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A Novel Ca^{2+}/Calmodulin-Dependent Protein Kinase and a Male Germ Cell-Specific Calmodulin-Binding Protein Are Derived from the Same Gene

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A cDNA representing a unique Ca^{2+}/calmodulin-dependent protein kinase has been cloned and sequenced from a rat brain cDNA library. This enzyme, expressed in brain, testis, and spleen, is only 32% identical to the various isoforms of Ca^{2+}/calmodulin-dependent protein kinase II. The sequence of the COOH-terminal 169 amino acids is identical to that of a previously described male germ cell-specific calmodulin-binding protein called calspermin (T. Ono, G. R. Slaughter, R. G. Cook, and A. R. Means, J. Biol. Chem. 264:2081–2087, 1989). This identity extends to the nucleic acid sequence and includes all but the first 130 nucleotides of the calspermin cDNA. Primer extension and sequence of a genomic fragment containing the unique calspermin sequence reveals that this mRNA is derived from the kinase transcription unit by germ cell-specific use of a unique exon. In situ hybridization was used to demonstrate that both kinase and calspermin mRNAs are expressed during spermatogenesis. The kinase mRNA is first detected in early meiotic cells and declines to a low level in haploid cells. Calspermin mRNA first appears in pachytene primary spermatocytes and continues to increase as cells complete meiosis and undergo terminal differentiation. These results show that differential utilization of a single gene during spermatogenesis is used to generate mRNAs that encode proteins with distinct functions.

Male germ cells express a large number of unique mRNAs at various stages of differentiation (24, 43). Whereas all of the common molecular regulatory mechanisms have been reported to be responsible in specific instances, the resultant unique proteins are frequently isoforms of the molecules found in somatic cells. One apparent exception to this generalization is the calmodulin-binding protein calspermin, which does not have a homolog in somatic tissues. This protein (M_r, 18735) is one of the most abundant calmodulin-binding molecules in adult mammalian testis (27). Calspermin is encoded by a 1.1-kb mRNA present predominantly in postmeiotic male germ cells and cannot be detected in any other mammalian tissue (29). The sequence of the NH_2-terminal half of the calmodulin-binding region (FNAR RKLK) is identical to the one present in all members of the family of multisubstrate calmodulin-dependent protein kinases called calmodulin kinase II (CaM kinase II), whereas the COOH-terminal half is unique (40). Translation of the 5′ nontranslated region of calspermin cDNA in the same reading frame as calspermin revealed, before a stop codon was reached, 33 amino acids that would be 58% identical to CaM kinase II α between residues 248 and 281.

Searching the available protein data bases for sequence similarity to calspermin yielded a single partial mouse cDNA called λ-ICM-1 that was obtained by screening a brain cDNA expression library with ^{125}I-calmodulin (35). Sikela et al. (36) subsequently reported that sequencing of an additional 200 nucleotides (nt) upstream of the published sequence revealed marked similarities to protein Ser-Thr kinases and suggested that this molecule be called Ca^{2+}/calmodulin-dependent protein kinase IV (CaM kinase IV). A paper documenting the presence of a novel CaM kinase that was enriched in granule cells of the rat cerebellum and that was claimed to be neuron specific was also published (25). A cDNA clone was obtained by screening a rat brain expression library with a monoclonal antibody to CaM kinase II. The sequence of this partial clone was identical to that of calspermin, with the exception of the first 120 nt of the calspermin cDNA that diverge completely from the corresponding sequence of the kinase. These analyses led us to postulate that calspermin might be derived from a transcription unit encoding a CaM kinase and be the rat homolog of CaM kinase IV (36). The unique 120 nt of 5′ nontranslated sequence of the rat clone could be due to the presence of an exon expressed only in meiotic or postmeiotic testicular germ cells. If so, then a molecular mechanism similar to that utilized to produce testicular angiotensin-converting enzyme (ACE) could exist, with the important distinction that the two protein products would encode functionally distinct molecules. In the case of testicular ACE, the transcript is initiated in an intron of the somatic gene and is translated into a protein identical to the COOH-terminal portion of somatic ACE, except for the first 66 amino acids (18). These intron sequences are located more than 7,200 bp downstream of the somatic promoter and seem to constitute an intragenic testis-specific promoter. However, testis ACE has been purified, and its kinetics are very similar to those of the larger somatic form of this enzyme.

Polymerase chain reaction (PCR) was utilized to extend the rat calspermin cDNA in the 5′ direction, and the sequence revealed further similarity to protein kinases. The PCR product was used to obtain a full-length cDNA from a rat brain λgt10 library. This clone encodes a protein kinase

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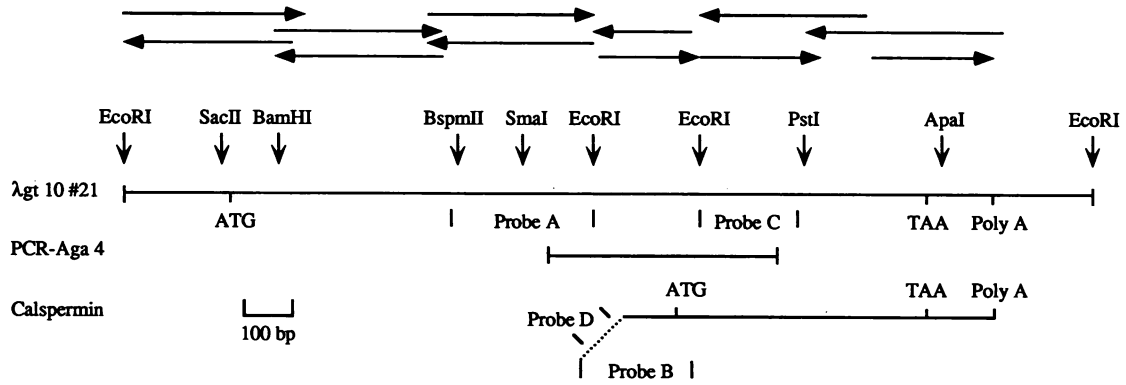

FIG. 1. Organization of the λ clone encoding a unique CaM kinase. The kinase clone λgt10#21 is illustrated schematically, and selected restriction endonuclease cleavage sites are shown above the lines. The arrows represent the sequencing strategy, and all of the clone was entirely sequenced on both strands. Below the line are depicted the approximate location of the initiation codon (ATG), termination codon (TAA), and site of polyadenylation (Poly A). PCR-Aga 4 represents the fragment of DNA obtained by PCR. The method is described in the text. The bottom line (Calspermin) is an illustration of the calspermin cDNA previously cloned and sequenced by Ono et al. (29). Above the line, the sites of translational initiation (ATG) and termination (TAA) are shown along with the polyadenylation site (Poly A). Probes A, B, C, and D represent restriction fragments used for library screening and/or Northern blot analysis. The dotted line (Probe D) depicts the only portion of calspermin cDNA that is unique to that molecule compared with the kinase.

that is only 32% identical to the α subunit of CaM kinase II (CaM kinase II α) over its entire length but, over the 70 amino acids that can be compared and that are proximal to the calmodulin-binding domain, is >90% identical to mouse CaM kinase IV (36) and is identical to the granule cell protein kinase (25). Because of these similarities and the obvious fact that this kinase is not neuron specific, we will comply with the nomenclature proposed by Sikela et al. (36) of Ca^{2+}/calmodulin-dependent protein kinase IV (CaM kinase IV). Whereas rat CaM kinase IV is expressed in several tissues, including testis, brain, and spleen, calspermin is restricted to testis. During spermatogenesis the kinase mRNA is first expressed in cells initiating the meiotic division and exists at only a low level in postmeiotic cells. Calspermin mRNA appears later in meiosis and continues to increase as haploid cells differentiate into immature spermatozoa. Our analyses are consistent with both mRNAs arising from the same gene and suggest differential transcriptional regulation of a single gene during germ cell development.

MATERIALS AND METHODS

Isolation of RNA. Tissues were frozen in liquid nitrogen immediately after dissection and stored frozen at −70°C. Total RNA was prepared as described by Chirgwin et al. (9). Poly(A)$^+$ RNA was isolated by the method of Aviv and Leder (2).

PCR. Poly(A)$^+$ RNA (2 μg) prepared from whole brains of 50-day-old rats was used to synthesize single-stranded cDNA in a total volume of 40 μl by the method of Krug and Berger (20). Random hexamer (Pharmacia) (10 μg) was next used as a primer. The reaction was carried out at 42°C for 140 min. Forty microliters of 0.3 M NaOH containing 30 mM EDTA was added to the reaction mixture, which was then boiled for 5 min. The solution was neutralized by addition of 28 μl of 0.5 N HCl. For the PCR, the following oligonucleotides were used:

(5′, sense strand) 5′ $C^{GT}_{TC}GAT^{T}_{C}TCAAGCC^{C}_{T}GAGAAC$ 3′

(3′, antisense strand) 5′ AGCGGTATCCATGTGGACAA 3′

The 5′ oligonucleotide was redundant to conform to a consensus region of CaM kinase II (nt 693 to 713 [40]). The 3′ oligonucleotide was the complement of nt 1145 to 1125 of calspermin cDNA (31). The reaction mixture contained 2 μg of 5′ oligonucleotide, 630 μg of 3′ oligonucleotide, and 3 μl of single-stranded cDNA in a total volume of 100 μl. The reaction mixture was first heated to 94°C for 5 min. Then 30 cycles of 94°C for 1 min, 45°C for 2 min, and 72°C for 3 min were carried out in a Perkin Elmer Cetus DNA Thermal Cycler. The mixture was then incubated at 72°C for 15 min. The specific PCR product (Fig. 1, PCR-Aga 4) was identified by Southern analysis with a synthetic oligonucleotide derived from the CaM kinase IV cDNA (nt 1029 to 1000; antisense strand). The sequence of this oligonucleotide is 5′ CCTTGGCATTTAAAGAAACTTCATCCAACC 3′. The product PCR-Aga 4 was purified from a low-melting-point agarose gel and blunt end ligated into the *Sma*I site of the pGEM3Zf(−) vector.

Library screening. A λgt10 library constructed from adult rat brain poly(A)$^+$ RNA was obtained from Rolf Joho (Baylor College of Medicine). One million plaques of this library were screened with the *Eco*RI fragment of PCR-Aga 4 as a probe. The screening protocol has been previously described by Ono et al. (29) and used *E. coli* C600H fl as host. Fifty positive plaques were obtained in the primary screen and 21 of these were eventually plaque purified. The cDNA inserts were mapped by restriction endonuclease digestion and Southern analysis. Eight phage clones appeared to have the same insert as that identified as λgt10#21 in Fig. 1.

Restriction endonuclease digestion and analytical gel electrophoresis. Enzyme digestions were carried out as described by the supplier. DNA digests were analyzed by 0.9% agarose or 5% polyacrylamide gel electrophoresis (PAGE) in TBE buffer (89 mM Tris borate [pH 8.3], 2 mM EDTA). DNA fragments were visualized by ethidium bromide staining and UV irradiation. DNA in selected blots was transferred to BIOTRANS nylon membranes (ICN Biochemicals, Inc.) in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Hybridization was carried out at 42°C in 50% formamide as described by Slaughter et al. (38). For experiments on analysis of RNA by Northern (RNA) blot-

ting, the samples of RNA were similarly separated by electrophoresis on a 1.5% agarose gel, transferred, and hybridized.

Radiolabeling of DNA fragments. Synthetic oligonucleotides were labeled with T4 polynucleotide kinase by using [γ-^{32}P]ATP as described by Maniatis et al. (23). DNA fragments were separated by electrophoresis on low-melting-point agarose gels, extracted with phenol-chloroform, and precipitated from ethanol. The purified fragments were labeled with [α-^{32}P]dCTP according to the random oligopriming method described by Feinberg and Vogelstein (11).

Sequencing. Single-stranded DNA was generated from pGEM3Zf(−) by the procedure recommended by Promega in which *E. coli* JM109 cells and R408 as a helper phage were used. Sequencing was carried out by the dideoxynucleotide method (32) with a modified T7 DNA polymerase (Sequenase kit; United States Biochemical Corp.) and α-^{35}S-dATP. Synthetic oligonucleotides as well as oligonucleotides complementary to the T7 and SP6 promoters were used as primers for the sequencing reactions.

Primer extension and direct dideoxynucleotide sequencing. Antisense synthetic oligonucleotides were end labeled with [γ-^{32}P]ATP and polynucleotide kinase. Adult rat testis poly(A)$^+$ RNA (10 μg) was mixed with the labeled oligonucleotide, heated to 85°C for 3 min, and then slowly cooled and annealed at 50°C for 45 min. cDNA synthesis was initiated in the presence of avian myeloblastosis virus reverse transcriptase and deoxynucleotides. Yeast tRNA was used as a negative template control. Direct sequencing of the testis poly(A)$^+$ RNA was performed by the method of Geliebter (12) under the annealing conditions described above. The samples were heat denatured and separated on 8% acrylamide sequencing gels.

Protein immunoblots. Rats were sacrificed by decapitation, tissues were rapidly removed, and 0.1 g of each tissue was homogenized in 1.0 ml of Laemmli sodium dodecyl sulfate (SDS)-containing sample buffer containing 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid] and the protease inhibitors phenylmethylsulfonyl fluoride (174 mg/ml), aprotinin (2 mg/ml), and leupeptin (2 mg/ml). The homogenates were boiled for 5 min and centrifuged for 15 min at 10,000 × *g* at 4°C. Protein concentrations of the resulting supernatant fluids were determined by the method of Bradford (6) by using purified gamma globulin (Bio-Rad) as the standard. To each lane of a 12.5% polyacrylamide gel was added a sample containing 150 μg of protein. After electrophoresis, proteins were transferred to an Immobilon-P membrane (Millipore) by a modification (14) of the original procedure described by Towbin et al. (42). For transfer, the electrode buffer contained 25 mM Tris, 192 mM glycine, 20% methanol, and 0.1% SDS. Transfer was carried out at 4°C at a constant voltage of 30 V for 1 h and then at 60 V for 7 h. The membrane was incubated with 5% nonfat dry milk for 1 h to block nonspecific antibody-binding sites. It was then incubated in 5% nonfat dry milk containing 30 μg of affinity-purified calspermin antibody per ml (28) for 2 h at room temperature. Immunoreactivity was detected by using ^{125}I-protein A as described by Guerriero et al. (14).

For the data shown in Fig. 3, SDS-PAGE was carried out with 20 μl of the nonlabeled in vitro-translated protein (calspermin-kinase or CaM kinase II). As a control, an aliquot of a 100,000 × *g* supernatant of rat brain (11 μg of protein) was loaded on the same gel. Transfer to Immobilon P (Millipore) was done at 60 V overnight in transfer buffer (25 mM Tris HCl, 192 mM glycine, 20% methanol, 0.01% SDS). The membrane was then blocked for 1 h in a solution containing 50 mg of dry milk per ml, 10 mM Tris HCl (pH 7.4), and 0.15 M NaCl and incubated with either an affinity-purified calspermin antibody or a polyclonal antibody of CaM kinase II (against peptide 281 to 302) for 3 h at room temperature. The membrane was then washed four times with a solution containing 10 mM Tris HCl, 0.15 M NaCl, and 0.05% Tween and incubated for 1 h with ^{125}I-protein A (10^6 cpm) at room temperature. Finally, the membrane was washed five times with a solution containing 10 mM Tris HCl (pH 7.4), 0.15 M NaCl, and 0.05% Tween, air-dried, and exposed for autoradiography.

Subcloning of CaM kinase IV cDNA into expression vector. The CaM kinase IV cDNA was previously cloned in λgt10 at its unique *Eco*RI site. *Sma*I digestion of this clone yielded a 3-kb fragment that contained 965 bp of the 3' end of the cDNA together with approximately 2 kb of lambda vector sequence. This fragment was isolated by excision of the band from an agarose gel. The remaining 5' 819 bp of the cDNA was contained in an independent *Eco*RI fragment previously inserted in a pGEM3Zf'(−) vector (Promega). *Sma*I digestion of this construct yielded a fragment of approximately 4 kb containing the 819-bp 5' portion of the cDNA and the plasmid vector. Blunt-end ligation of the 3 and 4-kb fragments yielded the clone CalspK-16, which includes the full-length CaM kinase IV cDNA downstream from the T7 promoter followed by 2 kb of λgt10 sequence.

In vitro transcription and translation. Purified double-stranded DNA from clone CalspK-16 was linearized at the 3' end of the cDNA by *Bgl*II digestion. The DNA was then transcribed by T7 RNA polymerase (Promega) as follows: 5 μg of DNA was added to a reaction containing in vitro transcription buffer (Promega), 10 mM dithiothreitol, 100 U of RNasin, 0.5 mM ribosomal nucleoside triphosphates, and 50 U of T7 RNA polymerase. After a 2-h incubation at 37°C, the DNA template was digested by RQ DNase (5 U) for 15 min at 37°C. Finally, the reaction was phenol-chloroform extracted and ethanol precipitated. Usual yields of RNA were 30 to 40 μg of RNA per 100-μl reaction. To verify the correct size of the transcript, 3 μg of RNA was run on a denaturing agarose gel. As a control reaction, in vitro transcription was also carried out by using a CaM kinase II α cDNA as a template.

For in vitro translation reactions, 1 μg of CaM kinase IV transcript was used in a 50-μl reaction containing 35 μl of nuclease-treated rabbit reticulocyte lysate (Promega), 4 U of RNasin, 20 μM of each amino acid except methionine, and 25 μCi of [^{35}S]methionine. Identical reactions were then performed except that the nonlabeled methionine was substituted at 20 μM for ^{35}S-labeled methionine. After a 60-min incubation at 30°C, a 5-μl aliquot was taken from the labeled reaction, mixed with SDS-Laemmli buffer, and subjected to SDS-PAGE. After adding ethylene glycol to a 10% final concentration, the reactions were stored at −70°C for further analysis. As a negative control, identical reactions were run with water instead of RNA.

Phosphorylation assays with in vitro-translated enzymes. Reactions (50 μl) were prepared as follows: 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5), 50 mM Mg acetate, 1 μM calmodulin, 400 μM ATP, 0.2 mM dithiothreitol, 0.2 mg of protein kinase A inhibitor per ml, and 20 μCi of [γ-^{32}P]ATP per reaction. Synapsin I was added to a final concentration of 30 μg/ml (0.36 μM). The in vitro-translated protein was diluted either 5- or 15-fold in a solution containing 50 mM HEPES, pH 7.5, 1 mg of bovine serum albumin (BSA) per ml, and 0.5 mM dithiothreitol. Of

this dilution, 10 μl was added to each reaction. After incubation at 30°C, the reactions were terminated by the addition of 17 μl of 4× SDS loading buffer. The mixture was then run on SDS-PAGE, and the gel was stained, destained, dried, and exposed for autoradiography.

RNA in situ hybridization. The specific [^{32}P]cDNA probes were hybridized to 5-μm testis sections from 60-day-old rats by the method of Slaughter and Means (37). Slides were prestained in Harris' hematoxylin, dipped in NTB3 emulsion, and exposed at −20°C for 3 to 5 days. Silver grains were visualized by using a Kodak D-19 developer and poststained in Harris' hematoxylin and eosin. Morphological characteristics were sufficiently preserved to permit tubules to be identified as one of the following stages on the basis of the criteria of Leblond and Clermont (21): I or II, III to V, VI, VII, VIII, IX, X, XI to XIII, or XIV.

Slides were photographed with a 40× objective (final magnification, ×125) on a Nikon Optiphot microscope. Silver grain distribution was analyzed semiquantitatively by counting the number of grains that were localized within a uniform-size circle overlaid onto photographs of cells at a defined stage of differentiation. Three to seven tubules from each stage were counted for each probe. Seven to 14 fields of each cell type were counted per tubule, determined by the number of fields of a cell type present. Grain counts were analyzed by using the Student's *t* test modified for samples with unequal *n* values (30). The differences between the means of grain counts from three different experiments were not statistically significant ($P < 0.1$). Data derived from this analysis are consistent with trends in grain localization assessed by two investigators who observed microscope slides from all experiments.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been assigned GenBank/EMBL data bank accession number M64757.

RESULTS

To determine if the calspermin cDNA represented the 3′ portion of a calmodulin-dependent protein kinase, the PCR was utilized. The 5′ oligonucleotide was redundant in four positions and synthesized to encode RDLKPEN, which is in consensus area VI of protein kinases (31). The 3′ oligonucleotide was an antisense sequence representing amino acids 30 to 38 (LGSASSSHT) of calspermin (29). Total rat brain poly(A)$^+$ RNA was utilized as template for the PCR, and a 480-bp fragment was synthesized, amplified by PCR, subcloned into pGEM3Zf(−) and sequenced. This fragment is represented as PCR-Aga 4 in Fig. 1. The 5′ unique portion of the PCR product (Fig. 1, Probe A) was used to screen a rat brain cDNA library prepared in λgt10. One clone, λgt10#21 (Fig. 1), was subcloned and sequenced with synthetic oligonucleotides by the strategy shown by the arrows in Fig. 1. The complete sequence of λgt10#21 is illustrated in Fig. 2. The clone contains a 218-nt 5′-nontranslated region, 1,425 nt of open reading frame, a 148-nt 3′-untranslated region, and a poly(A) tail. The encoded protein would consist of 474 amino acids with a calculated M_r of 53,159. This open reading frame contains all of the consensus amino acids characteristic of a protein kinase, as reported by Hanks et al. (15). These sequences which define the catalytic homology region begin with the ATP-binding site (amino acid 49) and end with the invariant Arg residue at position 284. We have assigned the initiator codon as the first ATG encountered in the open reading frame. The sequence from nt 816 (the third nucleotide in the codon for Leu 272) to the poly(A) tail is identical to that previously reported for calspermin (29), and the sequence from nt 963 is identical to the partial cDNA clone for the granule cell kinase reported by Ohmstede et al. (25).

Comparison of various protein and nucleic acid data bases revealed the expected similarities to protein Ser-Thr kinases. In general, the similarity was restricted to the catalytic homology region. Within this 270 to 280 amino acids of linear sequence, the identity ranged from 30 to 46% and included the α subunit of *Xenopus* S6 kinase (41%), the α subunit of phosphorylase kinase (43%), skeletal and smooth-muscle MLCKs (38 and 36%), protein kinase C α (33%), *nim1*$^+$ (34%), *cdc25* (34%), and cyclic AMP- and cyclic GMP-dependent protein kinases (34 and 32%, respectively). Somewhat surprising was the finding, shown in Fig. 3, that the new kinase was only 46% identical to the rat brain CaM kinase II α within the catalytic region and that the similarity decreased to 32% over the entire 425 amino acids of primary sequence. Similar percent identity exists between the kinase reported herein and the other three isoforms of CaM kinase II. Comparison of CaM kinase IV with the sequence of all isoforms of CaM kinase II reveals only three regions with linear identities of six or more amino acids. Two of these, namely, L-115VTGGELF-122 and V-156HRDLKPENLL-166, are in the catalytic homology region. The third region represents the NH_2-terminal half of the calmodulin-binding region domain F-315NARRKLK-322. Also shown in Fig. 3 is a comparison of the rat kinase with the mouse CaM kinase IV reported by Sikela et al. (36). From the first available amino acid of the mouse sequence to the end of the calmodulin-binding domain greater than 90% identity is observed. Distal to this region, the identity decreases to 70%. These data are compatible with the possibility that the kinase cloned here may represent one member of a class of calmodulin-dependent protein kinases that will phosphorylate multiple substrates, is distinct from the CaM kinase II isoforms, but is the homolog of the mouse clone obtained by Sikela et al. (36). Therefore, this rat enzyme should be called Ca^{2+}/calmodulin-dependent protein kinase IV or CaM kinase IV.

To ensure that the protein encoded by λgt10#21 was a calmodulin-dependent protein kinase, the amino acid coding region was subcloned into a pGEM 3 Zf′ (−) plasmid vector. In all cases data were compared to those obtained from a similarly subcloned cDNA representing the rat brain CaM kinase II α. Single-stranded mRNA was synthesized and translated in vitro in a rabbit reticulocyte lysate system. As shown in Fig. 4A, the protein translated from CaM kinase IV mRNA migrated on denaturing polyacrylamide gels at a M_r of 61,000 compared with the CaM kinase II, which was found at an M_r of 50,000. The calculated M_r of CaM kinase IV is 53,159, whereas that of CaM kinase II α is 54,111 (475 versus 478 amino acids, respectively). The aberrant migration of the CaM kinase IV may reflect the presence of a very Glu-rich region in its COOH-terminal region. Fig. 4B reveals that CaM kinase IV is recognized by affinity-purified calspermin antibodies, and Fig. 4C shows that CaM kinase II α is recognized by a polyclonal antibody to that subunit. However, under the conditions utilized to perform the immunoblot, these antibodies do not cross-react. The translated products of both CaM kinase IV and CaM kinase II α phosphorylate synapsin in a Ca^{2+}-dependent manner (Fig. 4D). It can also be seen that the reticulocyte lysate contains an endogenous enzyme capable of phosphorylating synapsin, although this activity appears to be Ca^{2+} independent. Finally, both translated enzymes could phosphorylate myosin light chains in the presence but not absence of Ca^{2+} (data

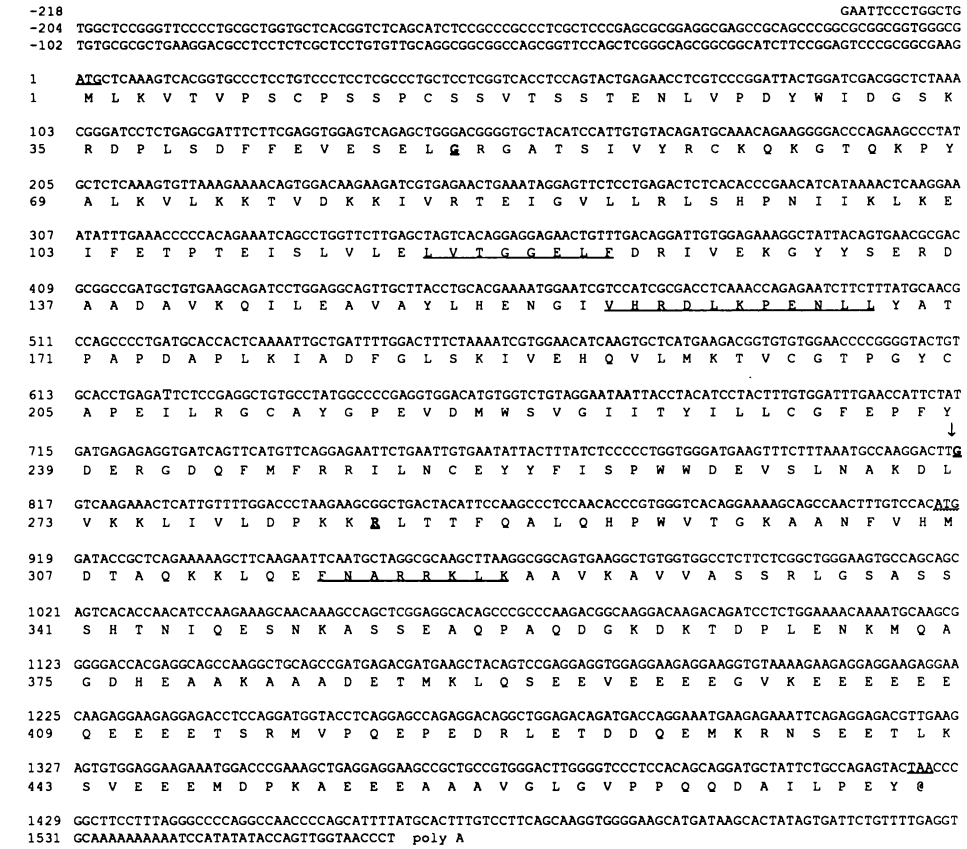

FIG. 2. Sequence of the unique CaM kinase. The nucleotide sequence is shown on the top line and the A of the ATG initiation codon (underlined) is nucleotide 1. The deduced amino acid sequence is illustrated on the second line with the single-letter nomenclature for amino acids. The initiation Met residue is designated amino acid 1. The three underlined amino acid sequences are those that are identical to sequences found in the isoforms of CaM kinase II (41). The kinase homology region as described by Hanks et al. (15) begins with the first G residue of the putative ATP-binding site (G-49 is in boldface type and underlined) and ends with the invariant Arg residue at amino acid 284 (R-284 is in boldface type and underlined). The ATG used to initiate calspermin translation is underscored with dots, and the guanine nucleotide representing the point of divergence between calspermin and the kinase is also underscored and marked by an arrow (G-816). Finally the termination codon TAA is underlined, and the site of polyadenylation is designated poly A.

not shown) as well as a synthetic peptide analog based on the sequence of glycogen synthase (GSK-10). It must be pointed out that under the conditions used here, autophosphorylation of CaM kinase II α is not evident. Since this reaction is a requisite part of the normal activation process, the failure to see the phosphorylated enzyme on the radioautograph must be due to the small amount of enzyme protein applied to the gel or to the possibility that the enzyme becomes phosphorylated in the lysate. Therefore, it is impossible to predict from our results whether CaM kinase IV undergoes autophosphorylation.

The tissue distribution of the kinase mRNA and protein was investigated by Northern hybridization and by Western immunoblot analysis. Poly(A)$^+$ RNA was isolated from several adult rat tissues, and 5 μg of each preparation was distributed on a polyacrylamide gel by electrophoresis (Fig. 5A). Under stringent hybridization conditions, signals were clearly present in cerebrum, cerebellum, testis, and spleen with a kinase-specific probe (Fig. 1, Probe A). The testis contained a single species of about 2.1 kb in size, whereas the mRNA in the other three tissues seems to be about 200 bp smaller. In addition to the 1.9-kb species a signal was also observed in cerebellum at 3.5 kb. Much fainter signals could also be observed in the 2-kb region in lung and small intestine. The strength of these signals was increased by decreasing the temperature of hybridization from 42 to 37°C (data not shown). It is not possible to conclude whether these two tissues express very low levels of the CaM kinase IV or whether the hybridization probe is recognizing a related species. Even under the less stringent hybridization conditions, no new mRNA species were detected. Thus, it is apparent that the CaM kinase IV probe does not cross hybridize to CaM kinase II subunit mRNAs.

Proteins that reacted with calspermin antibodies were only detected in the four tissues that contained a hybridizing species of mRNA and also in epididymis (Fig. 5B). Cerebrum, cerebellum, testis, and spleen contained a 61-kDa protein, whereas epididymis contained a single 64-kDa species that was also present in cerebellum and testis. The signal at about 32 kDa in the testis lane represents calspermin. On the basis of the data obtained by in vitro translation (Fig. 3A, lane 2) we suspect that the 61-kDa band represents the product of the CaM kinase IV gene. Since the cerebellum contains an additional hybridizing mRNA at 3.5 kb, it is possible that the 64-kDa protein may be the product of this species. This possibility, however, cannot explain the presence of the larger protein in testis, since no larger hybridizing mRNA was observed, or the presence of an immunore-

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CaMk IVr   PLSDFFEVESELGRGATSIVYRCKQKGTQKPYALKVLKKTVDKKIVRTEIGVLLR....LSHPNIIKLKEIFETPTEISL  112

CaMk IIα   RFTEEYQLFE---K--F-V-R--VKVLAGQE--A-IINTKKLSARDHQKLEREA-icrl-K----VR-HDSISEEGHHY-   88
CaMk IIβ   RFT-EYQLYEDI-K--F-V-R--VKLC-GHE--A-IINTKKLSARDHQKLEREA-icrl-K-S--VR-HDSISEEGFHY-
CaMk IIγ   RFT-DYQLFE---K--F-V-R--VK-TSTQE--A-IINTKKLSARDHQKLEREA-icrl-K----VR-HDSISEEGFHY-
CaMk IIδ   RFT-EYQLFE---K--F-V-R--MKIP-GQE--A-IINTKKLSARDHQKLEREA-icrl-K----VR-HDSISEEGFHY-

CaMk IVr   VLELVTGGELFDRIVEKGYYSERDAADAVKQILEAVAYLHENGIVHRDLKPENLLYATPAPDAPLKIADFGLSKIVEHQV  192

CaMk IIα   IFD--------ED--ARE----A--SHCIQ------LHC-QM-V----------L-SKLKG-AV-L-----AIE--GEQ  168
CaMk IIβ   -FD--------ED--ARE----A--SHCIQ------LHC-QM-V----------L-SKCKG-AV-L-----AIE-QGDQ
CaMk IIγ   -FD--------ED--ARE----A--SHCIH----S-NHI-QHD----------L-SKCKG-AV-L-----AIE-QGEQ
CaMk IIδ   -FD--------ED--ARE----A--SHCIQ----S-NHC-L----------L-SKSKG-AV-L-----AIE-QGDQ

CaMk IVr   LMK.TVCGTPGYCAPEILRGCAYGPEVDMWSVGIITYILLCGFEPFYDERGDQFMFRRILNCEYYFISPWWDEVSLNAKD  271
CaMk IVm                                                                                  22
CaMk IIα   QAWfGFA-----LS--V--KDP--KP--L-AC-V-L----V-YP--W--D.QHRLYQQ-KAGA-D-P--E--T-TPE---  247
CaMk IIβ   QAWfGFA-----LS--V--KE---KP--I-AC-V-L----V-YP--W--D.QHKLYQQ-KAGA-D-P--E--T-TPE--N
CaMk IIγ   QAWfGFA-----LS--V--KDP--KP--I-AC-V-L----V-YP--W--D.QHKLYQQ-KAGA-D-P--E--T-TPE--N
CaMk IIδ   QAWfGFA-----LS--V--KDP--KP----AC-V-L----V-YP--W--D.QHRLYQQ-KAGA-D-P--E--T-TPE---

CaMk IVr   LVKKLIVLD.PKKRLTTFQALQHPWVTGKA..ANFVHMDTAQKKLQEFNARRKLKAAVKAVVASSRLGS  337
CaMk IVm   ------CFGi----------..----------                                      89
CaMk IIα   -IN-MLTIN.-S--I-AAE--K---ISHRStv-SCM-RQETVDC-KK--------G-ILTTMLAT-NF-  315
CaMk IIβ   -INQMLTIN.-A--I-AHE--K----CQRStv-SMM-RQETVEC-KK--------G-ILTTMLAT-NF-
CaMk IIγ   -INQMLTIN.-A--I-AD---K----CQRStv-SMM-RQETVEC-RK--------G-ILTTMLV--NF-
CaMk IIδ   -IN-MLTIN.-A--I-ASE--K---ICQRStv-SMM-RQETVDC-KK--------G-ILTTMLAT-NF-
```

FIG. 3. Amino acid alignment of the catalytic and calmodulin-binding domains of the rat CaM kinase IV (CaMk IVr) with those of mouse CaM kinase IV (CaMk IVm) (36) and the α, β, γ, and δ subunits of CaM kinase II (4, 8, 16, 22, 41). Amino acids are represented by the single-letter code. Aligned identical amino acids are indicated by dashes, unaligned amino acids are represented by lowercase letters, and gaps inserted to maximize the alignment are shown by periods. Sequence alignment was performed by using the Doolittle program in the EuGene software package (20a). The first G of the ATP-binding homology (domain I [15]) and the invariant R in domain XI are marked by asterisks and highlighted in bold type. The putative calmodulin-binding site is overlined, and the T that becomes autophosphorylated in the CaM kinase II isoforms is also marked by boldface type and asterisks. The Arabic numbers to the right represent the amino acid numbering of the full-length proteins as referenced above. The single departure is the partial sequence of CaMk IVm, in which 1 is assigned to the first deduced amino acid as described by Sikela et al. (36).

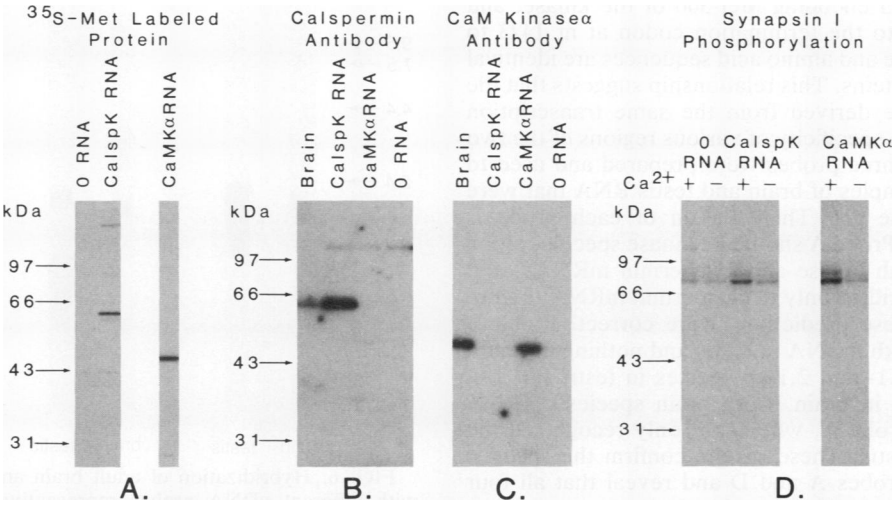

FIG. 4. Translation of CaM kinase IV and CaM kinase II α in rabbit reticulocyte lysate and properties of the enzymes. Single-stranded mRNA (in vitro transcribed) encoding either CaM kinase IV or CaM kinase II α was translated in vitro as described in Materials and Methods. (A) Radioautogram of the in vitro-synthesized proteins. (B and C) Immunoblot analysis of the in vitro-synthesized enzymes probed with either calspermin (B) or CaM kinase II (C) antibodies. Panels B and C also contain 11 μg of a 100,000 × *g* rat brain extract (Brain) as a positive tissue control. (D) Phosphorylation of synapsin by reticulocyte lysate (O RNA) or lysate containing calspermin kinase (CalspK) or CaM kinase II α (CaMKα). In each case reactions were carried out in the absence (−) or presence (+) of 1 mM Ca^{2+}. The numbers to the left of panels A, B, and D represent migration of protein standard molecular size markers.

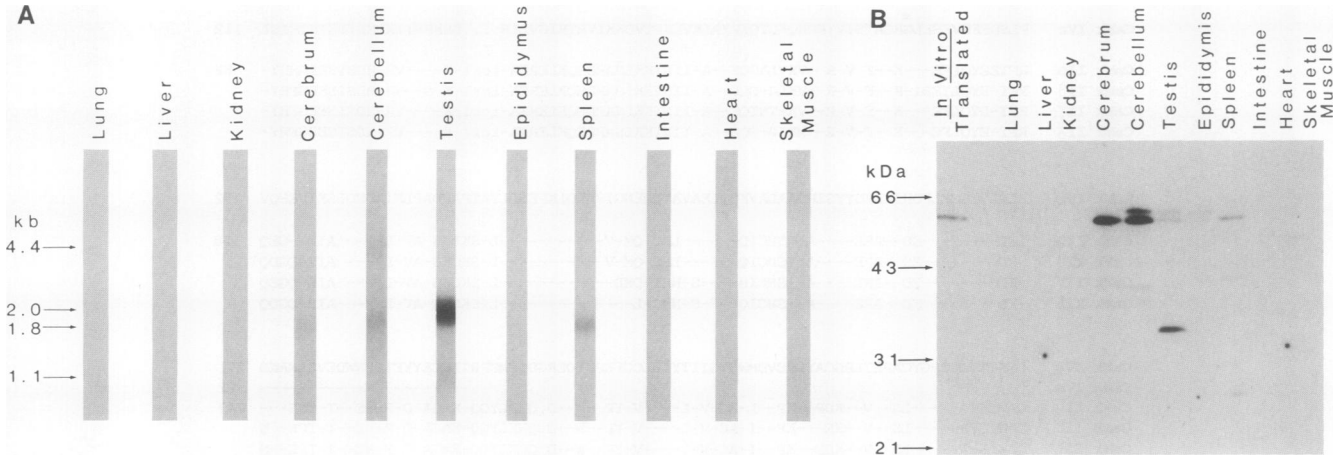

FIG. 5. (A) Rat tissue RNA blot hybridized with a cDNA probe specific for CaM kinase IV. Poly(A)$^+$ RNA was isolated from various fresh rat tissues as described in Materials and Methods, and 5 μg from each tissue was applied to a single lane of a 1.5% agarose gel. The RNA was transferred to a BIOTRANS membrane and hybridized with a radiolabeled cDNA probe (Fig. 1, Probe A) specific to the kinase. Nucleic acid size standards are shown by the number to the left of the lung lane (B) Rat tissue protein immunoblot probed with calspermin antibody. Rat tissues were prepared as described in Materials and Methods, and samples representing 150 μg of soluble protein were applied to single lanes of a 12.5% polyacrylamide gel containing SDS. After electrophoresis, protein was transferred to an Immobilon-P membrane and probed with ^{125}I-labeled affinity-purified polyclonal antibody to calspermin. Migration of protein size standards are shown by the numbers to the left and migration of in vitro-translated CaM kinase IV is shown in the first lane of the gel.

active protein in epididymis, since no hybridizing RNA at all was detected. It is likely that the epididymal protein is a component of sperm trapped in the epididymis, since mature sperm do not contain mRNA. If this is true, it seems likely that the epididymal preparation contains only the larger 64-kDa protein. Possibly the 61-kDa species is either shed with the cytoplast upon sperm release from the seminiferous tubule or is more subject to proteolysis than is the larger molecule.

With the exception of the first 121 nt of the calspermin cDNA (Fig. 1, dotted line called Probe D), the remaining nucleotide and amino acid sequence is identical to the corresponding portion of the kinase cDNA. Calspermin is initiated by the ATG encoding Met-306 of the kinase, and from the next Asp to the termination codon at nt 1423 to 1425, both nucleotide and amino acid sequences are identical between the two proteins. This relationship suggests that the two cDNAs may be derived from the same transcription unit. To examine the specificity of various regions of the two cloned sequences, three probes were prepared and used to detect mRNA in samples of brain and testis RNA that were all run on the same gel. The location of each probe is illustrated in Fig. 1. Probe A should be kinase specific, probe C should detect both kinase and calspermin mRNAs, and probe D should hybridize only to calspermin mRNA. Figure 6 illustrates that these predictions were correct. Probe D detected only a 1.1-kb mRNA in testis and nothing in brain. Probe C detected 1.1- and 2.1-kb species in testis but 1.9- and 3.5-kb mRNAs in brain. Both brain species of RNA were detected by probe A, whereas it only recognized the 2.1-kb mRNA in testis. These results confirm the species-specific nature of probes A and D and reveal that all four species of RNA must be closely related.

Primer extension of testis poly(A)$^+$ RNA with a synthetic oligonucleotide probe corresponding to the 5′ end of the common region (Fig. 1) revealed the unique nature of the first 121 nt of the calspermin cDNA relative to the kinase (data not shown) and appeared to extend farther 5′. To evaluate the site of transcriptional initiation, another oligonucleotide (primer B) was synthesized complementary to the very first portion of the 5′ untranslated region of the calspermin cDNA sequence (−205 to −176 from reference 4; underlined in Fig. 8). The results presented in Fig. 7A show that this oligonucleotide only results in a 9-nt extension of the calspermin cDNA sequence reported by Ono et al. (30) and appears to terminate with three residues, CAG. The sequence of the extended DNA is shown in Fig. 7B. Once the sequence of the genomic fragment containing these

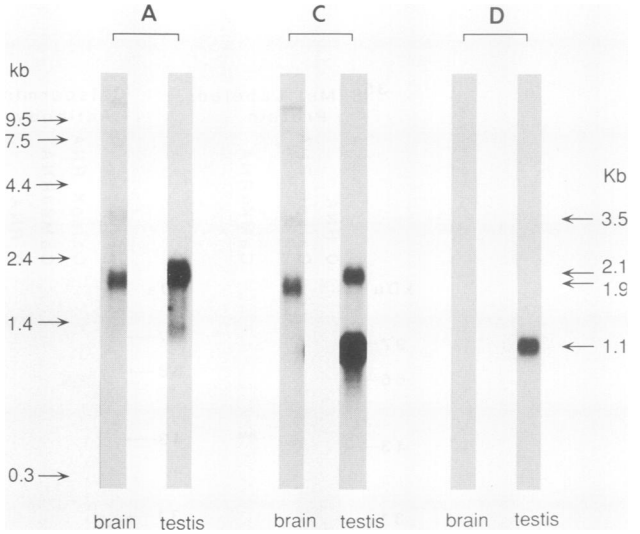

FIG. 6. Hybridization of adult brain and testis poly(A)$^+$ RNA with different cDNA probes representing calspermin and CaM kinase IV. Five micrograms of poly(A)$^+$ prepared as described in Materials and Methods was applied to a 1.5% agarose gel. Following electrophoresis, RNA was transferred to BIOTRANS and hybridized to radiolabeled probes A, C, or D as shown in Fig. 1. Migration of nucleic acid size standards are shown at the left, whereas the numbers on the right represent the calculated sizes of the four hybridizing bands.

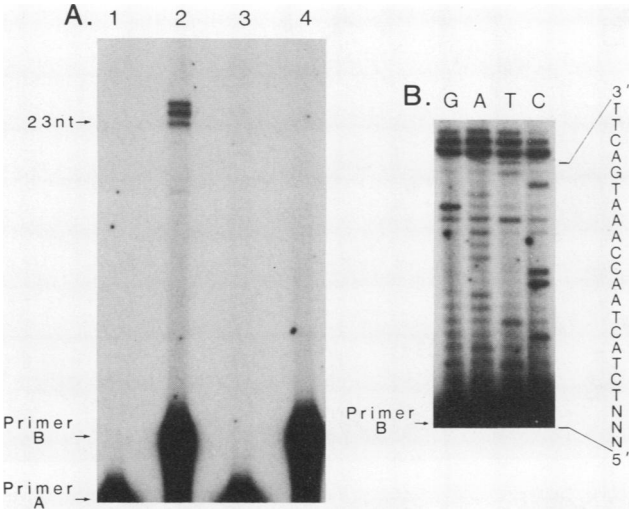

FIG. 7. Mapping the initiation site of the calspermin transcript by primer extension analysis. The protocols used are described in Materials and Methods. (A) The extended product for primer A and primer B (see Fig. 8) hybridized to adult rat testis poly(A)$^+$ RNA are shown in lanes 1 and 2. Lanes 3 and 4 display extension reactions for these primers hybridized to yeast tRNA. (B) Dideoxynucleotide sequencing of adult rat testis poly(A)$^+$ RNA using primer B and the resulting complementary DNA sequence are illustrated.

nucleotides was determined (see Fig. 8), another oligonucleotide (primer A) that overlapped the CAG residues was prepared. This oligonucleotide, whose sequence is also underlined in Fig. 8, was used in an extension reaction. The fact that this oligonucleotide failed to extend (Fig. 7B) confirmed the suspicion that the CAG may represent primary sites of transcriptional initiation.

A fragment of λgt10#21 common to both CaM kinase IV and calspermin cDNAs (Fig. 1, Probe C) was used to screen a rat genomic library packaged in Charon 4A bacteriophage. Three positive phage from the initial screen were plaque purified. One of these phage contained a 23-kb genomic DNA insert and was designated λch35. The insert was partially mapped by restriction endonuclease digestion, and fragments that hybridized to all four cDNA probes (A to D) illustrated in Fig. 1 were identified. The calspermin-specific probe D hybridized exclusively to a 390-nt *Eco*RI-*Hin*dIII fragment. This fragment was gel purified, subcloned into pGEM bluescript, and completely sequenced on both strands by the dideoxy method. Figure 8 presents this sequence. Beginning at the 5′ end, the fragment contains the *Eco*RI site followed within 20 nt by the CAG residues identified as the predominant transcription initiation sites by primer extension in Fig. 7. After 130 nt, which represents the entire sequence unique to calspermin cDNA, there is a consensus exon-intron junction sequence AG-GT. The intron is only 111 nt and ends with another consensus AG-GT. This is precisely at the point of divergence between the sequence of the kinase and calspermin (Fig. 2, highlighted G-816). The remaining sequence of the genomic fragment is identical to the cDNA sequence shown in Fig. 2 up to the *Hin*dIII site at nt 934 to 939 of the cDNA. These data show that the unique calspermin cDNA sequences are nested within the genomic DNA that contains the kinase transcription unit. The organization of the unique sequence relative to the common sequence favors the presence of an exon expressed in a germ cell-specific fashion.

Since both the kinase and calspermin are expressed in adult testis, in situ hybridization was employed to evaluate the cellular distribution of each species. Probes A and D (Fig. 1) were used to specifically localize mRNA of the kinase and calspermin, respectively. Figure 9 shows histological sections of an adult rat testis hybridized with either the kinase-specific probe (panels a and c) or the calspermin-specific probe (panels b and d). Spermatogenesis in the rat

```
   1 GAATTCTTCA GATGTTGTTT CAGCTCAGAA GTCATTTGGT TAGTAGATGA GCCAAGACCT
     EcoR1                      ***  Primer A

  61 AATTCTTTGA GCTCTGTAGA AAGAAATCTG TGAGTTTGGG GCCAGGGGTG CTGTGTGTCC
       Primer B
                                          Exon ⌈ Intron
 121 ATCTTCTGGA ACTCACTTAG CTGCTGCTTC CCTGAG⌉GTTG GTCCTACTGA TCGTGTAAAT

 181 ACTTGGTGCT GGTGGTAACA GAACAGCTGG ACTTTCACAA TGTGAGGGTA AGTCCAGTTT

                           Intron ⌈ Exon
 241 CTATAAGTTT ATTTTTTATG ATTGCAG⌉TC AAGAAACTCA TTGTTTTGGA CCCTAAGAAG
 273                               V   K  K  L   I  V  L  D   P  K  K

 301 CGGCTGACTA CATTCCAAGC CCTCCAACAC CCGTGGGTCA CAGGAAAAGC AGCCAACTTT
 284  R  L  T   T  F  Q  A   L  Q  H   P  W  V   T  G  K  A   A  N  F

 361 GTCCACATGG ATACCGCTCA GAAAAAGCTT
 304  V  H  M   D  T  A  Q   K HindIII
```

FIG. 8. Sequence of a fragment of the CaM kinase IV gene. The genomic clone was obtained by screening a rat genomic Charon 4A library with radiolabeled probe C (Fig. 1). A 390-nt *Eco*RI-*Hin*dIII fragment that hybridized to a calspermin-specific probe (Fig. 1, Probe D) was subcloned and sequenced. The oligonucleotide primers used in the primer extension experiments shown in Fig. 7 are underlined (Primers A and B), and the CAGs representing the primary transcriptional initiation sites are noted with an asterisk. The exon-intron and intron-exon boundaries are shown by vertical lines, and the G residue representing the point of divergence between calspermin and CaM kinase IV cDNAs (Fig. 2, G-816) is highlighted in boldface type and is underscored. The deduced amino acid sequence encoded by the second exon is listed below the nucleic acid sequence with the single-letter code for amino acids. These amino acids include 273 to 311 from Fig. 2. The ATG initiation codon of calspermin (M-306) is underscored with a wavy line.

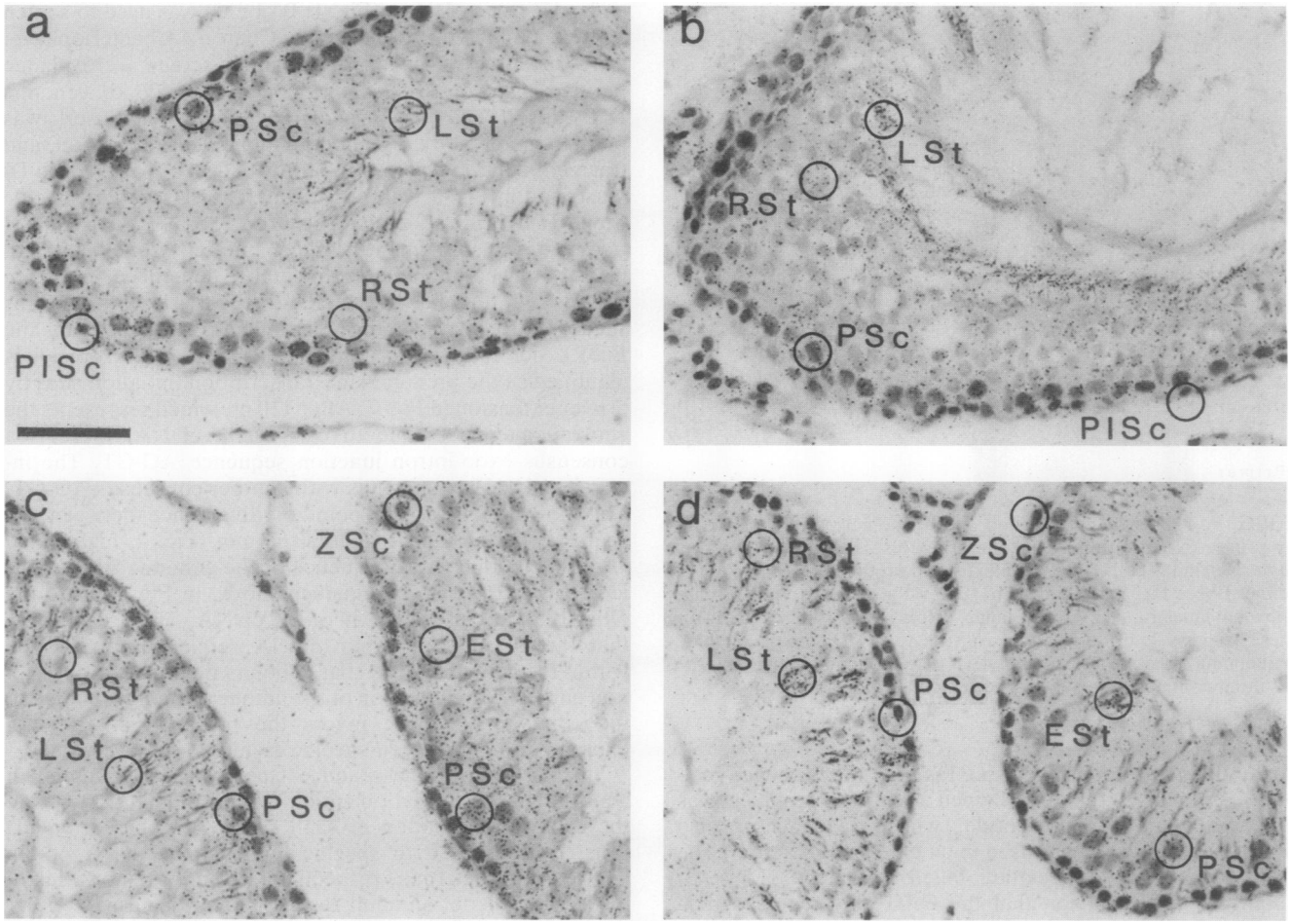

FIG. 9. In situ mRNA localization in rat testis. (a and c) CaM kinase IV RNA; (b and d) calspermin RNA; (a and b) stage VIII tubules; (c and d) stages III to V (left) and XI to XIII (right) tubules. A 7-mm circle was overlaid onto a field of cells at the indicated step in differentiation. The size bar represents 50 μm. Cell types in order of maturity: PlSc (preleptotene spermatocytes); ZSc (zygotene Sc); Psc (pachytene spermatocytes, stages III to V [early], VIII [mid], and XI to XIII [late]); RSt (round spermatids, stages III to V and VIII); ESt (early elongating spermatids); and LSt (late elongating spermatids, stages III to V and VIII).

has been divided into 16 stages (I to XVI) on the basis of the germ cell types present in the seminiferous epithelium (21). A major component of this classification is the morphology of the postmeiotic spermatids, which are characterized by 19 distinct cell shapes. Panels a and b represent stage VIII tubules. Cursory evaluation reveals a very different qualitative distribution of silver grains detected by the kinase (a) versus the calspermin (b) probes. More specific differences are emphasized by examination of the individual cells highlighted by the black circles. The preleptotene (PlSc) and pachytene (PSc) primary spermatocytes reveal numerous kinase-derived grains but few calspermin ones. On the other hand, both round (RSt) and late elongating (LSt) spermatids are intensely labeled with the calspermin probe but almost devoid of CaM kinase IV mRNA. Panels c and d show that these qualitative differences hold in tubules at other stages of spermatogenesis. In each of the lower panels the left tubule section is representative of stages III to V, whereas the right tubule is representative of stages XI and XII. The latter tubules contain zygotene spermatocytes (ZSc) that also contain many more kinase-derived transcripts than calspermin-derived transcripts. Conversely, the early elongating spermatids (ESt) present in the same epithelial area show much more abundant calspermin than kinase mRNA.

In order to quantify the in situ RNA hybridization data, the number of grains localized within a 7-mm circle were counted after the circle was overlaid onto photographs of cells at defined stages of differentiation. The details of this laborious procedure have been previously documented (37). Figure 10 presents the results of this analysis graphed as the number of silver grains per unit area as a function of those present in a specific cell type found in a defined tubule stage. The asterisk indicates no grains above background. Thus, type B spermatogonia (BSg), the last mitotic cell in the spermatogenic pathway, contained neither kinase nor calspermin mRNA. The kinase RNA is first detected in the early meiotic cell (the preleptotene spermatocyte [PlSc]), increases as meiosis progresses (LSc and PSc_M), decreases as meiosis nears completion (in the late pachytene spermatocyte [PSc_L]), and reaches a low level in the haploid cell (early round spermatid [RSt_E]) that is maintained throughout most of the remainder of spermatid differentiation (RSt_E through ESt_L).

Calspermin mRNA is first detectable at a very low level in

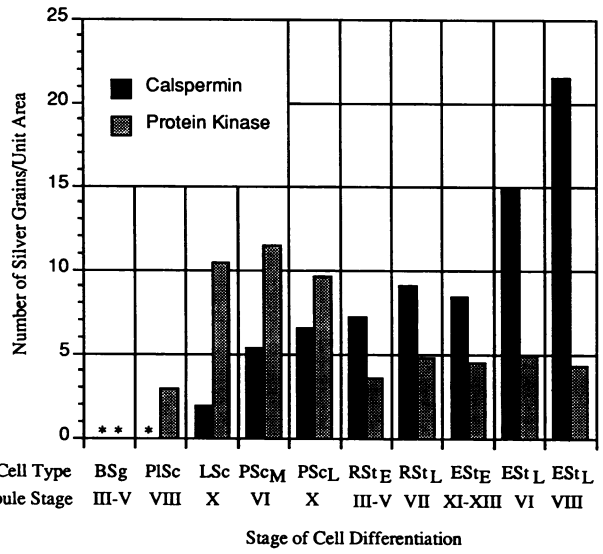

FIG. 10. Quantitative analysis of in situ RNA localization. The average number of silver grains present within a 7-mm circle were counted for each stage of germ cell differentiation. *, no grains above background level. Each set of values was derived by counting from 7 to 14 fields of cells at the same step in differentiation from five tubules at each of the indicated stages. Cell are progressively maturing from left to right. Cell types are denoted by the following abbreviations: BSg, B spermatogonia; PlSc, preleptotene spermatocytes; LSc, leptotene spermatocytes; PSc_M, mid-pachytene spermatocytes; PSc_L, late pachytene spermatocytes; RSt_E, early round spermatids; RSt_L, late round spermatids; ESt_E, early elongating spermatids; and ESt_L, late elongating spermatids.

leptotene spermatocytes (LSc). The concentration of this RNA increases considerably by the next morphologically distinct cell in the meiotic progression (PSc_M) and then continues to increase, reaching very high levels in the late elongating spermatids (ESt_L) present in stage VIII tubules. These cells have nearly completed differentiation and are soon to be released from the seminiferous tubules to begin their circuitous transport through the male reproductive system. Thus, the kinase RNA is more abundant in meiotic cells, whereas calspermin mRNA first appears later in development but continues to increase during spermiogenesis.

DISCUSSION

The archetypical multisubstrate calcium–calmodulin-dependent protein kinase is Ca^{2+}/CaM kinase II. Since the original discovery of this brain enzyme as an activity that phosphorylated site II on synapsin (34), five isoforms that represent the products of four genes have now been sequenced (4, 8, 16, 22, 41). At the amino acid level, these proteins are about 85% identical. All form heteromultimers, and autophosphorylation of an invariant internal Thr residue forms part of the activation mechanism. Once phosphorylated, the activity of these enzymes becomes independent of Ca^{2+}/CaM. CaM kinase IV may represent the first member of a new class of multisubstrate Ca^{2+}/CaM-dependent protein kinases. It is only 32% identical to members of the Ca^{2+}/CaM kinase II family and does not contain the RQET sequence that is frequently considered to constitute the primary autophosphorylation site of the CaM kinase II isozymes. Allowing for conservative substitutions, CaM kinase IV does contain the sequence HMFTAQ_KK which is similar to the expanded phosphorylation sequence RQETVDCKK in CaM kinase II. However, since under the conditions used we could not detect phosphorylation of CaM kinase II α, we cannot be certain whether CaM kinase IV is rapidly autophosphorylated and becomes independent of Ca^{2+}/CaM. Likewise, we cannot say with certainty whether CaM kinase IV forms a multimer. However, the primary sequence of the domain involved in subunit interaction in CaM kinase II is very different from that present in the equivalent region of CaM kinase IV.

After phosphorylation of the Thr-286, the CaM kinase II α undergoes additional autophosphorylation if Ca^{2+} is removed from the reactions. These additional phosphorylation sites are Thr-305, Thr-306, and Ser-314 (10, 32) (Fig. 3). Phosphorylation of residue 305 or 306 inhibits CaM binding, whereas phosphorylation of 314 does not. As previously noted (29), the CaM-binding region (overlined in Fig. 3) is predicted to form an amphipathic α-helix and binds CaM with high affinity, at least in the context of the calspermin protein. When the CaM-binding regions of CaM kinase IV and CaM kinase II are aligned, a Ser in the former (residue 337) exists in the same context as Ser-314 in the latter, that is, RxxS (41). This is a consensus phosphorylation site for CaM kinase II, so it might also be expected to undergo autophosphorylation in CaM kinase IV. Perhaps more interesting is the pair of Ser residues at positions 332 and 333. These Ser residues in CaM kinase IV are in a similar context to the pair of Thr residues in CaM kinase II α. This context is KxxxxTT (or -SS), where x represents hydrophobic amino acids. In the linear sequence of the enzymes, the phosphorylatable residues are offset by four amino acids. In CaM kinase IV the four residues are inserted before the Ser, whereas in CaM kinase II the four amino acids occur after the Thr (AAVK and MLAT, respectively). Such a similarity might suggest that CaM kinase IV could also undergo phosphorylation on Ser in a manner that would inhibit CaM binding. In addition, since calspermin also contains these Ser residues, CaM kinase IV could phosphorylate calspermin. Such phosphorylation could prevent CaM binding, thus inhibiting the only known function of calspermin. In CaM kinase II, the pair of phosphorylated Thr residues are readily dephosphorylated by protein phosphatase 1 or 2A (31). Testis-specific isoforms of both phosphatases exist in male germ cells and appear developmentally in a manner similar to calspermin (19). A similar specificity for phosphorylated Ser residues in CaM kinase IV could reverse the phenotype and restore CaM-binding capability. These possibilities can be readily tested once sufficient quantities of CaM kinase IV and calspermin are available.

There is a good possibility that CaM kinase IV is the same as or a close relative of the granule cell protein kinase cDNA isolated and characterized by Ohmstede et al. (25). These authors reported the sequence of an incomplete clone that began at an internal *Eco*RI site corresponding to nt 963 of our sequence. The partial sequence is identical to that of CaM kinase IV through the remainder of the amino acid coding region and for the first 142 nt of the 3′ nontranslated region. Whereas this is the site of polyadenylation in CaM kinase IV, the granule cell cDNA extends an additional 583 nt before the poly(A) tail. This raises the possibility that the two mRNAs could be derived from the same primary transcript by differential polyadenylation. Northern analysis by Ohmstede et al. (25) revealed 2.0- and 3.5-kb mRNAs in brain, which are of sizes very similar to those shown in Fig. 5A and 6 of the present paper. However, it is unlikely that

these two mRNAs encode the same protein and differ only in the length of the 3′ nontranslated region. We suggest that the ~2.0 kb mRNA encodes the CaM kinase IV and that the 3.5-kb species may originate from a separate gene, as is the case for four isoforms of CaM kinase II. Alternatively, the two mRNAs could be derived from the same transcription unit but be composed of differentially processed exons coupled with the use of alternate polyadenylation sites. This can only be resolved by analysis of the cloned gene(s).

Whereas testis contains a 2.1-kb mRNA, the species present in brain and spleen is about 200 nt smaller when analyzed on the same gel. It is possible that this difference represents only the number of A residues present in the poly(A) tail. A difference of this kind has recently been proven for *junB* mRNA in mouse testis by Alcivar et al. (1), and Schafer et al. (33) have shown that mRNAs transcribed in *Drosophila* spermatocytes acquire longer poly(A)$^+$ tails as they become translated later in development. Since CaM kinase IV is a brain clone and contains only 142 nt of the 3′ nontranslated sequence, it is equally possible that the testis mRNA is derived from use of an alternate polyadenylation site 200 nt downstream. Finally, we cannot rule out the possibility that the 2.1- and 1.9-kb species could be products of two closely related genes. In the unlikely event that this proves to be the case, then the apparent M_r of the proteins is likely to be identical since they migrate indistinguishably on denaturing polyacrylamide gels.

The CaM kinase IV cDNA sequence is identical to that previously reported by Ono et al. (29) for calspermin from nt 816 of the former sequence to its end at nt 1567. Since the calspermin protein begins with the Met encoded by nt 916 to 918, the nucleotide sequence from 816 to 917 represents 5′ nontranslated sequence in the calspermin mRNA but encodes amino acids in the kinase. The CaM kinase IV cDNA clone also contained 121 nt of 5′ nontranslated sequence that is unique to this species. Accordingly, the Northern blot in Fig. 6 shows that a probe derived from this region hybridizes exclusively to the 1.1-kb calspermin mRNA present in testis. Primer extension experiments revealed that the CAP site of the calspermin mRNA is only an additional 9 to 11 nt upstream of the reported cDNA sequence. Isolation and sequence of the genomic DNA containing this sequence unique to calspermin shows that it is contiguous with the sequence common to both calspermin and the kinase but is separated by a 111-nt intron. These results suggest that the sequences unique to calspermin mRNA must compose another exon. Transcriptional initiation would be specific to meiotic and/or postmeiotic germ cells and would be accompanied by an obligate unique splicing event to generate calspermin mRNA.

Precedence exists for male germ cell-specific gene expression. Best characterized are the protamine genes which only become transcriptionally active following completion of meiosis (26). A fragment of the 5′ regulatory region of a mouse protamine gene has been used to target expression of a transgene to spermatids in transgenic mice (7). More than 30 other examples exist for expression of germ cell-specific mRNAs (24). Molecular mechanisms responsible include differential transcriptional initiation of a gene expressed in somatic cells (13), expression of testis-specific genes encoding an isoform of a somatic cell counterpart (17), alternative choice of polyadenylation sites (3), change in the extent of mRNA polyadenylation (1, 33) and translational control of mRNA expression (5, 33). More rare are instances in which a germ cell-specific transcript is generated from a somatic cell transcription unit by transcriptional initiation within the usual amino acid-coding region of the gene. Two such examples are ACE and transferrin (18, 39). In each case the proteins are composed of identical functional domains. Germ cell-specific transcription results in a mRNA that would only encode the COOH-terminal domains of the protein.

Calspermin mRNA appears to be generated from the kinase gene by a mechanism similar to that used for ACE and transferrin. However, two unique features also exist. First, whereas the somatic gene encodes a protein kinase, calspermin is a calmodulin-binding protein that contains no other obvious functional domains. Thus, the function of the somatic and germ cell gene products is necessarily different. Second, both proteins are expressed in germ cells, but in distinct patterns. Results from the in situ hybridization experiments show that the kinase transcript is expressed prior to the calspermin mRNA during spermatogenesis. Whereas the kinase mRNA is first detected in cells undergoing the earliest stages of meiosis and the enzyme is only obvious in pachytene primary spermatocytes (data not shown), calspermin mRNA is first detected at a later stage of meiosis and calspermin is a prominent component of postmeiotic cells (29). Meiosis is a slow and very complicated process in mammalian spermatogenesis. The entire transition from a diploid to a haploid cell requires 13 days. It is tempting to speculate that the kinase plays a role in meiosis whereas calspermin primarily functions in haploid cells undergoing the spermiogenic differentiation program. The biological reagents to answer these and other questions concerning the mechanisms and consequences of differential gene expression during mammalian spermatogenesis are now available.

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