

Organization and Analysis of the Complete Rat Calmodulin-dependent Protein Kinase IV Gene*

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A 42-kilobase pair region of rat DNA containing the Ca^{2+} /calmodulin-dependent protein kinase IV (CaM kinase IV) gene has been cloned and characterized. The gene consists of 12 exons and 11 introns and is predicted to encode both β and α forms of CaM kinase IV as well as the testis-specific calmodulin-binding protein calspermin. The promoter utilized to generate the α -kinase isoform is located in intron 1, whereas the promoter utilized to produce the calspermin transcript is contained in intron 10. The calspermin promoter region which extends from –200 to +321 relative to the calspermin transcription initiation site that contains two cyclic AMP response elements (CRE) at –70 and –50 and has been shown previously to be inactive in NIH3T3 cells (Sun, Z., Sassone-Corsi, P., and Means, A. R. (1995) *Mol. Cell. Biol.* 15, 561–571) was ligated to the *lacZ* reporter gene and used to generate transgenic mice. The promoter was expressed exclusively in postmeiotic testis where β -galactosidase was found predominantly in elongating spermatids. The cell and developmental specificity of transgene expression was very similar to the pattern shown by the endogenous gene. Although the transgene promoter was silent in somatic tissues, β -galactosidase expression could be restored in primary cultures of skin fibroblasts by introduction of vectors encoding CREM τ and CaM kinase IV.

with relative M_r of about 62,000 (α) and 64,000 (β) (Ohmsted *et al.*, 1989). The cDNA of the smaller α isoform, which has a calculated M_r of 53,159, has been expressed in *Escherichia coli* and baculovirus systems, and many properties of the enzyme have been determined (Cruzalegui and Means, 1993; Okuno and Fujisawa, 1993; Mosialos *et al.*, 1994; Kitani *et al.*, 1994; Enslen *et al.*, 1994; Tokumitsu *et al.*, 1994; Selbert *et al.*, 1995). The relationship between the α and β isoforms is controversial. It has been suggested that β is a post-translational modification of α (Mosialos *et al.*, 1994). However, McDonald *et al.* (1993) provided evidence that β could not be converted to α by dephosphorylation and argued for an independent gene product. The latter contention was strengthened by Sakagami and Kondo (1993) who obtained a cDNA from a rat brain library thought to encode the larger β isoform. The calculated M_r was 55,705, and it differed from the α isoform only by a unique 28-amino acid N-terminal extension. Whereas the β mRNA was distributed differently from α in parasagittal sections of the brain, the properties of CaM kinase IV β have yet to be reported. The α form of CaM kinase IV requires phosphorylation on a threonine residue in the “activation loop” (Thr¹⁷⁷) by a CaM kinase IV kinase for full activity (Selbert *et al.*, 1995). This phosphorylation stimulates subsequent autophosphorylation on several serine residues, resulting in a significant amount of Ca^{2+} /calmodulin-independent activity (McDonald *et al.*, 1993; Selbert *et al.*, 1995).

Ca^{2+} /calmodulin-dependent protein kinase IV (CaM kinase IV,¹ also known as CaM kinase Gr) is a monomeric multifunctional enzyme expressed in a tissue-restricted manner. It is particularly abundant in T-lymphocytes, granule cells of the cerebellum and meiotic male germ cells (Ohmsted *et al.*, 1991; Means *et al.*, 1991; Hanissian *et al.*, 1993; Jones *et al.*, 1991; Miyano *et al.*, 1992). Although CaM kinase IV is the product of a unique gene in both mouse and human (Sikela *et al.*, 1989, 1990), two isoforms of the enzyme are present in the cerebellum

The C-terminal 169 amino acids of CaM kinase IV comprise a separate protein called calspermin that is expressed exclusively in postmeiotic male germ cells (Ono *et al.*, 1989; Means *et al.*, 1991). This is the most abundant calmodulin-binding protein in spermatozoa but is of unknown function. In heterologous cell lines, the transcript of calspermin can be initiated from a promoter within an intron of the CaM kinase IV gene (Means *et al.*, 1991; Jones *et al.*, 1991; Ohmsted *et al.*, 1991; Sun *et al.*, 1995) and, with the exception of the first 130 nucleotides, is identical to the corresponding region of the CaM kinase IV transcript (Means *et al.*, 1991). Transcription from this promoter in NIH3T3 cells requires two cyclic AMP response elements (CRE) at –70 and –50 bp relative to the transcription start site (Sun *et al.*, 1995). These CREs bind the transcription factors CREB or CREM τ and either factor markedly stimulates activity of the basal promoter defined by Sun *et al.* (1995) as the genomic DNA fragment extending from nucleotide –80 to nucleotide +361. However, the addition of 120 bp to the 5' end of the basal promoter markedly inhibits transcription in NIH3T3 cells. Some activity could be restored by co-transfection of CREM τ (or CREB) together with CaM kinase IV (or protein kinase A). We have suggested that the –200 to +361 fragment might be sufficient to produce germ cell-specific transcription.

We have cloned the entire CaM kinase IV gene which spans greater than 42 kbp. The organization of this gene suggests that CaM kinase IV β and α and calspermin are all produced by

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X91964.

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¹ The abbreviations used are: CaM kinase IV, Ca^{2+} /calmodulin-dependent protein kinase IV; CRE, cAMP response element; X-gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; kbp, kilobase pair(s); bp, base pair(s); CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; PBS, phosphate-buffered saline.

alternative transcriptional initiation. The longest transcript would produce CaM kinase IV β . The CaM kinase IV α promoter is located within the first intron, whereas the promoter and first exon of caldesmon are located in intron 10. We also show the -200 to +321 region of the caldesmon gene is sufficient to target expression of a reporter gene in transgenic mice. Expression of the transgene is restricted to postmeiotic male germ cells and shows the correct developmental pattern during the initiation of spermatogenesis. However, transgene expression can be induced in primary cultures of skin fibroblasts by transfection of expression vectors encoding CREM7 and CaM kinase IV.

EXPERIMENTAL PROCEDURES

Cloning and Characterization of the Rat CaM Kinase IV Gene—Genomic clones were obtained by screening two rat genomic libraries with cDNA probes derived from various portions of the full-length cDNA (Means *et al.*, 1991). One library was in Charon 35 and the other was a λ -DASH library from Stratagene. Subgenomic *EcoRI* fragments were subcloned into either pGEM or pUC plasmids for sequencing. The location of the *EcoRI* sites is shown in Fig. 1B. Nucleotide sequence was obtained using sequencing kits from Amersham Corp. In many cases sequencing was extended by the use of synthetic oligonucleotides based on the previous sequencing reaction results as primers. All exons, intron/exon junctions, and 1100 bp of 5'-flanking DNA were completely sequenced on both strands. Some of the large introns were not completely sequenced, so the 42 kbp is probably an underestimate of the gene size. The sequence of 5'-flanking region presented in Fig. 3 has been deposited in the EMBL data base under accession number X91964.

Construction and Analysis of the Promoter-CAT Reporter Gene—Different fragments of CaM kinase α promoter were amplified from a 6-kbp genomic DNA fragment by PCR according to the conditions described by Boehringer Mannheim for its *Taq* DNA polymerase. In order to subclone the PCR fragments into pCAT basic vector (Promega), the 5' primers (synthesized using an Applied Biosystem 392 DNA/RNA synthesizer) were designed to contain a *PstI* site, and the 3' primer was designed to contain a *XbaI* site. PCR products (amplified by a Perkin-Elmer DNA thermal cycler), which were digested by *PstI* and *XbaI*, were then subcloned between the *PstI* and *XbaI* sites of the pCAT basic vector. In all cases the PCR-derived fragments were sequenced to ensure that no errors had been introduced. The oligonucleotide sequences used in the construction of different length promoter fragments are available upon request. Transfections and CAT assays were performed as described by Sun *et al.* (1995).

Construction of the β -Galactosidase Plasmid for Generation of Transgenic Mice—The β -galactosidase plasmid was created by cloning the -200 to +321 DNA fragment of the caldesmon gene into the *XbaI* and *PstI* sites of pUC19/AUG β -galactosidase (a gift from Dr. Eric N. Olson, Department of Biochemistry and Molecular Biology, M. D. Anderson Cancer Center, Houston, TX). The *KpnI* fragment containing the -200 to +321 promoter fragment, the β -galactosidase gene, the SV40 splicing junction, and poly(A) addition site was microinjected into the male pronuclei of one-cell stage embryos, and transgenic founder mice were generated by the Transgenic Core Facility of the Duke Comprehensive Cancer Center.

β -Galactosidase Assays—X-Gal β -galactosidase assay: fresh tissues were removed from mice and then fixed for 30 min at 4 °C in a solution containing 100 mM sodium phosphate, pH 7.3, 2% paraformaldehyde, 0.2% glutaraldehyde, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40. After fixation, tissues were stained for 5 h at 37 °C in a solution containing 100 mM sodium phosphate, pH 7.3, 1.3 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$, and 1 mg/ml X-gal. β -Galactosidase converts X-gal into a water-insoluble blue product which allows visual identification of the tissues containing β -galactosidase activity.

X-Gal β -Galactosidase Assay of Cultured Cells—Cultured cells were washed twice with PBS and then fixed for 5 min at room temperature in 2% paraformaldehyde, 0.2% glutaraldehyde, 100 mM sodium phosphate, pH 7.3. The cells were washed twice with PBS after fixation and stained in X-gal solution at 37 °C for 24 h. X-Gal solution contains 100 mM sodium phosphate, pH 7.3, 1.3 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$, and 1 mg/ml X-gal.

Chlorophenol- β -D-galactopyranoside β -Galactosidase Assay—100 μ g of protein from homogenized fresh tissues of transgenic mice were used for β -galactosidase activity assay. Protein concentration was determined by the Bradford method (Bradford, 1976). 100 μ g of protein from the supernatant was added to 600 μ l of Z buffer (60 mM Na_2HPO_4 ,

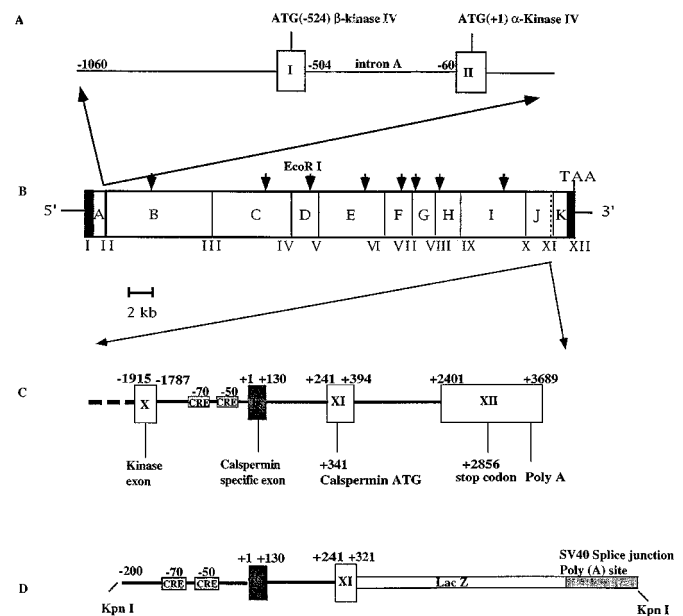


FIG. 1. Schematic representation of the organization of the rat CaM kinase IV gene. A, schematic representation of the organization of exons I and II as well as intron A. The CaM kinase IV α initiation codon is nucleotide +1 which is in exon II. The CaM kinase IV β initiation codon is at nucleotide -524 which is in exon I. Both isoforms of the kinase share exons II–XII illustrated in B. B, organization of the CaM kinase IV gene. Exons are indicated by Roman numerals, and introns are indicated by capital letters. The dotted line between intron J and exon XI represents the location of the testis-specific exon involved in generation of the caldesmon transcript. The arrows indicate the location of *EcoRI* restriction sites that were used to clone and order the genomic fragments of DNA. C, caldesmon gene structure. Exons are indicated by boxes, and the black box is the testis-specific caldesmon exon (Ts). +1 is the caldesmon transcription initiation site. The numbers indicate the positions of intron and exon junctions. Two CRE-like motifs are located at -50 and -70 relative to the transcriptional initiation site. D, structure of the construct used to generate transgenic mice. The -200 to +321 region of the caldesmon promoter was linked to a lacZ reporter gene.

40 mM NaH_2PO_4 , 10 mM KCl, 1 mM $MgSO_4$, 50 mM β -mercaptoethanol, and 4 mM chlorophenol- β -D-galactopyranoside). After 20-min to 1-h incubation at 37 °C, the reaction was terminated by placing the samples on ice. If β -galactosidase activity was present, the solution became a red color, which could be readily visualized. The optical density was recorded at 420 nm, and enzyme activity was calculated as described (Macgregor *et al.*, 1991).

Immunohistochemistry—Antibody to β -galactosidase was obtained from 5 Prime \rightarrow 3 Prime, Inc., (Boulder, CO). Immunohistochemistry was performed using the staining procedure provided by Vector Laboratory (Burlingame, CA). The frozen sections were fixed in 4% paraformaldehyde for 5 min, and the endogenous peroxidase activity was quenched by incubating the sections for 30 min in 0.3% H_2O_2 . After washing the sections with phosphate-buffered normal saline solution (PBS) for 20 min, the sections were incubated for 20 min with diluted normal blocking serum, which was prepared from the species in which secondary antibody was made. The blocking serum was washed away with PBS, and the sections were incubated at 37 °C with β -galactosidase antibody which was diluted 500 times with 1 \times PBS, 0.02% sodium azide, 1% bovine serum albumin. The slides were washed in PBS for 15 min and incubated with diluted secondary antibody solution at room temperature for 30 min. After washing the sections for 15 min with PBS, the sections were incubated with streptavidin-conjugated peroxidase for 30 min at room temperature. The sections were washed again with PBS for 15 min and then the sections were incubated in peroxidase substrate, 3',3'-diaminobenzidine with 0.3 mg/ml nickel, for 2–7 min or until the desired stain intensity developed. The sections were washed for 5 min in water and counterstained with hematoxylin for 30 s to 5 min.

Preparation of Fibroblasts from Transgenic Mice—The skin from a 2-day-old transgenic mouse was cut into 1-mm small pieces and transferred to a 60-mm tissue culture dish. The skin tissue was air-dried for

Junction	Sequence	Exon	Intron	AA Interrupted
I/A A/II	GGCAACGATCAGGTCTCTTCTCA CCTGTGTTGCAGGGCGGCCAGC	I	A	Q8 A9 CAG/GCG
II/B B/III	AGAGCTGGGACGCTAAGGCGAGGG TTCCCCCTCAAGGGGTGCTACATC	II	B	R50 CG/G
III/C C/IV	TTAAAGAAAACAGTAAGTTTATTT TTACCTTCTAGGTGGACAAGAG	III	C	T76 V77 ACA/GTG
IV/D D/V	CACCCGAACATCGTAAGTGGCTCT TCTTCCCTCAGATAAACTCAAG	IV	D	I97 I98 ATC/ATA
V/E E/VI	ACTGTTTGACAGGTAAGTCGGACC TATTTTCTTCAGGATTGTGGAGAA	V	E	R125 AG/G
VI/F F/VII	GAGGCAGTTGCTGTAAGTACAATC TTCCCTCTGTAGTACCTGCACGAA	VI	F	A149 Y150 GCT/TAC
VII/G G/VIII	CACCTAAAATTGGTAAGCAAGTCT TTGTACTTCTAGCTGATTTTGGAC	VII	G	A180 G/CT
VIII/H H/IX	CGGGGTACTGTGTAAGCTCTGAT CCTTCTTTTTCAGCACCTGAGATT	VIII	H	A205 G/CA
IX/I I/X	TACCTACATCCCTGTAAGTGACAAA GTTTCCCTGCAGACTTTGTGGATT	IX	I	L230 CT/A
X/J J/XI	GCCAAGGACTTGGTAAGTGTGACA TTATGATTGCAGGTCAAGAACTC	X	J	L272 TT/G
XI/K K/XII	CGCAAGCTTAAGGTAAGTGTGTG CTCTCTAATCAGCGCGCAGTGAAG	XI	K	K323 A324 AAG/GCG
		XII		

FIG. 2. The sequences of intron-exon junctions of the CaM kinase IV and caldesmon gene. The last column indicates the amino acid codons interrupted by each intron.

10 min in the culture dish before adding the medium. This step ensures that the skin tissue attaches to the culture dish so it will not float away when the medium is added. Five ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum was added into each culture dish and maintained in a 5% CO₂ atmosphere at 37 °C for several days. It usually took 1–2 days for fibroblasts to migrate out of the skin and attach to the culture dish. The medium was changed every 3 or 4 days until the cells became confluent, which required 1–2 weeks. After that the fibroblasts were passaged as regular cell lines.

Transfection of the Fibroblasts—The transfections were performed with a Bio-Rad gene pulser transfection apparatus. 10 µg of DNA and 0.8 ml of cell suspension (1 × 10⁶ cells in Dulbecco's modified Eagle's medium) were placed in a 0.4-cm gene pulser cuvette. The DNA and cells were thoroughly mixed by pipetting. This mixture was incubated for 10 min at room temperature prior to electroporation. The cuvette was placed into the chamber and electroporated once at 250 V and 960 microfarads. A pCMV-CAT construct was cotransfected as an internal control. The cells were then incubated at room temperature for 10 min before being plated into culture dishes. The cells were grown for 4 days for the purpose of the β-galactosidase and CAT assays.

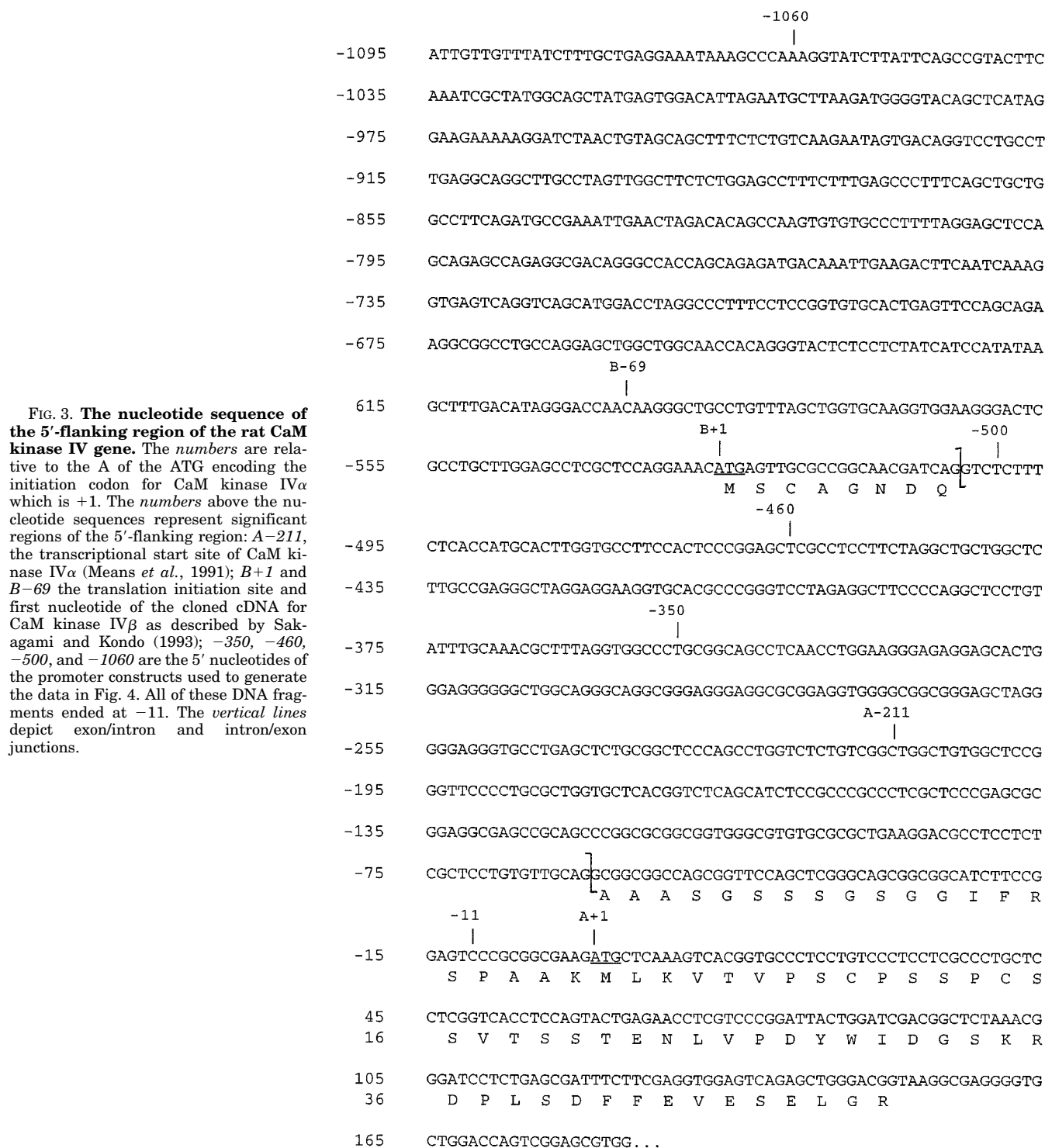
RESULTS

Organization of the CaM Kinase IV Gene—The organization of about 42 kbp of genomic DNA representing the entire CaM kinase IV gene is shown in Fig. 1B. It is comprised of 12 exons (Roman numerals) and 11 introns (capital letters). An expanded diagram representing the translational initiation codons of the β and α isoforms, based on cDNA sequences of Sakagami and Kondo (1993) and Means *et al.* (1991), respectively, is shown in Fig. 1A. This analysis predicted that the promoter for the α-kinase would reside in intron A. The region in intron J containing the promoter and testis-specific exon required to produce the caldesmon transcript is illustrated in Fig. 1C. The nucleotide sequence of −1930 to +361 has been previously published by Sun *et al.* (1995). The nucleotide sequence of all exon/intron junctions and the amino acid sequence of the cDNA interrupted by each are shown in Fig. 2. The sequence of 1100 nucleotides of the region flanking the translation initiation codon of the α-kinase isoform (where the A of the ATG is designated as +1), the sequence of exon II, encoding the first 50 amino acids of the α-kinase, and 34 nucleotides of

intron B are shown in Fig. 3. The A of the ATG predicted to encode the initiating Met of the β-kinase by Sakagami and Kondo (1993) is at −524 in what we have termed exon I. Exon I encodes the first 8 amino acids of β-kinase and ends with the first exon/intron junction at −504. This intron A contains the transcription initiation site of the α kinase at −211 as defined by Means *et al.* (1991). The boundary between intron A and exon II occurs at −60. Whereas the sequence from −60 to +1 is noncoding in the α-kinase, it encodes amino acids 8 through 28 of the β-kinase. The remainder of the α- and β-kinase sequence is identical and Met¹ of the α isoform would be Met²⁹ of the β isoform.

Identification of a Transcriptionally Active Promoter Element Immediately Upstream of CaM Kinase IVα—Since the ATG site for CaM kinase IVα is separated from the predicted CaM kinase IVβ ATG by an intron (intron A, Fig. 1B), we tested whether this intron might contain a promoter that could be used to drive transcription of CaM kinase IVα. Different segments of DNA upstream of the ATG site for CaM kinase IVα were amplified by PCR and linked to a CAT reporter gene. The sequence of the DNA fragments linked to CAT is shown in Fig. 3. The ability of each construct to direct expression of the CAT gene was analyzed following transfection into HeLa cells by the CaPO₄ method. The results shown in Fig. 4 were obtained 48 h after transfection. For quantitation of CAT activity, an internal control, pRSV-β-gal, and a negative control, pCAT basic (a promoterless CAT), were included. Although the longer DNA fragments (−460/−11, −500/−11, and −1060/−11) did not generate significant CAT activity, the DNA from −350 to −11 resulted in CAT activity similar to that produced by the SV40 promoter, suggesting that the upstream region of the CaM kinase IVα harbors a promoter element.

Testis-specific Expression of the lacZ Gene in Transgenic Mice Driven by the −200 to +321 Region of the Caldesmon Promoter—Our previous studies demonstrated that DNA extending from −80 to +361 (Fig. 1C) relative to the caldesmon transcription initiation site had strong promoter activity in



NIH3T3 cells (Sun *et al.*, 1995). However, promoter activity was severely inhibited in these cells by extending the 5'-flanking sequence to -200, unless CREM τ or CREB expression vectors were cotransfected. We tested whether this -200 to +321 promoter region could be expressed in testis and was sufficient to produce germ cell-specific transcription. As illustrated in Fig. 1D, the -200 to +321 caldesmon promoter fragment was linked to a *lacZ* reporter gene. The *Kpn*I restriction fragment was microinjected into the male pronuclei of one-cell stage embryos to generate transgenic mice. Five positive founder mice were identified by Southern blot analysis (data not shown). In order to localize β -galactosidase activity,

extracts from various tissues were prepared from postpubertal transgenic and nontransgenic littermates. Enzyme activity was determined as described under "Experimental Procedures," and a typical result is shown in Fig. 5. Offspring from the three surviving lines of transgenic mice showed high levels of β -galactosidase activity in testis, and testis from the transgenic mice was the only tissue analyzed that specifically expressed elevated levels of β -galactosidase activity. Different absolute levels of β -galactosidase activity were measured from testes of mice from the different transgenic lines which was likely the result of integration of the transgene into different locations in the genome and/or the presence of different copy

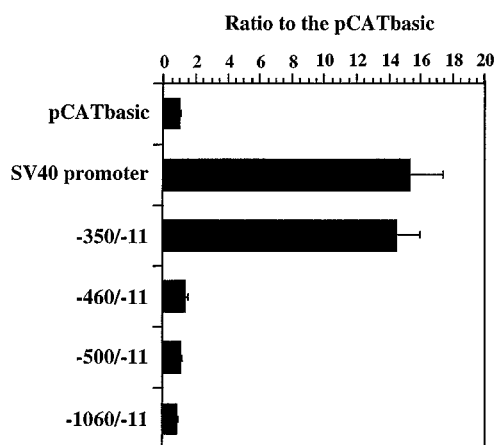


FIG. 4. **CaM kinase IV α promoter activity as a function of DNA length.** HeLa cells were transfected with 10 μ g of DNA containing different portions of the CaM kinase IV α promoter ligated to CAT (the sequence of the promoter regions used in each construct is shown in Fig. 3). CAT activity is expressed as the ratio of that obtained from the construct to be tested relative to pCAT basic which is a promoterless CAT construct. The results shown are the mean values of at least three independent experiments \pm S.E.

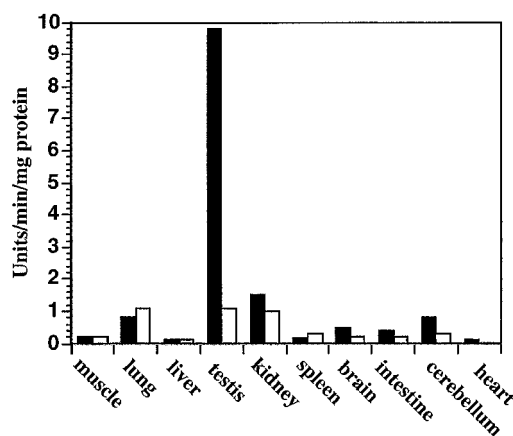


FIG. 5. **β-Galactosidase activity from various tissues.** Black bars indicate β-galactosidase activity from tissues of postpubertal transgenic mice. White bars indicate β-galactosidase activity from tissues of non-transgenic age-matched littermates. The experiment was repeated at least three times with animals from each of the three transgenic mice. These data are the results of a typical experiment.

numbers.

Testes from postpubertal transgenic mice were removed, briefly fixed, and then soaked in an X-gal solution for 5 h. The transgenic testis stained an intense blue as shown in Fig. 6A. A testis from an age-matched nontransgenic littermate was treated in an identical way and is shown in Fig. 6B. The control testis remained unstained and thus contained little β-galactosidase activity. Immunohistochemistry was performed to determine which cell types expressed β-galactosidase in postpubertal testis as shown in Fig. 6. The immunoreactive β-galactosidase protein was stained black as described under "Experimental Procedures," and the tissue sections were counterstained with hematoxylin. β-Galactosidase was localized primarily in the central portion of the seminiferous tubules in testis from transgenic mice as indicated by the arrow (in Fig. 6C). Fig. 6E is a higher magnification of one representative tubule. β-Galactosidase is present predominantly where elongating spermatids are located. No β-galactosidase expression was detected in testis from nontransgenic mice (Fig. 4D). These results reveal that β-galactosidase is expressed from the cal-spermin promoter specifically in postmeiotic germ cells during

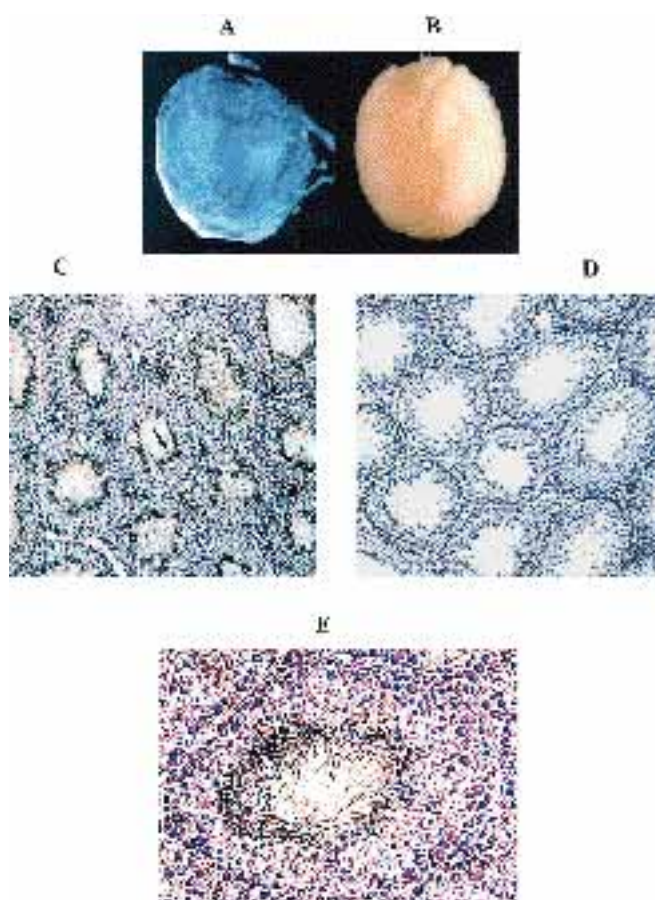


FIG. 6. **Localization of the β-galactosidase transgene product in testis.** A, a testis of a postpubertal transgenic mouse was removed and stained in a solution containing X-gal for 5 h. The blue color indicates the presence of β-galactosidase which catalyzed hydrolysis of X-gal into an insoluble blue product. B, a testis from a nontransgenic age-matched littermate treated in an identical manner to the testis described in A. C and D, immunohistochemical localization of β-galactosidase protein in seminiferous tubules. Immunoreactive β-galactosidase is stained black. The tissue sections are counterstained with hematoxylin. C, section of testis from a postpubertal transgenic mouse. β-Galactosidase is primarily located in the central portion of the seminiferous tubules as indicated by the arrow. D, section of testis from a nontransgenic age-matched littermate. This section was treated identically to the section in Fig. 3C. Note the complete absence of β-galactosidase reaction product. E, a higher magnification of one seminiferous tubule from the testis of a transgenic mouse (the magnification is $\times 200$). β-Galactosidase is restricted to the central region of the tubule that contains postmeiotic elongating spermatids.

the late stages of spermiogenesis.

Expression of the transgene was also examined as a function of postnatal development. The first cycle of spermatogenesis begins at birth and is completed at about day 34 (Russell, 1990). Spermatogonia constitute the predominant germ cell population in 6–8 day mice. Leptotene and zygotene spermatocytes appear at day 10, pachytene spermatocytes at day 14, round spermatids at day 19–20, and elongating spermatids at day 23–25 (Bellve *et al.*, 1995). β-Galactosidase activity of testis from transgenic mice of various postnatal ages was measured as shown in Fig. 7. There was no significant change in β-galactosidase activity before day 19. The first obvious increase of β-galactosidase activity occurred between days 22 and 24, correlating with the initial appearance of elongating spermatids. β-Galactosidase activity continued to increase until day 35 when the first round of spermatogenesis is completed. Similar changes of β-galactosidase activity during testis development were observed in the other two lines of transgenic mice (data not shown). Collectively the observations suggest that expres-

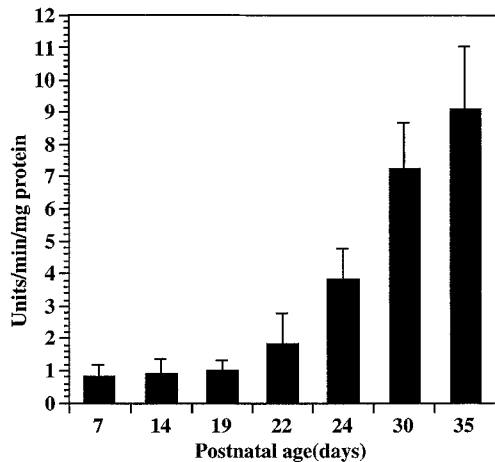


FIG. 7. β -Galactosidase activity from testes of transgenic mice of different postnatal ages. Testes from 7-, 14-, 19-, 22-, 24-, 30-, and 35-day-old transgenic mice were removed and the β -galactosidase activity was measured as described under "Experimental Procedures." The experiment was repeated at least three times on animals from each of the three transgenic lines. The data shown are the results of a typical experiment and represent the mean values of triplicate samples from each of three mice \pm S.E.

sion of the *lacZ* transgene is primarily restricted to postmeiotic germ cells.

Transgene Expression Can be Rescued in Skin Fibroblasts of Transgenic Mice by Expression of CREM τ —To test the hypothesis that the lack of expression of the -200 to $+321$ caldesmon promoter in somatic tissues was due to an insufficient concentration of CREM τ (Sun *et al.*, 1995), we prepared primary cultures of skin fibroblasts from transgenic mice. As shown in Fig. 8, these cells did not express β -galactosidase, confirming the *in vivo* results. The remainder of Fig. 8 reports results of a typical experiment designed to restore transgene expression. Expression vectors for CREM τ , CaM kinase IV, or both were transfected into the fibroblasts. After 4 days the cells were stained for X-gal and the number of blue cells within 10 random microscopic fields were counted. Whereas neither CREM τ or CaM kinase IV alone was effective, cotransfection of CREM τ and CaM kinase IV resulted in β -galactosidase expression in all transfected cells. Since cAMP dependent protein kinase can also phosphorylate CREB and CREB is present in the fibroblasts, cell cultures were treated with forskolin to elevate cAMP. This treatment also failed to restore transgene expression. These results demonstrate that high levels of transcriptionally competent CREM τ produced by phosphorylation by CaM kinase IV are sufficient stimulus to restore expression of the caldesmon promoter-driven transgene in a somatic cells that is otherwise unable to express the normally testis-specific promoter.

DISCUSSION

The rat CaM kinase IV gene spans at least 42 kbp of DNA and contains 12 exons. With the exception of the second (362 bp) and last (456 bp) exons, the other nine average only 82 bp in length. This complicated structural organization, if the norm, might explain why other genes encoding calmodulin-dependent enzymes from multicellular organisms have yet to be sequenced in their entirety. The location of the introns in the coding region seems to disrupt at random, and without other CaM kinase gene structures to compare, little else can be concluded.

The unusual aspect of the CaM kinase gene organization is that alternative promoters exist in the first and penultimate introns. The intron 1 promoter presumably directs transcrip-

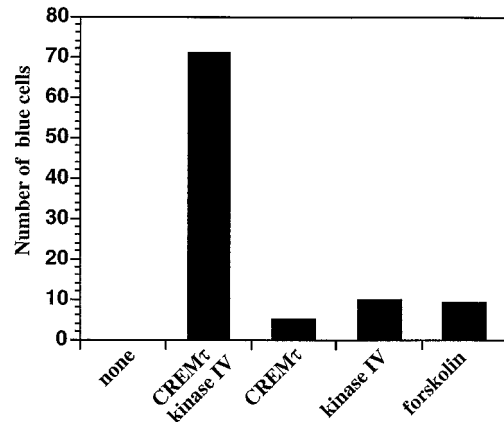


FIG. 8. Restoration of β -galactosidase activity in skin fibroblasts of transgenic mice. The cells were prepared from 2-day-old transgenic mice and were either transfected with expression vectors as indicated or treated with forskolin. The fibroblasts were stained with a solution containing X-gal 4 days after transfection. The data presented represent the total number of blue cells present within 10 random microscopic fields. A pCMV-CAT construct was cotransfected to control for transfection efficiency. All cells transfected with CREM τ and CaM kinase IV were forced to express β -galactosidase. The experiment was repeated three times with cells prepared from each of the three transgenic lines. The data shown are representative of all experiments.

tion of the smaller or α form of CaM kinase IV which is the enzyme on which most of the biochemical characterization has been performed. We show that a fragment extending about 150 bp from the transcriptional start site at -211 (Fig. 3) can function as a promoter in HeLa cells. This 150-bp fragment contains GC-rich regions typical for those present in a variety of genes but does not contain a TATA box. In the only other published study evaluating regulation of the expression of a CaM kinase gene, Olson *et al.* (1995) show the importance of a TATA box at -162 of the CaM kinase II α gene. Extending the 150-bp promoter fragment of the CaM kinase IV gene 100 bp or more at the 5' end severely reduces transcription in HeLa cells. Since CaM kinase IV α expression is generally limited to thymic lymphocytes, cerebellum, and testicular germ cells undergoing meiosis, perhaps the silencing due to addition of additional 5' sequences reflects such cell specificity. Alternatively, it is possible that expression of CaM kinase IV requires regulation, and HeLa cells do not contain such putative regulatory factors. For example, a preliminary report suggested that expression of the enzyme in an embryonic stem cell-derived neuronal culture required thyroid hormone and the thyroid hormone receptor α (Larson *et al.*, 1995). The availability of the 5'-flanking region of the CaM kinase IV gene will allow a mechanistic approach to this problem.

The organization of the CaM kinase IV gene also allows a plausible explanation for how the β and α isoforms of the enzyme are generated. Sakagami and Kondo (1993) reported the isolation of a rat brain cDNA that encoded a different form of CaM kinase IV than that cloned by Means *et al.* (1991). The only differences were the presence of a 92-bp extension of unique sequence at the 5' end and the absence of 151 bp of 5'-nontranslated region present in the clone obtained by Means *et al.* (1991). The 92-bp extension was predicted to change the start site of translation resulting in an additional 28 N-terminal amino acids not present in the CaM kinase IV α . The first 8 amino acids would be encoded by the last 24 bp of the unique sequence, whereas the remaining 20 amino acids would be encoded by nucleotides present as part of the 5'-nontranslated sequence of the α cDNA. Whereas Sakagami and Kondo (1993) did not formally show the presence of a protein containing the extra 28 amino acids, they did utilize *in situ* hybridization to

reveal that the unique segments of the two cDNAs differently hybridized to anatomical structures present in parasagittal sections of rat brain. As shown in Fig. 3, the flanking region of the CaM kinase IV gene contains all the unique nucleotides present in both α and β CaM kinase cDNA clones. The 92 bp unique to β are separated from the initial common segment, also suggested to be present by Bland (1993), by an intervening 464 bp. We have interpreted this intervening segment to represent an intron due to the presence of donor and acceptor splicing sites. The fact that the first 151 bp of the α cDNA constitute the last segment of this intron implied that additional sequences in the intron might function as a promoter. Preliminary evidence supporting this possibility is shown in Fig. 4. Whereas our data cannot prove the existence of two CaM kinase IV isoforms derived from one gene, they do support the contention of Sakagami and Kondo (1993) that α and β are the products of distinct mRNAs.

What our results have proven is that the 10th intron of the CaM kinase IV gene contains the testis-specific promoter responsible for the production of the caldesmon protein. The -200 to $+321$ region of the caldesmon promoter fragment was sufficient to target expression of β -galactosidase to postmeiotic germ cells in a cell- and developmentally specific manner that mimics expression of the endogenous gene judged from *in situ* hybridization analyses (Means *et al.*, 1991). Sun *et al.* (1995) had shown that the -200 to $+361$ region of the caldesmon gene contained at least three elements that influenced expression. First the -80 to $+361$ fragment could be expressed in a variety of cultured cells. One required element included the two CREs at -70 and -50 . The second element was the 111-bp intron sequence separating the 3' end of the testis-specific caldesmon exon from the 5' end of exon XI that is common to both CaM kinase and caldesmon (Fig. 1C). This intron was subsequently shown to function in an orientation-dependent but distance-independent manner and was required for stimulation of transcription by CREM τ (Sun and Means, 1995). The third element was contained in the DNA between -200 and -80 , since the addition of this 120 bp largely inhibited transcriptional activity in NIH3T3 cells. We postulated that these negative elements could contribute to the inhibition of caldesmon gene expression in somatic tissues *in vivo*. The fact that the transgene driven by the -200 to $+321$ is not expressed in any somatic tissues examined in the present studies supports this suggestion (Fig. 5).

Although the -200 to $+361$ caldesmon promoter had very little activity when acutely transfected into NIH3T3 cells, some activity could be restored by cotransfection of expression vectors encoding CREB or CREM τ together with a protein kinase capable of specifically phosphorylating the transcription factors on Ser¹³³ or Ser¹¹⁷, respectively (Sun *et al.*, 1995). This modification is required for the transactivation function (deGroot *et al.*, 1993). Since NIH3T3 cells contain CREB, which is found at low levels in most cells, we questioned whether an important factor in the expression of the caldesmon promoter might be the concentration of CRE-binding protein. Indeed the pattern of expression of CREM τ is very similar to that of caldesmon and the caldesmon promoter transgene. The mRNA for the transcriptional activating form of the CREM gene is first produced in very late meiotic cells (Foulkes *et al.*, 1992), but the protein does not appear until later and accumulates to very high levels in cells undergoing the late stages of differentiation during spermiogenesis (Delmas *et al.*, 1993). Therefore high concentrations of CREM τ are present in the same cells that transcribe the endogenous caldesmon gene (Means *et al.*, 1991) or the transgene driven by the -200 to $+321$ portion of the caldesmon promoter (Fig. 6). We reasoned

that the transgene would have integrated in a random fashion and conducted preliminary experiments, suggesting that the site of integration was different in all three transgenic lines (data not shown). Thus, if our hypothesis was correct, transgenic expression should be restored in somatic cells by simply increasing the cellular content of CREM τ . The data in Fig. 8 show, indeed, that the transgene can be expressed in primary skin fibroblasts but only when the cells are transfected with expression vectors for CREM τ and CaM kinase IV. Although the data shown are from a single transgenic line, similar results were obtained with cells derived from all three lines (data not shown). We would argue that one critical factor responsible for the cell-specific expression of the caldesmon gene is the availability of high levels of CREM τ in those cells at the developmentally correct time.

Clearly, the abundance of CREM τ cannot be the only factor required for caldesmon gene expression, or its presence would be sufficient to activate the endogenous gene in somatic cells which is not the case (Fig. 8 and Sun *et al.*, 1995). This has been aptly shown for the testis-specific promoter of the angiotensin converting enzyme gene which also contains a requisite CRE that binds CREM τ (Goraya *et al.*, 1995). Although this 85-bp regulatory region is sufficient to faithfully target testis-specific expression in transgenic mice (Howard *et al.*, 1993), it is not active in heterologous cells even when cotransfected with CREM τ . As shown by Goraya *et al.* (1995), mutation of the cryptic TATA box (TCTTATT) to a consensus TATAATT results in a promoter that can be expressed in heterologous cells and is responsive to CREM τ . Thus a second important factor for germ cell-specific expression could be the nature of the basal promoter elements. A third likely contributing factor could be a reorientation or modification of the gene that occurs as chromatin is remodeled following meiosis. One of the mechanisms determining the accessibility of promoters to transcription factors is DNA methylation. DNA methylation is thought to play a role in the regulation of tissue-specific gene expression (Cedar, 1988). Methylation inhibits gene expression by interfering with transcription factor binding to DNA. Studies on the DNA methylation patterns of testis-specific genes reveal a very good correlation between undermethylation and gene expression. The testis-specific histone H2B (Choi and Chae, 1991), TP1 (Trasler *et al.*, 1990), and PGK2 (Ariel *et al.*, 1991) genes are undermethylated in testis, but more methylated in somatic tissues where they are not expressed. It would be interesting to study the methylation patterns of the caldesmon transgene and the endogenous caldesmon gene and evaluate the accessibility of CREM τ to the CRE motifs in the promoter regions of both genes.

Since CRE motifs are conserved within promoters of many postmeiotic germ cell specific genes (Howard *et al.*, 1993; Delmas *et al.*, 1992), it is tempting to speculate that they also are exposed during meiosis by chromatin rearrangement and/or demethylation and in this state also respond to the high levels of CREM τ . Were this true, then the rate-limiting signal could be the stimulus that results in the production of the CREM τ transcript from the CREM gene primary transcript. That this event is controlled by follicle-stimulating hormone (Foulkes *et al.*, 1993) might provide one explanation for the requirement of this hormone for complete spermatogenesis (Means *et al.*, 1976).

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