Expression of a nonmyristylated variant of the catalytic subunit of protein kinase A during male germ-cell development

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The catalytic subunits of protein kinase A are transcribed in all mouse tissues from two distinct genes that code for the Cα and Cβ isoforms. Alternative promoters exist for the Cβ gene that are used in a tissue-specific fashion and give rise to variants that differ in their amino-terminal sequences. We have characterized an alternative promoter that is present in the first intron of the Cα gene and is transcriptionally active in male germ cells. Transcription from this promoter is coincident with the appearance of pachytene spermatocytes and leads to a Cα protein (Cα2) that contains a distinctive 7 amino acid amino-terminus differing from the 14 amino acid amino-terminus of Cα1. The Cα2 protein does not contain the myristylation signal present on Cα1 and migrates at a lower molecular weight on SDS/PAGE gels. By Western blotting, we estimate that most or all of the Cα protein present in mature sperm is Cα2. The amino-terminal sequence of Cα2 is similar to that of ovine sperm C as previously reported [San Agustin, J. T., Leszyk, J. D., Nuwaysir, L. M. & Witman, G. B. (1998) *J. Biol. Chem*. 273, 24874–24883], and we show by cDNA cloning that human sperm also express a highly related Cα2 homolog. The Cα2 subunit forms holoenzymes with either RIIα or RIα, and both activate at the same concentration of cyclic nucleotide. Because protein kinase A is thought to play a pivotal role in sperm motility and capacitation, the distinctive biochemical properties of the unmyristylated Cα2 may be essential for fertility in the male.

The mouse genome contains two genes coding for the catalytic (C) subunit, Cα and Cβ (1) of protein kinase A (PKA). A processed pseudogene, Cx, which is not transcribed, also has been cloned (2), and its sequence is highly similar to the sequence of the Cα cDNA, although they diverge at the amino terminus. The Cβ gene codes for at least three isoforms (Cβ1, Cβ2, and Cβ3) that arise from the use of three different first exons, each of which contains a distinct initiator methionine codon (3). Whereas Cα and Cβ1 are widely expressed, Cβ2 and Cβ3 are neural-specific isoforms. All three Cβ isoforms have similar activity in gene induction, and they all interact equally well with regulatory (R) subunits and the heat-stable PKA inhibitor, PKI (3).

The C subunit of PKA and calcineurin B were the first proteins shown to be blocked at their amino-terminal glycine by myristylation (4, 5). A number of eukaryotic proteins subsequently have been shown to be *N*-myristylated on glycine and a consensus sequence has been suggested (6, 7). Although the amino-terminal sequence of the C subunit does not fit perfectly with this consensus sequence, it is recognized by the *N*-myristyltransferase. In other proteins, the presence of myristate at the amino terminus helps target the protein to membranes and has been shown to be essential for the function of a number of kinases (8), including Src (9), c-Abl (10), and cGMP-dependent protein kinase (11). The accessibility of the myristic group for membrane binding is regulated in some proteins by a switch mechanism that can be triggered by phosphorylation, calcium binding, cleavage of the myristylated protein, or GTP binding (see refs. 8 and 12 for reviews). C subunit *N*-myristylation is conserved across species ranging from *Caenorhabditis elegans* to mammals, suggesting an essential function for this cotranslational modification. However, *N*-myristylated C isoforms coexist in an organism with nonmyristylated isoforms. For example, the mouse Cβ1 is *N*-myristylated, whereas Cβ2 and Cβ3 are not (3). In *C. elegans*, the C subunit consists of at least 12 different isoforms that derive from amino-terminal alternative-splicing events in combination with a carboxyl-terminal splicing event and generate myristylated and nonmyristylated variants (13, 14). For the mouse Cα subunit, it has been shown that the myristate group contributes to the structural and thermostability of the enzyme (15) but no evidence for myristate-dependent membrane association has been found.

Recently, San Agustin *et al*.(16) reported the purification of the C subunit from ovine sperm. They showed that the sperm C (Cs) is smaller than the somatic form, and sequence analysis revealed that the first 14 amino acids found in Cα are replaced by six different amino acids. Cs no longer contains the consensus myristylation motif and was shown to be acetylated on its amino-terminal alanine. Because the region of similarity between Cs and Cα begins at the exon 1/exon 2 boundary in Cα, San Agustin *et al*. suggested that Cs may result from the use of an alternate first exon in the Cα gene. By using a degenerate oligonucleotide primer deduced from the amino-terminal sequence of the ovine Cs, we report the cloning and characterization of a mouse Cα isoform we have called Cα2. This isoform arises from an alternative promoter and first exon for the Cα gene. We have mapped the transcriptional start site of this nonmyristylatable isoform within intron 1 of the Cα gene, and we have studied its stage-specific expression in testis. A homolog Cα2 isoform also is shown to exist in human testis.

Experimental Procedures

Characterization of Genomic Clones Containing Cα2 Sequences. The mouse Cα gene was cloned previously from an European Molecular Biology Laboratory (EMBL)3 lambda phage library with three overlapping clones (MCG-3, -5, and -1) covering 25 kb (17). A 10.6-kb *Bam*HI/*Bam*HI fragment from the 5′ end MCG-3 insert was subcloned into a pBluescript KS(+) vector (Stratagene) and designated as pB2 in Fig. 1. Restriction mapping and Southern blot analysis using a degenerate primer (5′-ATGGCIAGYAAYCCIAAYGAYGGT-3′, where Y is C or T) based on the amino-terminal sequence of the ovine Cs

Abbreviations: AKAP, A-kinase anchoring protein; Cs, sperm C; R subunit, regulatory subunit of cAMP-dependent protein kinase; PKA, protein kinase A; RACE, rapid amplification of cDNA ends; P*n*, postnatal day *n*.

Data deposition: The sequences reported in this paper have been submitted to the GenBank database (accession nos. AF224718 and AF224719).

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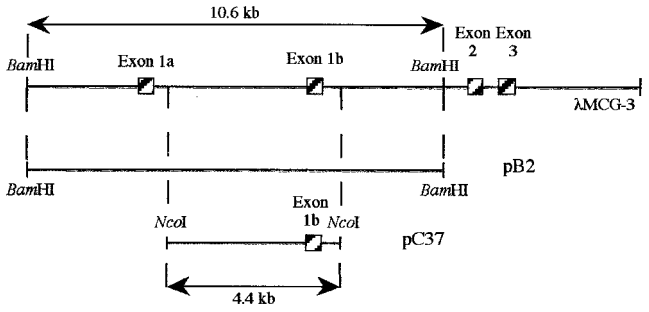

Fig. 1. Sequencing and cloning strategy of exon 1b. The first four exons of the Cα gene (exons 1a, 1b, 2, and 3) are shown and indicated by hatched boxes. Genomic fragments pB2 and pC37 of the phage λMCG-3 insert were subcloned into plasmid vectors.

[ASNPNDV (16)] identified a 4.4-kb *Nco*I/*Nco*I fragment containing a homologous sequence. The *Nco*I fragment was subcloned for further analysis and the clone was designated pC37 (Fig. 1).

Isolation of Proteins and Total RNA. Adult tissues and testis from 10-day-, 19-day-, and 42-day-old C57BL/6 mice were obtained fresh, rapidly frozen, and stored at −80°C before use. Total RNA was extracted by using guanidine hydrochloride as previously described (3). Protein was extracted either from testis or from sperm (isolated from the cauda epididymus) as previously described (18).

Reverse Transcription–PCR Amplifications. Single-stranded cDNA was generated from 0.5 μg of total RNA using the First-Strand cDNA Synthesis Kit (CLONTECH) and random hexamers. The efficiency of the cDNA synthesis was estimated by PCR using two specific primers of the glyceraldehyde-3-phosphate dehydrogenase cDNA. PCR amplification was carried out in 50-μl reaction volumes containing 5 μl of the first strand cDNA, 0.25 mM dNTPs, 15 pmol of each primer, 2 units of *Taq* polymerase (Boehringer-Mannheim), and PCR buffer (final concentration, 10 mM Tris·HCl/1.5 mM $MgCl_2$/50 mM KCl). After electrophoresis, the amplified products were either purified for subcloning into a T/A cloning vector or transferred for analysis by Southern blot using a Cα2-specific internal oligonucleotide (5′-TACTCAAAGGTCAGGACGATC-3′) as a probe.

DNA Sequence Analysis. The plasmid inserts were sequenced on an ABI Prism 377 (Perkin–Elmer), and the mouse genomic sequence and the human cDNA sequence reported in this paper have been deposited in GenBank with accession nos. AF224719 and AF224718, respectively.

Primer Extension. Primer extension was performed as described (19) with an antisense primer from exon 1b (5′-TCAGGGCACTAG-CATTACGGT-3′) end-labeled with [γ-^{32}]ATP. Total mouse RNA from testis and total RNA from yeast (Sigma) were used. Extension products were purified, precipitated, and dissolved in 4 μl of Tris/EDTA (10 mM Tris·HCl/1 mM EDTA, pH 8.0) and in 6 μl of sequencing reaction stop solution (United States Biochemical). The samples were denaturated and size-fractionated on a 6% polyacrylamide/7 M urea sequencing gel (Sequagel-6; National Diagnostics) alongside a sequencing ladder.

5′ End Amplification of cDNAs. First-strand cDNA from human testis total RNA (1 μg) obtained from CLONTECH and first-strand cDNA from mouse testis total RNA (1 μg) were synthesized with the 5′ rapid amplification of cDNA ends (RACE) kit from Boehringer Mannheim and the two 31-bp antisense oligonucleotides 5′-GTTCCCGGTCTCCTTGTGTTTCACCAG-CATC-3′ [nucleotides 254-284; accession no. NM 00273 (20)] and 5′-CTTCTGCTTGTCTAAGATCTTCATGGCGTAG-3′ [nucleotides 111–141; accession no. M19953 (17)], respectively. The cDNAs were purified and terminal transferase was used to add a homopolymer A-tail to the 3′ end of the cDNAs. Tailed cDNAs were amplified by PCR using the oligo(dT)-anchor primer and the antisense human specific primer 5′-GTGC-CGAGGGTCTTGATTCGTTCA-3′ (nucleotides 212–235) or the antisense mouse specific primer 5′-GCATCACTCGC-CCAAAGGAGC-3′ (nucleotides 62–82). A second round was performed on diluted human PCR product by using the anchor primer coupled to the specific human antisense primer 5′-TGGGCTGTGTTCTGAGCGGGA-3′ (nucleotides 179–199). The PCR products were subcloned to a T/A vector. Several clones were screened by PCR, and clones with the longest inserts were sequenced.

Northern Blots. RNA samples (20 μg) were electrophoresed on a 1% agarose/formaldehyde denaturating gel, transferred overnight to membranes (Nytran Plus; Schleicher & Schuell, Keene, NH), and hybridized with specific probes (3). The probes used were the 500-bp *Eco*RI Cα cDNA [MC8 cDNA clone (1)], a 250-bp fragment obtained by amplification of pC37 and encompassing exon 1b using the primers Cα29 (5′-CCAA-GAGGGTCTTCCCAGGC-3′) and Cα30 (5′-CATCGTTG-GAGCTGGAAGCC-3′), the 2-kb *Eco*RI Tenr cDNA (kindly provided by R. E. Braun, University of Washington; ref. 21), and the 0.8-kb *Sma*I/*Hin*dIII fragment of RIIα cDNA.

Western Blots. Proteins (5 μg per lane) were separated by 10% SDS/PAGE gels and transferred onto nitrocellulose (Schleicher & Schuell). Blots were blocked overnight and were probed with a polyclonal antiserum against the murine Cα at 1:10,000 (provided by S. S. Taylor, University of California, San Diego) as previously described (18). Proteins were visualized by using an enhanced chemiluminescence kit (Amersham Pharmacia).

Kinase Activity Assay. Protein homogenates from cauda epididymal sperm were prepared in a homogenization buffer [250 mM sucrose/100 mM sodium phosphate, pH 7.0/150 mM NaCl/1 mM EDTA/4 mM EGTA/4 mM DTT/1.0% Triton X-100/2 μg/ml leupeptin/3 μg/ml aprotinin/0.2 mg/ml soybean trypsin inhibitor/1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride] from adult wild-type and RIIα knockout mice (18). Protein kinase activity was assayed with Kemptide (Sigma) as a substrate and with [γ-^{32}P]ATP, as described previously (22). Cyclic IMP was added at concentrations of 10 nM to 100 μM.

Results

Genomic Mapping and Cloning of a New Alternatively Spliced Cα Isoform. Recent work on the C subunit of PKA that is expressed in ovine sperm suggested that this subunit might be transcribed from an alternate 5′ exonic region in the Cα gene (16). We designed a 24-bp degenerate primer based on the ovine Cs and used it to map a putative new exonic region within a 4.4-kb *Nco*I/*Nco*I insert cloned from the first intron of the mouse Cα gene (Fig. 1). Sequencing revealed a 24-bp sequence (underlined on Fig. 2*A*) that is 83% similar to the sequence of the 16-fold degenerate primer and contains an ATG codon within a strong consensus sequence for translation initiation (23). This 5′ exonic region (exon 1b) encodes a variant of mouse Cα that is not related to the 5′ sequence of the mouse Cα gene or the human Cγ retrogene (24). To determine whether the putative exonic region is a coding region and whether this new putative exon is spliced to exon 2, we performed PCRs on mouse cDNA from brain and testis using a 5′ exon 1b-specific primer and a 3′ primer located within the last exon of Cα. The expected fragment of 1.1

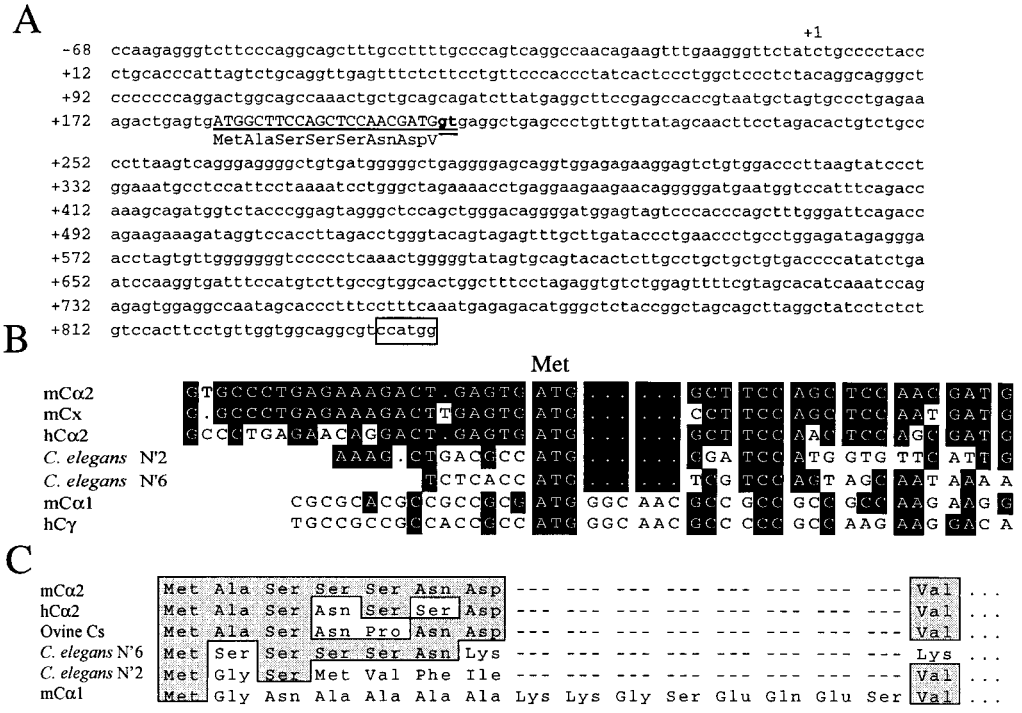

Fig. 2. Nucleotide and amino acid sequences of the 5′ region encompassing exon 1b. (*A*) Sequence surrounding the Cα1b exon. Numbering is relative to the transcriptional start site (+1). The *Nco*I restriction site is boxed. The sequence matching the degenerate primer Cα12 is underlined. The splice donor site is in bold and double underlined. (*B*) Comparison of the 3′ end of the mouse exon 1b (mCα2) and human exon 1b (hCα2), a part of the 5′ end region of mouse pseudogene Cx (mCx), a part of the 5′ end of the human Cγ retrogene (hCγ), the 3′ end of exon 1a of the Cα gene (mCα1), and 5′ sequences of *C. elegans* exons N′2 and N′6 (see *Results*). Dots indicate gaps introduced in the sequence for alignment purposes. The position of the initiating methionine (Met) is indicated. (*C*) Comparison of the deduced peptide sequence of mouse and human exon 1b, the deduced amino acid sequences of N′2 and N′6 (*C. elegans*), and the amino-terminal sequences of Cs and the mouse Cα1. Conserved amino acids are boxed.

kb in length was amplified only in testis, purified, and cloned into a T/A vector. The sequence of this PCR fragment demonstrates that exon 1b is spliced to exon 2, giving rise to another isoform we termed Cα2. The previously described Cα cDNA will be referred to as the Cα1 isoform. The two cDNA isoforms are identical downstream of their first exon. The ORF of exon 1b codes for the heptapeptide MASSSND, which does not contain the essential amino-terminal glycine for *N*-myristylation. The ORF upstream of the ATG has termination codons in all three frames, showing that exon 1b contains the Met initiation codon of Cα2.

It has been shown recently that the gene coding for the C subunit of PKA in *C. elegans* gives rise to at least six transcripts with six different first exons, resulting in a multiplicity of nonmyristylatable isoforms (14). Two of these isoforms, termed N′2 and N′6, show similarities at the nucleotide level (Fig. 2*B*) or at the peptide level (Fig. 2*C*) with the mouse Cα2 isoform.

We previously cloned a mouse pseudogene (Cx) related to Cα and located on the X chromosome (2). This processed retropseudogene is closely related to Cα except for the 5′ end, which diverges in sequence and, as shown in Fig. 2*B*, is highly similar to Cα2. We conclude that Cx is a processed retroposon derived from a Cα2 transcript that was inserted on the X chromosome.

Mapping of the Transcriptional Start Site of Cα2. Primer extension analysis with an antisense primer located at the 3′ end of exon 1b results in one major extension product of 203 bp (Fig. 3*A*), identifying the transcription start site of exon 1b as shown in Fig. 2*A* as "+1." We carried out a RACE-PCR experiment using total RNA from mouse testis, and among the clones we sequenced, the longest begins at +1 in accordance with the primer extension analysis. Furthermore, a series of reverse transcription-PCRs using primers both 3′ and 5′ of the putative transcription start site on mouse testis RNA gave results consistent with the start site for exon 1b.

Expression of Cα2 in Mouse Tissues. To determine the tissue distribution of Cα2, a Northern blot containing total RNA from brain, heart, kidney, and testis was hybridized with either the 250-bp Cα2-specific probe or a Cα probe (which recognized both Cα1 and Cα2). An abundant transcript of 2.4 kb was detected only in testis with the Cα2 probe (Fig. 3*B*). The Cα probe shows a strong band in the four tissues tested (Fig. 3*C*). We next investigated the expression of Cα2 in other tissues by using a more sensitive method. We performed reverse transcription–PCR using total RNA from various tissues and analyzed the PCR product by Southern blotting using an internal primer as a probe (Fig. 3*D*). The Cα2 isoform was detected by ethidium bromide staining and Southern blotting in testis only. In each case, the quality of the cDNA was verified by amplification of the glyceraldehyde-3-phosphate dehydrogenase cDNA (data not shown). The results of both Northern blotting and PCR analysis indicate that the Cα2 isoform is testis-specific.

Conservation of Cα2 in Human. 5′ RACE reactions were carried out to confirm the 5′ end of the mouse Cα2 and to try to identify its putative human homolog. The longest mouse cDNA is 336 bp in length and its sequence confirms our primer extension result, although a single g→c nucleotide substitution was found in the RACE cDNA. This substitution is likely to be an error of the reverse transcriptase or the *Taq* polymerase. By using the 5′ RACE protocol, we cloned a 120-bp human cDNA that has a

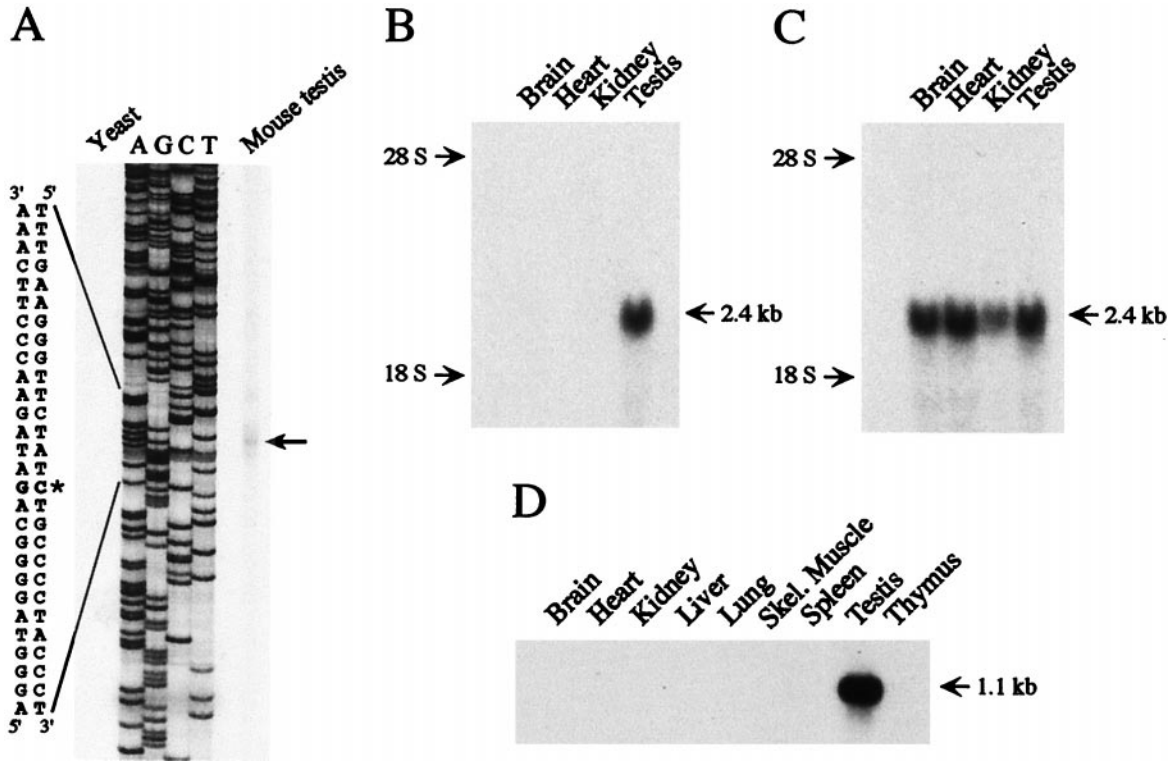

Fig. 3. Identification of the Cα2 transcription start site and expression of Cα isoforms. (*A*) Primer extension products obtained from yeast (control) and mouse testis RNA were electrophoresed next to sequencing reactions with the same primer and pC37 as template. Total RNA (20 μg) was isolated from adult tissues, electrophoresed under denaturating conditions, and transferred to nylon membrane. (*B* and *C*) The membrane was hybridized to ^{32}P-labeled specific Cα2 probe (*B*) and MC8 *Eco*RI insert (Cα probe) (*C*). The positions of 18S and 28S RNA are shown to the left of the blots, and the sizes of the transcripts are shown to the right. (*D*) Reverse transcription–PCR also was performed on total RNA from various tissues and the products then were analyzed by Southern blotting using an internal primer as a probe. The amplified product is 1.1 kb in length.

73-bp 3′ region identical to the human Cα cDNA (nucleotides 127–199). The remaining 5′ end sequence (126 bp) of our human cDNA clone contains a new sequence of 47 bp that is not identical to any nucleotide sequence in the EMBL/GenBank. Its 3′ end sequence is highly similar to the 3′ end sequence of the mouse exon 1b, showing that the exon1/exon2 junction is conserved between mouse and human and that a splice variant similar to the mouse Cα2 exists in human testis. The human cDNA we characterized has a methionine codon in frame with the previously published human Cα cDNA. This frame is closed 6 bp upstream of the new ATG codon (Fig. 2*B*) and codes for an amino-terminal heptapeptide (MASNSSD) that is missing the essential glycine for *N*-myristylation and is similar to the N terminus of mouse Cα2 and ovine Cs (Fig. 2*C*). We conclude that human testis also expresses an mRNA that codes for a Cα2-related protein. It is likely that the human Cα2 also is testis-specific, but the small Cα2-specific (47 bp) fragment we have identified was not sensitive enough as a probe to detect transcripts on a Northern blot of human tissues.

Developmental Expression of Cα2 mRNA in Testis. Adult testis contains germ cells in many different stages of development. Spermatogenesis occurs in three successive phases: mitotic, meiotic, and postmeiotic, which occur in mouse for $\approx$10, $\approx$11, and $\approx$14 days, respectively (25). To determine which cell types express Cα2 mRNA in testis, we took advantage of the fact that the first wave of germ-cell development occurs synchronously and begins at approximately postnatal day 11 (P11). Northern blotting was performed on testis isolated at P10, P19, and P42. Cα2 mRNA expression was compared with Tenr, RIIα, and a probe that recognizes both Cα1 and Cα2 (Fig. 4). Tenr is a male germ-cell-specific transcript expressed from midpachytene (meiotic phase) through midround spermatid stage (21). RIIα is processed into a 6-kb mRNA in somatic cells, but in testis, the intensity of this 6-kb band decreases with time and a 2.2-kb germ-cell-specific transcript begins to appear at the round spermatid stage (26). In the experiment shown in Fig. 4, the 2.2-kb RIIα mRNA is not transcribed until P42. Cα2 and Tenr cDNA probes show a similar developmental pattern of expression. No band is visible at day 10 but both mRNAs, which are 2.4 kb in length, are detected at P19. At P42, a dramatic increase in the levels of both Tenr and Cα2 mRNA is observed because of the proliferation of germ cells. The Cα cDNA probe (Cα1 and Cα2) revealed a 2.4-kb band present in all RNA samples. These data demonstrate that the Cα2 transcript is germ-cell-specific and that it appears coincident with pachytene stage spermatocytes.

Expression of Cα2 Protein. To provide evidence that the Cα2 mRNA is translated into protein, we performed Western blot analysis. As shown in Fig. 5, the polyclonal C subunit antibody detects a band of 40 kDa in protein extracts from the adult heart, brain, and testes from mice (10-, 19-, and 42-day-old). This 40-kDa protein corresponds to the somatic Cα1 subunit. A second protein with a molecular mass of 39 kDa is revealed only in testis at P42 (Fig. 5) and correlates with the expression of Cα2 mRNA at P42. The same antiserum used against a protein extract from sperm released from the cauda epididymus recognized only the 39-kDa band (Fig. 5), suggesting that the lower band detected in testis comes from male germ cells and that Cα2 is the only isoform of Cα present in fully mature sperm.

Activation of PKA Holoenzyme Containing Cα2. PKA holoenzyme containing the Cα1 subunit has increased sensitivity to cAMP

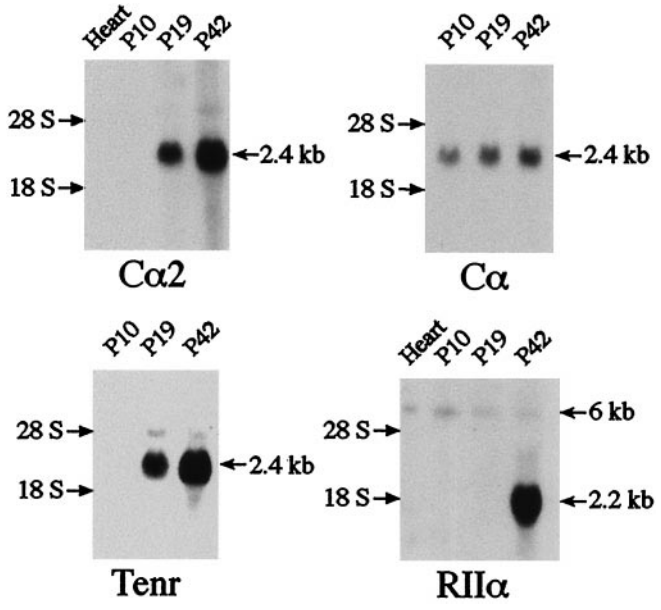

Fig. 4. Developmental expression of Cα2 in comparison to Cα, RIIα, and Tenr. Northern blots were loaded with 20 μg of total RNA from mouse heart and testis harvested at 10, 19, or 42 days after birth. Blots were hybridized with a specific Cα2 probe, a Cα probe that recognizes both Cα1 and Cα2 transcripts, a Tenr probe, and an RIIα probe.

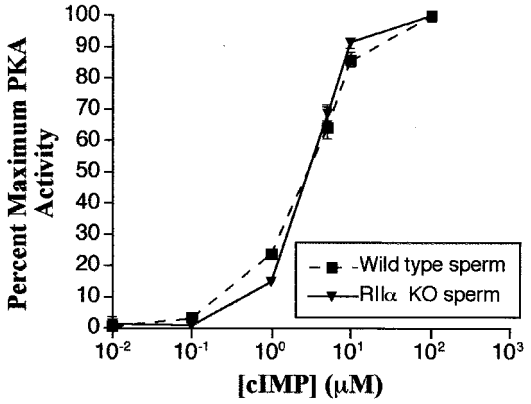

Fig. 6. Activation of PKA holoenzyme containing Cα2. A cIMP activation curve for the PKA holoenzyme containing Cα2 and RIIα (as found in wild-type mouse sperm) is compared with that obtained for a Cα2/RIα holoenzyme [as found in sperm from mice with a targeted disruption of the RIIα gene (RIIα KO)]. Results are the average of triplicate data points. Error bars for SD are shown.

when bound by RIα versus RIIβ (27). To determine possible differences in the activation of type I versus type II PKA holoenzyme containing the Cα2 subunit, a dose–response curve was generated by assaying the kinase activity in sperm samples that express either RIα or RIIα with concentrations of cIMP between 10 nM and 100 μM (Fig. 6). Cyclic IMP was used instead of cAMP because it has a 15-fold lower affinity for R subunits and, therefore, the amount of free cyclic nucleotide is not affected by the concentration of holoenzyme in the assay. Type II PKA holoenzyme containing Cα2 and RIIα was prepared from wild-type mouse sperm, a cell that predominantly expresses type II PKA (28), and type I PKA holoenzyme containing Cα2 and RIα was prepared from RIIα-deficient mouse sperm, a cell that only expresses type I PKA (18). No differences were observed in kinase activation by cIMP between the type I and type II PKAs containing the Cα2 subunit.

Discussion

There are numerous examples in which genes are differentially transcribed or spliced to yield multiple protein products with increased functional diversity. The genes coding for the C subunits of PKA seem to make use of multiple, tissue-specific promoters to provide both increased expression and alternative amino-terminal protein sequences. The mouse Cβ gene recently has been shown to initiate transcription from both constitutive and neural-specific promoters and, in this case, the alternate first exons give rise to changes in the amino-terminal sequence of the protein that may have functional significance (3). The Cα gene also has been suggested to give rise to alternative products and, recently, a C subunit was sequenced from ovine sperm in which the first 14 amino acids were replaced by a novel 6 amino acid stretch. In this report, we have characterized a similar mouse Cα variant and identified the genomic sequences that give rise to this distinct product. The mouse Cα variant, termed Cα2, is transcribed from a promoter within the first intron of the previously described Cα gene (17), and this Cα2 transcript encodes an amino-terminal heptapeptide with strong sequence similarity to the ovine C subunit.

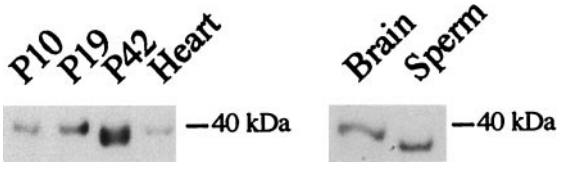

Fig. 5. Western blot analysis of testis. Protein (5 μg) from mouse heart, brain, sperm, and P10, P19, or P42 testis was separated by SDS/PAGE (10%), transferred to membrane, and probed with Cα antiserum, showing the somatic catalytic isoform with a molecular mass of 40 kDa in heart and in testis and a smaller subunit in testis at P42. The antiserum also reveals a smaller catalytic isoform in sperm in comparison to the somatic 40-kDa C isoform in brain.

The Cα2 transcript is testis-specific and the developmental time course strongly argues that it is not expressed in Leydig, Sertoli, or other somatic cells, because these cell types are abundant in testis at P10 before the induction of Cα2 mRNA and protein. The similarity in time course of expression between Cα2 and a previously characterized germ-cell-specific protein, Tenr, suggests that Cα2 transcription is germ-cell-specific and initiates at the premeiotic pachytene stage as well. The protein is then synthesized and becomes the major C isoform in mature mouse sperm.

The Cα2 variant described in this paper helps to explain two observations that we had made previously in studies on the mouse Cα gene. One is the slightly faster mobility on SDS/PAGE of testis Cα compared with Cα in all other tissues (Y. Huang and G.S.M., unpublished data). The other is the origin of Cx, a processed pseudogene that is integrated on the X chromosome and diverged from the Cα sequence at the exon1/exon2 boundary (2). We originally had suggested that this variant arose from reverse transcription of an incorrectly spliced Cα transcript. However, the near perfect similarity between 5′ sequences upstream of the Cα2 exon 2 and the corresponding region of Cx suggests that Cx arose as a processed product from the Cα2 transcript that was then inserted on the X chromosome.

A large body of data suggests that PKA is involved in sperm function, including possible roles in motility and capacitation. Considerable attention has focused on both the overall level of PKA expression and its subcellular localization within the developing and mature sperm cell. During postmeiotic maturation, the level of RIIα mRNA and protein increases dramatically, and RIIα becomes the major sperm R subunit (29). At the same time, a small group of well characterized RII subunit binding proteins

termed A-kinase anchoring proteins (AKAPs) are synthesized that are thought to anchor the PKA holoenzyme to specific sperm components. AKAP-84 is expressed in round spermatids and may anchor PKA to mitochondria (30), whereas FSC-1 (AKAP-82) is expressed in mature sperm and is thought to anchor PKA to the fibrous sheath of the flagellum (31). We recently have shown that RIIα itself is not required for motility or fertility but that mice deficient in RIIα maintain normal levels of PKA and show a compensatory increase in expression of RIα (18). The subcellular targeting of PKA to the flagellum was disrupted in the RIIα knockout mice, but a role for PKA in either development or maturation and capacitation cannot be ruled out by these studies.

Is there a specific functional role for Cα2 in male germ-cell development? One rationale for expression of Cα2 from a germ-cell-specific promoter would be to facilitate increased transcription of Cα mRNA in a cell-type-specific fashion. An increase in Cα mRNA has been observed during germ-cell development in rat (32) that might correspond to the activation of the Cα2 promoter. A second possibility is that the altered amino-terminal sequence confers distinct biological properties to Cα2 that are essential for function in spermatogenesis. The amino-terminal 14 amino acids of Cα1 contain a sequence that is myristylated at the terminal glycine and is followed by an α-helix, designated the A-helix. This A-helix occupies a hydrophobic surface traversing the small and large lobes of the kinase core. The myristyl group binds within an acyl binding pocket on the core structure and thus the entire A-helix shields an extended hydrophobic region of the C subunit. Previous studies have shown that this anchoring of the A-helix by the myristyl group confers thermodynamic stability to the purified Cα protein (33). Cα2 no longer has the myristylation consensus and also is missing the first 8 amino acids of this region. This might lead to thermo-instability of the protein, although the Cα2 protein apparently is abundantly expressed in mature sperm. Alternatively, the Cα2 protein may now have part of the core hydrophobic surface exposed giving the Cα2 subunit a potentially new protein or membrane-binding interface that could change its subcellular localization. The earlier studies on the ovine Cs demonstrated that the sperm C subunit had unusual solubility properties that conferred detergent-sensitive binding of the C subunit to sperm structures that did not depend on interaction with R subunits and presumably AKAPs (16).

Truncations of the amino terminus of Cα have been shown to differentially affect the holoenzymes formed with either the RIIα (type II) or RIα (type I) subunits, producing an increase in the association constant K_a for activation by cAMP. This increase in K_a for cAMP was much greater for the type II holoenzyme as compared with the type I holoenzyme (33), suggesting that a switch from the somatic form of Cα (Cα1) to the germ-cell form (Cα2) might differentially affect the formation and activity of type II versus type I holoenzyme. We have examined this question by measuring the activation of sperm PKA isolated from cauda epididymus. In wild-type sperm, the majority of sperm PKA is a holoenzyme of Cα2 and RIIα, whereas in RIIα knockout mice, the sperm PKA is made up of Cα2 and RIα, which compensates for the loss of RIIα (18). As depicted in Fig. 6, holoenzymes that contain Cα2 activate at similar concentrations of cyclic nucleotides regardless of the R subunit partner, suggesting that the change in the amino terminus in Cα2 is not preferentially affecting interactions with specific R subunits.

The mouse Cα2 protein and the ovine homolog, Cs, are the major C isoforms present in sperm. Furthermore, we have cloned a new Cα isoform from human testis RNA that is similar to the mouse Cα2 and the ovine Cs, demonstrating conservation of this isoform during evolution and suggesting an important function in sperm. The expression of both myristylated and nonmyristylated variants of Cα and Cβ further increases the diversity of the PKA system. These results highlight the need for a better understanding of the functional role of the C subunit amino terminus and its myristate modification in both the subcellular localization of the C subunit and the assembly of R subunit-specific holoenzymes.

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