 units of Takara Ex Taq polymerase (Takara Shuzo Co., Otsu, Shiga, Japan). This was followed by a final extension step of 72 °C for 10 min. The amplified fragments of correct size were excised from the agarose gel and purified by a gel extraction kit (Qiagen, Inc., Chatsworth, CA) for subcloning and sequencing.

**cDNA Cloning**—The FL-rsAC fragment generated by PCR from rat testis cDNA using rsAC-1f and rsAC-1r primers (Table I) was cloned in frame with the V5 epitope of the pcDNA3.1/V5-His/TOPO mammalian expression vector using a Eukaryotic TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA) following the manufacturer’s directions.

The C1C2-rsAC was generated by PCR amplification using rsAC-2f and rsAC-4r primers and FL-rsAC plasmid as a template and cloned in frame with the V5 epitope of the pcDNA3.1/V5-His-TOPO mammalian expression vector. The nucleotide sequences of the inserted fragments were determined by DNA sequencing based on the dideoxy chain ter-
FIG. 4. Expression of the recombinant RsAC in HEK293 cells. A shows the Western blot analysis of cytosolic extracts from HEK293 cells transfected with an empty vector (Mock), C1C2-rsAC, and FL-rsAC pcDNA3 plasmids. Soluble extracts were fractionated by electrophoresis on 8% SDS-PAGE and probed with an anti-V5 antibody. B shows the adenylyl cyclase activity measured in the same cytosolic extracts in the presence of 5 mM MnCl₂. The data shown are representative of at least 3 different experiments.

transcription method or automatic sequencing in the DNA sequencing facility at Stanford University.

Northern Blot Analysis—Total RNA was extracted from testis as described above. Equivalent amounts of RNA (20 μg) were fractionated by electrophoresis through denaturing agarose gel containing formaldehyde and transferred to Biorex Nylon membrane (ICN Pharmaceuticals, Aurora, OH), fixed by ultraviolet illumination, and processed for Northern blot analysis. The membrane was prehybridized with Express Hyb hybridization solution from CLONTECH Laboratories, Inc. (Palo Alto, CA) for 1 h followed by hybridization for 1 h with a labeled cDNA probe at 68 °C. Rat sAC cDNA containing a C1C2 catalytic domain was digested from the FL-rsAC-V5-pcDNA-TOPO construct by EcoRI and XbaI, and a 1445-bp fragment was recovered from the gel. This fragment was labeled to a specific activity of 10⁶ cpm/μg of DNA using [α-³²P]deoxyctydine triphosphate and the random primers DNA label-ling system (Life Technologies, Inc.) following the manufacturer’s protocol. Twenty micrograms of total RNA were hybridized with 10⁶ cpm ³²P-labeled probe overnight at 42 °C. Unprotected single strand RNA was further digested with RNase, and protected fragments were fractionated on a 5% urea/acylamide gel.

Transcript Transfection of HEK293 Cells—Expression constructs were transiently transfected into HEK293 cells by the calcium phosphate method. Briefly, HEK293 cells (2 × 10⁶ cells) were seeded in 35-mm dishes and grown in DMEM containing 10% fetal bovine serum at 70% confluence. Twenty μg of DNA of each construct were transfected to HEK293 (2 × 10⁶ cells) by CaCl₂ precipitation (125 mM) in 2-(bis[2-hydroxyethyl]-amino)ethane sulfonic acid. Cells were harvested 24 h after transfection with lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 0.7 mg/ml pepstatin A, 50 mM benzamidine, and 1 mM phenylmethysulfonyl fluoride) and disrupted by homogenization in a Dounce homogenizer by 20 strokes. Cell homogenates were centrifuged first at 14,000 × g for 20 min at 4 °C. Supernatants were again centrifuged at 100,000 × g for 20 min at 4 °C to obtain the soluble fraction.

Size-exclusion Gel Chromatography—Size-exclusion gel chromatography was performed in a fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech) using a Sephacryl S-200 column (Superdex 200 high load column, 16 × 60 cm, Amersham Pharmacia Biotech). The column was equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 1 mM DTT. Protein samples (2 ml) were loaded onto the column and run with a flow rate of 0.75 ml/min. The column was calibrated with the following gel filtration standards: catalase (232 kDa), aldolase (158 kDa), ovalbumin (43 kDa), chymotrypsin (25 kDa), and blue dextran (2000 kDa). Aliquots (2 ml) of 100,000 × g supernatant of rat testis, recombinant T-rsAC, or FL-rsAC was used for each injection. Fractions (1 ml) were collected and analyzed for AC activity and for immunoprecipitation.

Adenylate Cyclase Assay—In vitro adenyl cyclase assay was performed as described by Alvarez and Daniels (29) with some modifications. AC activity was measured from the enzyme prepared (cytosolic fractions as described above) from cells expressing an empty plasmid (pcDNA3.1), different RsAC constructs, or sAC-immunoprecipitated samples. Briefly, enzyme preparations were incubated in a reaction buffer containing 40 mM Tris-HCl, pH 7.5, 5 mM NaCl, or 5 mM MgCl₂, 0.2 mM cAMP, 10 mM phosphoenol pyruvate, 3 units of pyruvate kinase, 10 μM GTP, 1 mM ATP, and 2 μCi of [³²P]ATP for 20 min at 37 °C. Reaction was terminated with the addition of 20 μl of 2.2 N HCl containing [³²P]cAMP (0.01 μCi) followed by boiling for 4 min and then cooling in an ice-water bath. Labeled cAMP was added to estimate and correct for recovery of the cyclic nucleotide during column chromatography following the manufacturer’s standard protocol. Twenty micrograms of total RNA were hybridized with 10⁶ cpm ³²P-labeled probe overnight at 42 °C. Unprotected single strand RNA was further digested with RNase, and protected fragments were fractionated on a 5% urea/acylamide gel.
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**FIG. 5.** Adenylyl cyclase activity of recombinant FL and T-sAC and from rat cauda sperm extracts. HEK293 cells were transfected with empty vector (Mock) or vectors containing FL-rsAC and C1C2-rsAC cDNAs. After 24 h cells were harvested, and soluble fractions were prepared as described under “Experimental Procedures.” The adenylyl cyclase activity was measured in the presence of either 5 mM MgCl₂ (A) or 5 mM MnCl₂ (B) in the absence (empty bars) or presence (filled bars) of 50 mM sodium bicarbonate. Note the 1000-fold difference in the scale of the ordinate between A and B. The data are the mean ± S.E. from at least three separate experiments. *, p < 0.05; and **, p < 0.0001 compared with basal (no bicarbonate) activity of same enzyme preparation.

**A**

- HCO₃⁻
- + 50 mM HCO₃⁻
- 5 mM MgCl₂

**B**

- HCO₃⁻
- + 50 mM HCO₃⁻
- 5 mM MnCl₂

Enzyme preparations

Mock C1C2-rsAC FL-rsAC Cauda sperm

Enzyme preparations

C. Ethanol in the supernatants was evaporated using vacuum centrifugation at room temperature, and the pellets were reconstituted with phosphate-buffered saline, pH 7.4, then cAMP accumulation was measured by RIA (32) after acetylation of the samples and the appropriate dilutions.

**Generation and Purification of sAC Antibody**—Two rabbit polyclonal antisera were used. One antiserum was against a synthetic peptide of 22 amino acids with the sequence NH₂-CIKYKDRPTNLQRKVTLLDDK-COOH. This sequence corresponds to amino acids 571–592 of rsAC, a region located after the C1C2 catalytic domain. A second antisera was raised against a fusion protein of 210 amino acids of the carboxyl terminus end of rsAC (amino acids 1399–1608) fused to glutathione S-transferase, purified with the peptide or the GST-rsAC fusion protein immobilized on an activated CH-Sepharose 4B column. Selectivity for the full-length sAC and specificity of the antisera were confirmed by Western blotting and enzyme-linked immunosorbent assay (data not shown).

**Immunoprecipitation**—Recombinant and native sAC from different cells or tissue extract was immunoprecipitated using the sAC antibody (peptide or carboxyl terminus) or V5 antibody (Invitrogen) immobilized on protein A-Sepharose beads. Extracts were incubated either with preimmune serum (sAC and V5 antibodies) or preimmune to protein A-Sepharose beads overnight at 4 °C by gentle mixing. At the end of incubation, the samples were centrifuged at 1000 × g for 3 min. The supernatant containing the nonsorbed sAC were removed and saved for an AC assay. The pellets were washed three times with TBS (20 mM Tris-HCl and 14 mM NaCl, pH 7.6), pellets were resuspended in a similar volume of TBS, and an aliquot was used for the AC assay.

**Western Blotting**—Cytosolic extracts and immunoprecipitated samples were diluted in 1× sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 0.7 M β-mercaptoethanol, 0.0025% (w/v) bromphenol blue) for Western blot analysis.

**RESULTS**

**RT-PCR Amplification, Cloning, and Sequencing of sAC Transcript from Rodent Testis**—PCR amplification was performed on first-strand cDNA synthesized from adult rat testis total RNA using primers specific for the 5' and 3' region of the rat sAC sequence (20). First-strand cDNAs were amplified using two different polymerases (Taq plus precision and Takara Ex Taq) on two RNA preparations from different rats. This strategy yielded PCR products of the expected size. These fragments were subcloned in pCDA3.1/V5-His/TOPO mammalian expression vector. Sequencing of five different sAC clones uncovered a deletion of 56 nucleotides in two out of three clones amplified by the Taq plus precision polymerase enzyme, and one out of two clones amplified by the Takara Ex Taq polymerase enzyme. It should be noted that a similar clone containing this deletion had also been retrieved by screening of a testis cDNA library (20). This preliminary observation indi...
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Figure 6. Characterization of the anti-sAC antibody. A, schematic representation showing the coding region of sAC, the C1C2 domain, the V5 and His tag, and the location of epitope recognized by the sAC antibodies. B, immunoprecipitation of FL-rsAC using anti-sAC (peptide) and anti-V5 antibodies. The cytosolic fraction of HEK293 cells transfected with FL-rsAC was immunoprecipitated with the anti-sAC antibody or the anti-V5 antibody. As a control for specificity, immunoprecipitation was performed with preimmune serum or IgG, respectively. The immunoprecipitated samples were fractionated by SDS-PAGE, transferred to Immobilon membrane, and probed with anti-V5 antibody (when immunoprecipitation was done with anti-sAC antibody) or with anti-sAC peptide antibody (when immunoprecipitation was done with anti-V5 antibody). C, immunoprecipitation of the recombinant FL-rsAC activity with increasing concentrations of carboxyl-terminal sAC antibody. The immunoprecipitation conditions are detailed under “Experimental Procedures.” Input activity in the immunoprecipitation was 5 pmol/min FL-sAC activity.

cated that a transcript containing an alternated spliced exon may be expressed in the testis and that the cDNA with the deletion previously reported may not be a cloning artifact.

Northern Blot and RT-PCR Analysis of sAC Transcript in Rat and Mouse and during Testis Differentiation—A Northern blot analysis of mouse and rat total testis RNA demonstrated the presence of a major mRNA species of ~5.3 kb (Fig. 1A). In rat, this transcript was present in 31- and 61-day-old rats but undetectable in 11- and 21-day-old rats (Fig. 1B), a finding similar to that previously reported for mouse (33). The presence of an equal amount of RNA in the blot was confirmed by the comparable intensity of the β-actin band (Fig. 1B, bottom). Hybridization with a 3.5-kb cDNA probe corresponding to the 3′ end of rsAC (3′ to the C1C2 domain) yielded identical results (data not shown). Although a broad mRNA band was sometimes obtained (see Fig. 1A), the presence of two distinct transcripts could not be ascertained by this method.

To determine further whether transcripts with the 56-bp deletion were expressed at any significant level during spermatogenesis, a PCR strategy was used. Primers flanking the region deleted in some of the clones (Fig. 1C, rsAC-3f and rsAC-3r) were used for RT-PCR of RNA from testes of different ages. This amplification yielded two PCR products of 445 and 389 bp (Fig. 1D) with mobility identical to that of fragments generated when cDNAs with or without the deletion were used as a template (Fig. 1D). Both PCR products were absent at 11 days of age, appeared between 20 and 30 days of age, and were retained in the adult testis (Fig. 1D). The ratio between the two amplified bands during testis differentiation was estimated by densitometry as 4:1, 7:1, and 8:1, with the transcript for the full length being more abundant. Identical results were obtained with both rat and mouse testis RNA, with different reverse transcriptase, and with first-strand generated either by oligo(dT) priming (data not shown) or with gene-specific primer rsAC-2r (Fig. 1E). Controls omitted reverse transcriptase or cDNA did not yield PCR products (Fig. 1D, 1st lane), whereas PCR products of the correct size of 445 bp were present when FL-rsAC was used as a template (Fig. 1D, 6th lane, and E, 4th lane) and 389 bp when T-rsAC was used as a template (Fig. 1D, 7th lane, and E, 3rd lane). These data ruled out the possibility that the amplified fragments arise from residual genomic DNA or a contaminating cDNA. Similar results were obtained with a second set of primers (rsAC-4f and rsAC-2r) different from the one used in Fig. 1C in both rat and mouse (data not shown). Taken together, these findings demonstrate that two transcripts with a deletion are not a peculiarity of rat but are also present with a similar ratio in mouse.

To confirm that the PCR amplification indeed represented the amplified FL-sAC and T-sAC cDNA, products were gel-purified, excised, and sequenced. Sequence analysis of both the PCR products showed long and short fragments contain identical sAC sequences except for the deletion of 56 nucleotides in the shorter clone (Fig. 2). This deletion had identical boundaries in PCR products derived from rat and mouse RNA (Fig. 2).

Comparison of the deleted sequence with the human genomic sequence available through GenBank™ (PAC 295C6 on chromosome 1q24) indicates that the second transcript (T-sAC) lacks exon 11 causing a frameshift that introduces two in-frame serine (threonine in the mouse) and cysteine codons followed by a stop codon, thus producing a premature termination of the open reading frame after the catalytic domain (Fig. 2). It should be noted that the splicing rule for acceptor and donor sites is conserved in the T-sAC of both species (i.e. the deletion is followed by standard donors site, “GT,” Fig. 2).

Identification and Quantitation of Splice Variants Using RNase Protection Assay—To verify the presence of the sAC transcripts by an independent method, and to better quantify the relative abundance of FL-sAC mRNA to T-sAC in rat testis, we used a more sensitive and specific RNase protection strategy for simultaneously detecting FL-sAC and T-sAC mRNA splice variants in testis.

As shown in Fig. 3A, the region of the RNA probe complementary to the FL-sAC sequence, if completely protected by endogenous FL-sAC transcript, would yield a protected RNA fragment of 463 nucleotides in length. Conversely, T-sAC mRNA would not completely protect the RNA probe yielding two products of 344 and 63 nucleotides in length. Total RNA
from rat testis of different ages was thus used for RNase protection with this probe. As shown in Fig. 3B, RNA from 11-day-old testis yielded two fragments of the RNA probe with the expected sizes of 344 and 63 bases (Fig. 3B, 3rd and 5th lanes). In agreement with the RT-PCR data, RPA performed with RNA isolated from rat testis of different ages showed the appearance of protected fragments corresponding to both FL-sAC and T-sAC transcripts at 21 days of age and thereafter (data not shown). Densitometric quantification of the signal corresponding to the 463 FL-sAC and the 344-base T-sAC-specific RNase-protected bands demonstrated a ratio of 4:1, a figure in good agreement with the RT-PCR data. Thus, the RNase protection data confirm the expression of two sAC transcripts in the testis.

**Properties of the Proteins Encoded in the Two sAC Transcripts**—To determine the properties of the proteins encoded by the two transcripts, the two cDNAs were inserted into a mammalian expression vector and expressed in HEK293 cells. Cytosolic fractions (100,000 g supernatant) of HEK293 cells transfected with an empty vector showed minimal AC activity in the presence of 5 mM MnCl$_2$ (1.1 ± 0.8 pmol/min/mg protein, n = 8), whereas those transfected with FL-sAC and T-sAC demonstrated a significant increase in AC activity (FL-sAC 74.6 ± 12.9 pmol/min/mg protein, n = 8; T-sAC 10.8 ± 2.6 pmol/min/mg protein, n = 5) with the T-sAC ~150 times more active than the FL-sAC. To determine whether this difference in recovered activity is due to differences in the level of expression of the two proteins, tagged constructs corresponding to FL-sAC and T-sAC minus the two terminal amino acids (C1C2-rsAC) were expressed in HEK293 cells. In good agreement with the 48 and 187 kDa calculated from the sequence, immunoreactive bands of 46 ± 1 and 171 ± 12 kDa (Fig. 4A, 1st and 3rd lanes) were detected by the anti-V5 antibody. On the other hand, no signal was detected when cells were transfected with empty plasmid (Fig. 4A, mock, 2nd lane). These constructs yielded activities similar to those described above for the untagged constructs (Fig. 4B). Because the expression of FL-sAC is ~1/12th that of C1C2-rsAC, it was estimated that the recombinant C1C2-rsAC is 20–25 or 10 times more active than the recombinant FL-sAC, when assayed with MnCl$_2$ or MgCl$_2$, respectively.

Both C1C2-rsAC and FL-rsAC were active when Mg$^{2+}$ or Mn$^{2+}$ was used as cation in the assay (Fig. 5). Addition of bicarbonate (50 mM) in the presence of Mg$^{2+}$ produced a variable (5–20-fold) activation of FL-rsAC and C1C2-rsAC (Fig. 5A), whereas only a 3-fold activation of both FL-rsAC and C1C2-rsAC was observed in the presence of Mn$^{2+}$ (Fig. 5B). Bicarbonate also stimulated both Mg$^{2+}$ (~2-fold) and Mn$^{2+}$-dependent (~3-fold) sAC activities derived from mature cauda sperm (Fig. 5).

**Western Blot Analysis of the Expression of Recombinant and Native sAC in Rat Testis**—In order to distinguish between the activity of the native FL-sAC and T-sAC, an antipeptide antibody against a carboxyl-terminal epitope (carboxyl-terminal to the C1C2 domain) of sAC (Fig. 6A) was generated. Cytosolic fractions of HEK293 cells transfected with FL-rsAC were immunoprecipitated either with the anti-sAC antibody or an anti-V5 antibody, and Western blot analysis was performed with both antibodies on the immunoprecipitated fractions. Both antibodies identified an immunoreactive band of 171 kDa (Fig. 6B). In contrast, no band was detected when immunoprecipitation was done with preimmune serum (as negative control for the sAC antibody) or IgG (as negative control for the V5 anti-
immunoprecipitated with both anti-sAC and anti-V5 antibodies, whereas little activity was recovered after immunoprecipitation with preimmune serum or IgG (data not shown). Bicarbonate ions stimulated (approximately 2-fold) the immunoprecipitated sAC activity (data not shown) indicating that the immunoprecipitated activity corresponds to that of the sAC molecule. The T-sAC activity could not be immunoprecipitated by the sAC antibody (data not shown). A second antibody specific for the FL-sAC was generated against the carboxyl terminus of the protein. Similar to the peptide antibody, this second antibody quantitatively immunoprecipitated the recombinant AC activity in a concentration-dependent manner (Fig. 6C).

When soluble fractions from total germ cell preparations or spermatozoa were immunoprecipitated with the peptide sAC antibody, between 25 and 30% of the input AC activity was immunoprecipitated (Fig. 7A). Moreover, specific immunoprecipitation of sAC activity was observed with extracts from 31- and 61-day-old testis, whereas no cyclase activity was immunoprecipitated from the immature testis (Fig. 7B). Western blot analysis performed with the sAC antibody on the immunoprecipitated fractions confirmed the presence of the immunoreactive band of 171 kDa only in extracts from the more mature testes. The specificity of this band was confirmed by the absence of a band with the fractions immunoprecipitated with preimmune serum (Fig. 7C). Bicarbonate stimulated this immunoprecipitated activity confirming the identity of FL-sAC (Table II).

Size-exclusion Gel Chromatography of the Endogenous sAC Demonstrates the Presence of Two Forms—To confirm that both FL-sAC and T-sAC transcripts are translated into active protein, cytosolic fractions (100,000 × g supernatant) of 60-day-old rat testis were fractionated on size-exclusion Sephacryl S-200 gel chromatography. Measurement of adenylyl cyclase activity of eluted fractions identifies two distinct peaks of activity, one peak eluting close to the void volume (V_o) and the other before the ovalbumin marker (43 kDa) (Fig. 8). The elution of the peak in the void volume is identical to that of a recombinant FL-sAC (Fig. 8C), whereas the elution of the second peak coincided with the elution of the recombinant T-sAC (Fig. 8B). The activity of the high molecular weight peak was 20–40% of that of the second peak, in good agreement with the immunoprecipitation data (see Fig. 7A). More importantly, both the antipeptide and carboxyl-terminal sAC antibodies, which recognize only FL-sAC but not T-sAC proteins, immunoprecipitated the first but not the second peak of activity (Fig. 8A, inset). The fractionation and immunoprecipitation studies, therefore, confirm that two sAC species are expressed in the testis and that they correspond to FL-sAC and T-sAC on the basis of their size and immunoreactivity.

sAC Is Active in Intact Developing Germ Cells—The effect of bicarbonate on the activation of sAC was further tested by monitoring cAMP accumulation in intact HEK293 cells transiently expressing FL-rsAC and T-rsAC cDNAs. As shown in Fig. 9A, addition of sodium bicarbonate to the medium of these transfected cells results in nearly a 2-fold increase in the cAMP levels, whereas HEK293 cells transfected with the empty vector (mock) did not respond to bicarbonate.

**Fig. 8.** Identification of FL and T-sAC protein expression in testis using FPLC size-exclusion chromatography. A, cytosolic fraction (2 ml) of testis (60-day-old rat) was fractionated by FPLC-Sephacryl S-200 column as described under “Experimental Procedures.” An aliquot of each fraction was assayed for AC activity in presence of 5 mM MnCl₂. The data reported are the total amount of AC activity (pmol/min) recovered in each eluted fraction. Arrows indicate the elution of the following markers: 232 kDa, catalase; 158 kDa, aldolase; 43 kDa, ovalbumin; and 25 kDa, chymotrypsin. V_o was determined by the elution of blue dextran (2000 kDa). Inset, immunoprecipitation of AC activity in the fractions of the first peak (Fraction 46) and second peak (Fraction 79) of AC activity. Immunoprecipitation of the indicated fractions were performed as described under “Experimental Procedures” with an anti-sAC peptide antibody (P-AB) and carboxyl-terminal sAC antibody (C-AB), both specific for FL-sAC. Input activity in the immunoprecipitation was 5 pmol/min AC activity for both peak fractions. The data are representative of three different experiments performed. B, elution pattern of recombinant T-rsAC activity; C, elution pattern of FL-rsAC activity on the same FPLC column.
In order to determine the effect of bicarbonate on cAMP accumulation in germ cells, total germ cell preparations were incubated with different concentrations of sodium bicarbonate for 1 h in the presence or absence of rolipram (a PDE4 inhibitor), and intracellular cAMP levels were measured. Bicarbonate caused an increase in cAMP levels in germ cells in a concentration-dependent manner (Fig. 9B). Inhibition of germ cell phosphodiesterase with 50 μM rolipram further amplified the bicarbonate stimulation (EC50 NaHCO3 = 2.5 mM) (Fig. 9B). This effect of bicarbonate on cAMP accumulation was observed as early as in 25-day-old germ cells (Fig. 9C), confirming the presence of an active sAC in round spermatids and possibly pachytene spermatocytes. The bicarbonate concentration that was used (50 mM) corresponds to the level of bicarbonate found in testicular and seminal fluids of mammals (34–36).

**DISCUSSION**

Our study provides evidence that two distinct transcripts encoding full-length and truncated sAC, as well as the corresponding proteins, are present in mouse and rat germ cells. When expressed in a heterologous system, these proteins have distinct properties. In addition, we demonstrate that the full-length sAC is active and contributes to the control of cAMP levels in developing germ cells and in mature spermatozoa. Given the high concentration of bicarbonate ions in the seminiferous tubule fluid, we propose that this cation controls intracellular cAMP concentration not only in mature spermatozoa but also in differentiating spermatids.

The characterization of the properties of the soluble testis adenylyl cyclase indicated that this activity resides in a polypeptide of 46–70 kDa in rat testis (21, 26), 46 kDa in ram sperm (37), and 48–50 kDa in human testis (22, 38). With the cloning of the sAC cDNA and the realization that the protein coded by this cDNA is much larger (187 kDa), it has been proposed that posttranslational proteolytic processing generates the lower molecular weight form (20, 39). Indeed, Stengel et al. (37) have shown that chymotrypsin treatment of ram spermatozoon membrane yields a soluble cyclase with a molecular mass of 46 kDa, even though some additional physicochemical properties of this proteolytic product were not identical to those of the testis-derived form. Additional attempts to generate a proteolytically cleaved soluble cyclase that is activated by bicarbonate have generated conflicting results (40).

Our data provide evidence that an additional mechanism may generate the low molecular weight sAC. Because a cDNA clone coding for sAC with an exon deletion had been isolated during the screening of a rat testis cDNA library (20), by using an independent approach with rat and mouse testis RNA, we have demonstrated that this deletion is retrieved in a significant number of clones (3 out of 5 sequenced clones). The high frequency with which these clones were retrieved suggests that it is not a fortuitous splicing event or a cloning artifact. PCR and RNase protection analyses of RNA from testes of mouse and rat at different stages of development confirmed that transcripts containing the deletion account for ~10–25% of the total sAC mRNA. Assuming that these transcripts are translated at comparable rates, it can be predicted that 75–90% of the sAC protein is full length whereas 10–25% is the truncated
variant. Given the finding that T-sAC is ~10 times more active than FL-sAC, one would predict that 70% of the adenyl cyclase activity in the testis cytosol is due to T-sAC, whereas 30% is attributable to FL-sAC. This estimate on the relative abundance of the two forms is consistent with the data on both the immunoprecipitated sAC activity from germ cells and the gel filtration-eluted fractions of testis. By using an antibody that recognizes only the full-length form, we show that FL-sAC is not expressed in the immature testis devoid of meiotic germ cells, and its expression is detected when spermatogenesis progresses to the round spermatid stage. Thus, our data provide evidence for an alternative mechanism for the genesis of full-length and truncated sAC. Without excluding the possibility that proteolysis also generates the short form as suggested by others (20), we propose that this splicing event accounts for the presence of the two sAC proteins.

Several lines of evidence demonstrate that the FL-sAC is active in germ cells. Significant cyclase activity was recovered after expression of the full-length clone or after immunoprecipitation with an antibody that recognizes only FL-sAC. Both endogenous immunoprecipitated FL-sAC and recombinant FL-sAC are stimulated by bicarbonate, confirming the identity of FL-sAC. More importantly, gel filtration chromatography of testis-soluble extracts provides evidence for the presence of an active sAC form (FL-sAC) with a molecular mass higher than 150 kDa. It should be noted that this finding of the presence of two distinct active peaks is consistent with several reports published earlier (21, 26, 37). Finally, cAMP levels measured in intact cells were significantly increased when the FL-sAC was transfected in HEK293 cells.

The recombinant T-sAC eluted as a single symmetrical peak with an approximate mass of 49 kDa with little activity recovered in the void volume. This finding, together with the distinct immunological properties of the two peaks, rules out the possibility that the peak in the void volume is generated by aggregation of T-sAC. The gel filtration data suggest that, unlike T-sAC that behaves as a monomer, the FL-sAC exists as an oligomer of either two identical subunits (187 kDa) or as a complex with an unknown protein. This finding again points to the differences in properties between the FL-sAC and the T-sAC. It should be noted that we cannot formally exclude the possibility that FL-sAC and T-sAC exist as heterodimers and that immunoprecipitation with an antibody that recognizes only the FL-sAC also precipitates the more active T-sAC, nor that the full-length sAC is rapidly proteolyzed and activated during the AC assay incubation. Although the presence of a heterodimer FL-sAC/T-sAC is inconsistent with the gel filtration data, this possibility warrants further study.

Because our data provide evidence that both sAC molecules are active in germ cells, the possibility needs to be entertained that FL-sAC has functions other than serving as an inactive precursor for T-sAC generation. In addition to bicarbonate, which apparently interacts directly with the catalytic domain, we propose that FL-sAC may be regulated by other signals. Thus, it is possible that the large carboxyl-terminal end of FL-sAC contains inhibitory domains for catalysis and domains involved in the reception of regulatory signals. These signals activate the full-length but not the truncated protein by promoting a change in conformation of the enzyme. The large increase in activity following deletion of the carboxyl terminus including the putative P site is consistent with this view.

When epididymal spermatozoa were used as a source of sAC, about 30% of the soluble activity could be immunoprecipitated, indicating that the full-length sAC is retained during the terminal differentiation of spermatids and recovered in spermatozoa, a finding consistent with the Western blot analysis reported by others (25). This observation reinforces the conclusion that both full-length and truncated sAC play a crucial role in the activation and capacitation of ejaculated spermatozoa.

All the data thus far generated demonstrate that sAC gene expression is switched on during meiosis and that two active sAC proteins are present at least in round spermatids and possibly pachytene spermatocytes. This finding strongly suggests that sAC functions are not limited to the control of cAMP levels in mature spermatozoa but that sAC is active in generating cAMP earlier during germ cell differentiation. This conclusion is further supported by the data showing that bicarbonate anion stimulates cAMP levels in germ cells derived from 25- and 50-day-old rat testis, at a stage of development when no or few mature spermatozoa are present.

It has long been recognized that seminiferous tubule fluid has a composition distinct from that of the interstitial fluid, potassium being present at considerably higher levels than in plasma. In the seminiferous tubule fluid, bicarbonate reaches concentrations between 20 and 40 mM (34, 35, 41, 42), which is 5 times higher than that in the interstitial fluid. These concentrations are sufficient to activate sAC in a cell-free system or in intact germ cells as shown for cAMP accumulation. In addition, carbonic anhydrase has been detected as mRNA, protein, or activity in the testis, epididymis, and in spermatozoa (43–47). Human and rat germ cells express unique carbonic anhydrase transcripts (45), and high levels of carbonic anhydrase activity have been localized in Sertoli cells of several species (43, 47). Taken together, all these findings, and our observation that sodium bicarbonate stimulates cAMP accumulation in isolated immature germ cells, strongly suggest that bicarbonate controls cAMP accumulation during spermatid differentiation. Although it is not known whether bicarbonate concentration fluctuates during the seminiferous tubule cycle, we propose that bicarbonate released in the seminiferous tubule fluid is part of an autocrine or paracrine loop to maintain intracellular cAMP concentrations permissive for spermatid differentiation.

In summary, the data described above provide evidence that the two active sAC proteins are generated through a splicing mechanism and are suggestive of FL-sAC functions distinct from those of the truncated sAC. In addition, we provide evidence that bicarbonate is a signal that regulates cAMP levels in developing spermatids. It remains to be determined whether this regulation has an impact on survival and differentiation of these cells as well as on the CREM-dependent program of gene transcription.

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