MOLECULAR AND CELLULAR BIOLOGY, Jan. 1995, p. 561–571 Vol. 15, No. 1
0270-7306/95/$04.00+0

Calspermin Gene Transcription Is Regulated by Two Cyclic AMP Response Elements Contained in an Alternative Promoter in the Calmodulin Kinase IV Gene

ZUOMING SUN,[1] PAOLO SASSONE-CORSI,[2] AND ANTHONY R. MEANS[1]*

Department of Pharmacology, Duke University Medical Center, Durham, North Carolina 27710,[1] and Laboratoire de Génétique Moléculaire des Eucaryotes, U184, Centre National de la Recherche Scientifique, Faculté de Médecine, 67085 Strasbourg, France[2]

Received 10 August 1994/Returned for modification 9 September 1994/Accepted 28 October 1994

The transcript for the high-affinity Ca^{2+}/calmodulin-binding protein calspermin is generated from the gene encoding Ca^{2+}/calmodulin-dependent protein kinase IV only in postmeiotic germ cells during spermatogenesis. We demonstrate that this testis-specific calspermin transcript can be produced in heterologous cells by utilization of a promoter located in an intron of the calmodulin (CaM) kinase IV gene. Critical motifs within this promoter are two cyclic AMP response element (CRE)-like sequences located about −70 and −50 bp upstream of the transcriptional initiation site. Both CRE motifs are footprinted by the authentic testis-specific transcriptional activator CREMτ or by CREMτ present in adult testis nuclear extract. Whereas a 2.1-kb DNA fragment containing the calspermin promoter is inactive when transfected into NIH 3T3 cells, activity can be restored by cotransfection of CREMτ and protein kinase A or CaM kinase IV but not CaM kinase IIα. Restoration of activity is greatly reduced by mutation of the two CRE motifs. Since CRE-like motifs have been identified in many genes uniquely expressed in postmeiotic germ cells, which contain abundant CREMτ protein, we suggest that CREMτ may function as one transcription factor responsible for the expression of postmeiotic germ cell-specific genes.

Spermatogenesis is a cyclic and continual process by which spermatogonia are produced from renewable stem cells (32). Each stem cell divides mitotically, and the daughter cells remain associated by a cytoplasmic bridge. Subsequent mitotic divisions yield a cohort of interconnected cells that undergo two meiotic divisions, resulting in haploid spermatids. These postmeiotic cells then initiate a remarkable differentiation process termed spermiogenesis that produces the immature spermatozoa. Both meiosis and spermiogenesis require a large number of unique proteins not found in the mitotically dividing germ cells (21, 46). A number of such proteins arise by the activation of genes expressed only in a cell-specific manner. Considerable attention is now focused on the sequence of regulatory elements that impart germ cell specificity and on identification of the cognate transcription factors (2, 7, 14, 15, 40, 42, 49). This quest is made much more difficult since no permanent line of postmeiotic germ cells has been established.

In the absence of an appropriate cell line, transgenic mice have been employed to evaluate germ cell-specific regulatory regions of genes expressed only subsequent to late meiosis (18, 29, 31). In such studies a 113-bp fragment of the mouse protamine I gene promoter (48) and a 91-bp piece of the angiotensin-converting enzyme (ACE) gene promoter (14) have been shown to be sufficient to target expression to haploid germ cells. Both promoter fragments contain a cyclic AMP response element (CRE)-like element that can be protected by testis nuclear extract in a DNase I footprint assay. However, the cognate factor has not been identified, and the CRE-like element has not been proven to be functionally important by either mutagenesis or deletion analysis. We previously identified two CRE-like elements that occur −70 and −50 bp upstream of the transcriptional initiation site of the calspermin gene which is also expressed only in postmeiotic germ cells (21). Calspermin is a high-affinity Ca^{2+}/calmodulin-binding protein that is present exclusively in postmeiotic male germ cells, including spermatozoa (28). The calspermin transcript is derived from a gene encoding a Ca^{2+}/calmodulin-dependent protein kinase called CaM kinase Gr or CaM kinase IV (16, 22, 26). Whereas the kinase mRNA is present in several somatic tissues and in early- to mid-phase meiotic primary spermatocytes, the calspermin transcript is restricted to late primary spermatocytes and increases in abundance throughout spermiogenesis (22). Because the 5′ end of the calspermin mRNA was found in an intron of the CaM kinase IV gene, we postulated that this short region constituted a germ cell-specific exon and was utilized only when the immediately upstream region served as a promoter in the appropriate germ cells. The two CRE-like elements were suggested to play a regulatory role in this transcriptional process (21).

We now demonstrate that the region of the intron immediately upstream of the germ cell-specific exon can serve as a promoter. Optimal transcriptional activity in NIH 3T3 cells requires the 111-bp intron that exists between the 3′ boundary of the germ cell-specific exon and the 5′ boundary of the first exon that is common to calspermin and CaM kinase IV transcripts. The two CRE-like motifs are required for this promoter activity and can be protected by testis nuclear extract as well as by the testis-selective CRE transcriptional activator CREMτ. The activity of the basal promoter can be markedly stimulated by cotransfection of CREMτ and protein kinase A (PKA) or CaM kinase IV but not CaM kinase IIα. Moreover, cotransfection of CREMτ and either constitutively active kinase will restore activity of a longer DNA fragment that is completely inactive in NIH 3T3 cells. Since CRE-like motifs are present at similar positions in several genes expressed in a postmeiotic germ cell-specific manner (14, 27, 42, 49), we suggest that this factor may participate in the activation of a

* Corresponding author. Phone: (919) 681-6209. Fax: (919) 681-7767.

number of genes expressed at a similar time during spermatogenesis.

MATERIALS AND METHODS

DNA constructs. (i) Construction of chloramphenicol acetyltransferase (CAT) reporter gene. A 6-kb genomic DNA fragment which contains the calspermin gene promoter region was previously cloned into a pGEM-3Zf(−) vector (22). Different fragments of the calspermin promoter were amplified from the 6-kb 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 the pCAT basic vector (Promega), the 5′ primers (synthesized with an Applied Biosystems 392 DNA/RNA synthesizer) were designed to contain a *Pst*I site and the 3′ primer was designed to contain a *Xba*I site. PCR products (amplified with a Perkin-Elmer Cetus DNA thermal cycler), which were digested with *Pst*I or *Xba*I, were then subcloned between the *Pst*I and *Xba*I sites of the pCAT basic vector. In all cases, the PCR-derived fragments were sequenced to insure that no error had been introduced. The oligonucleotide sequences used in the construction of different-length promoter fragments are available upon request.

(ii) Expression vector for CaM kinase IV. CaM kinase IV cDNA (22) was used as a template to amplify the DNA fragment which encodes CaM kinase IV M-1 to Q-314 by PCR. This PCR fragment was then subcloned into the *Xba*I site of pRc/CMV (Invitrogen). The PCR primers used were sense 5′TCCGGAGTCTA GACGGCGAAGATGCTCAAAGTC3′ and antisense 5′CTTGCGCCTAGTC TAGAATTCTTAAAGCTTTTTC3′. The inactive kinase IV expression vector was created by mutating K-71 of the above-mentioned kinase IV construct to M with a Clontech Transformer site-directed mutagenesis kit. The mutant oligonucleotide sequence was 5′CCCTATGCTCTCATGGTGTTAAAGAAAAC3′, and the selection oligonucleotide sequence was 5′CTTCGGTCCTCCAATC GATGTCAGAAGTAAG3′, which incorporated a *Pvu*I-to-*Cla*I change.

(iii) Expression vector for CaM kinase IIα (pRSV-CaM KII). A CaM kinase IIα cDNA fragment encoding amino acids (aa) 1 to 290 was cloned into the pRSV expression vector. The inactive CaM kinase II was created by mutating Lys-42 of the ATP binding site to Met (K42M).

(iv) Expression vectors for the catalytic subunit of protein kinase A and its inactive counterpart and CREB. Expression vectors for the catalytic subunit of protein kinase A and its inactive counterpart as well as CREB (39) were gifts from Richard A. Maurer (Oregon Health Sciences University, Portland).

Mutagenesis. The two CREs were mutated as recommended by Clontech for use of its Transformer site-directed mutagenesis kit. The sequence for the −50 CRE mutant oligonucleotide was 5′GAGTTCTAAGAGTCCTGACAATATG GGC3′, and the sequence for the mutant −70 CRE was 5′CTGAGGGAATCA TGACTTTTGAGTTC3′. The sequence for the selection oligonucleotide used for the mutation was 5′ATCATGTCTGGATCCCCCGGAATTC3′, which incorporated a *Kpn*I-to-*Bam*HI change.

Transfection and CAT assay. NIH 3T3 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. Cells were plated at an initial density of 10^6 cells per culture dish (diameter, 60 mm) and maintained in a 5%-CO_2 atmosphere at 37°C. The medium was changed 3 h prior to transfection. DNA-$CaPO_4$ coprecipitation was used to transfect 10 μg of plasmid DNA in each plate as described by Promega for use of the ProFection mammalian transfection system. Cells were incubated in medium containing DNA precipitate for 48 h for the purpose of the CAT assay. Harvested cells were sonicated (Branson Sonifier 250) in an Eppendorf tube to rupture plasma membranes. Released cytosol was used for the CAT assay as described previously (12) with [dichloroacetyl-1-^{14}C]-chloramphenicol. Cytosolic protein (100 μg) was incubated for 1 h at 37°C with 2 μl of chloramphenicol (0.0025 Ci/ml)–10 μl of *n*-butyryl coenzyme A (5 mg/ml)–88 μl of 0.25 M Tris (pH 8.0). The reaction was stopped with 300 μl of xylene, and the mixture was then extracted three times with 100 μl of 0.25 M Tris buffer, pH 8.0. Activity was quantified in a 200-μl aliquot of the xylene phase by liquid scintillation spectrometry. A pCAT basic vector from Promega was used as a negative control, and pRSV-β-gal was used as the internal control in all assays. The CAT activities reported in the figures are the means ± the standard errors of the means (SEMs) from at least three experiments.

Nuclear extract preparation. Testis nuclear extracts were prepared as described by Howard et al. (14). Male Sprague-Dawley rats over 2 months old were used for nuclear extract preparation. The final concentration of nuclear extract was adjusted to 10 μg of protein per μl.

NIH 3T3 cell nuclear extracts were prepared as described by Shapiro et al. (34). The cells were harvested by scraping them into cold phosphate-buffered saline and collected by microcentrifugation in an Eppendorf tube. Cells were then resuspended in 5 volumes of PCV hypotonic buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 10 mM KCl, 1 mM dithiothreitol]) and allowed to swell on ice for 10 min before a second centrifugation. Cells were again resuspended in 2 volumes of PCV hypotonic buffer, transferred to a Dounce homogenizer, and stroked three to ten times with the Dounce B pestle to lyse the cells. After completeness of lysis was checked by microscopy, 0.1 volume of a sucrose-containing buffer (9 volumes of 75% sucrose with 1 volume of 10× salt solution, which consists of 0.5 M HEPES [pH 7.5], 7.5 mM spermidine, 1.5 mM spermine, 0.1 M KCl, and 2 mM EDTA) was added and the suspension was mixed by two strokes of a loose-fitting Dounce A pestle. The mixture was then centrifuged at 16,000 × *g* for 30 s after reaching the maximum speed. The supernatant was carefully decanted, and the pellet was resuspended in nuclear resuspension buffer (3 ml/10^9 cells) in an ultracentrifuge tube. Nuclear resuspension buffer contains 1 volume of saturated ammonium sulfate with 9 volumes of 20 mM HEPES (pH 7.9)–0.75 mM spermidine–0.15 mM spermine–0.2 mM EDTA–2 mM EGTA–25% glycerol. The sample was agitated by rocking the tube at 4°C for 30 min and then centrifuged at 150,000 × *g* for 90 min at 4°C. The supernatant was removed, and 0.33 g of ammonium sulfate per ml of supernatant was slowly added. After agitation by rocking the tube for 20 min at 4°C, the sample was centrifuged at 85,000 × *g* for 20 min at 4°C. The pellet was redissolved in nuclear dialysis buffer at a concentration of 1 ml/10^9 cells (20 mM HEPES [pH 7.9], 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM dithiothreitol). The samples were dialyzed twice for 90 min against nuclear dialysis buffer, and the final concentration of the protein was adjusted to 10 μg/μl.

DNase I footprint analysis. (i) Probe preparation. Ten micrograms of the plasmid DNA containing the region to be footprinted was digested with an appropriate enzyme designed to cut 25 to 100 bp away from the region to be footprinted (in this case, *Xba*I was used). The DNA was labeled with [α-^{32}P]-dCTP with Klenow fragment as described by Sambrook et al. (33). The labeled DNA was precipitated by adding ethanol. The pelleted DNA was digested with a second enzyme which would cut 150 to 600 bp away from the site to be footprinted (in this case, *Pst*I was used). The digested DNA was separated on a 5% polyacrylamide gel (in 1× Tris-borate-EDTA [TBE] buffer) at 300 V for 2 h. The expected size of the DNA probe was located by exposing the gel to X-ray film for 1 min. The area on the gel corresponding to the appropriate band on the film was cut out. The probe was then electroeluted from the gel in 1× TBE buffer and was precipitated by adding ethanol. The pellet was dissolved in Tris-EDTA (TE) buffer to yield about 10,000 cpm/μl.

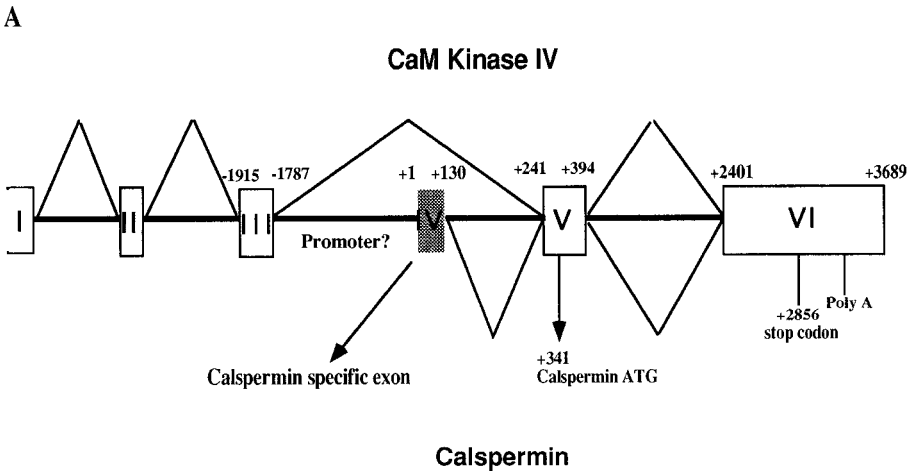
(ii) DNase I footprinting. The DNA probe (10^5 cpm) was incubated for 20 min at room temperature with 50 μg of nuclear extract protein in a 50-μl reaction mixture containing 5 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 5% glycerol, and 2 μg of dI-dC. Competition assays were performed by preincubating cold oligonucleotides with nuclear extract for 20 min before the labeled probe was added. At the end of the incubation, 5 μl of 2-μg/ml DNase I (which was diluted from a 2-mg/ml concentrated stock enzyme with a solution containing 25 mM $MgCl_2$ and 25 mM $CaCl_2$) was added to the sample and mixed well. After a 1-min digestion, the reaction was stopped by 100 μl of stop buffer (0.2 M NaCl, 0.03 M EDTA, 1% SDS, 100 μg of tRNA per ml). Samples were extracted once with phenol-chloroform (1:1) and precipitated by adding ethanol. The resulting pellet was resolved on a 6% sequencing gel, which was then exposed to X-ray film overnight.

Gel mobility shift assay. The synthetic oligonucleotide was annealed in 20 mM Tris (pH 7.0)–10 mM $MgCl_2$–50 mM NaCl by heating to 100°C for 5 min followed by cooling for 2 min on ice. The oligonucleotide (100 ng) was labeled with [γ-^{32}P]-dCTP by using T4 DNA polynucleotide kinase as described by Sambrook et al. (33). The labeled DNA oligonucleotide (5,000 cpm) was incubated for 20 min with 10 μg of nuclear extract protein in a 10-μl reaction mixture which contained the same components as the footprint buffer. The DNA and protein complexes were then resolved on a 5% native polyacrylamide gel in 1× TBE buffer. Competition assays were performed by preincubating cold oligonucleotide with nuclear extract for 20 min before the labeled probe was added. The sequence of the oligonucleotide probe for the −50 CRE was 5′GAGTTCTA AGTGACCTCACAATATGGGC3′. The oligonucleotide probe sequence for the −70 CRE was 5′ACTGAGGGAATGATGTCATTTGAGTTCT3′.

RESULTS

Structural organization of the CaM kinase IV and calspermin gene. Calspermin and CaM kinase IV transcripts are derived from the same gene (16, 22, 26). The organization of about 13 kb of genomic DNA that encodes all of calspermin and part of the kinase is shown in Fig. 1A. Primer extension analysis had been used previously to map the calspermin transcriptional initiation site (22). Six exons and five introns have been identified in the DNA fragment. Exons I, II, and III encode regions of the kinase not present in calspermin, whereas exons V and VI are common to calspermin and kinase. Exon IV is the only calspermin-specific exon. The calspermin transcript contains two introns. The first one is only 111 bp (+130 to +241), whereas the second one is about 2 kb (+394 to +2401). There are 230 bp of a 5′ untranslated region in the calspermin transcript. Calspermin-specific exon IV encodes the first 130 nucleotides (nt), and the remainder consists

A



B

-1930

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TAAATGATTGGTGGCATCTTATTTTACTGGACCAATGTCTAGAGAGACCGTGGAGTCCTCCCAGTGCAGGATGTAAGATGAGCTTCCACATGATGTAGAC
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TTGGACCCTAAGAAGCGGCTGACTACATTCCAAGCCCTCCAACACCCGTGGGTCACAGGAAAAGCAGCCAACTTTGTCCACATGGATACCGCTCAGAAAA
AGCTTCAAGAATTCAATGCTA(+361)
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FIG. 1. (A) Schematic representation of the CaM kinase IV and calspermin gene. Exons are indicated by rectangles. +1 is the transcriptional initiation site of the calspermin transcript, and the numbers indicate the positions of intron-exon junctions relative to the transcriptional initiation site. (B) Nucleotide sequence of the calspermin promoter, extending from −1930 to +361. Two CREs are indicated by rectangles. The arrow indicates the transcriptional initiation site, and the intron-exon junctions at +130 and +242 are denoted by brackets. The translational initiation codon ATG at +341 is underlined.

of the 100 nt immediately upstream of the calspermin translation initiation codon (ATG, at position +341), which is in exon V and encodes amino acids in the kinase. The calspermin translation initiation codon also specifies M-308 of the kinase (nucleotide 936 of the kinase cDNA). Calspermin shares the stop codon (at position +2856) and poly(A) site in exon VI with the kinase. Therefore, calspermin is composed of amino acids that are identical to the last 166 residues of the kinase. Thus, all but the first 130 nt of the 5′ untranslated region of the calspermin mRNA are common to calspermin and the kinase. We suspected that the regulatory elements controlling transcription of the calspermin gene would reside in the intron immediately upstream of the transcriptional initiation site. Figure 1B shows the DNA sequence extending from −1930 to +361.

Promoter consensus sequences upstream of the calspermin gene. In order to identify possible promoter elements and transcription factor binding sites upstream of the calspermin gene, we used a computer to analyze 1,787 nt of sequence immediately upstream of exon IV. A sequence, CTCAGAAG TC, immediately surrounding and including the calspermin transcriptional initiation site was found to be homologous to the initiator sequence which has previously been shown to function as a weak promoter in other genes (37, 38). This initiator sequence is preceded by two potential CREs at positions −50 and −70. Whereas the consensus CRE is an 8-nt inverted repeat, TGACGTCA, the middle 2 nt differ in both sequences found in the calspermin upstream DNA. Thus, the sequence at −50 is TGACCTCA, whereas that at −70 is TGATGTCA. In addition, at position −140 there is a growth hormone factor 1 binding site (CTATAAATCTC) (20). At position +170, which is just in the middle of the calspermin intron, there is a glucocorticoid receptor binding site (TGTT CT) (43). This putative regulatory region contains neither a TATA-like motif nor a CCAAT element, which are sequences found in many eucaryotic promoters.

Identification of a calspermin promoter. In order to determine whether portions of the intron function as a promoter,

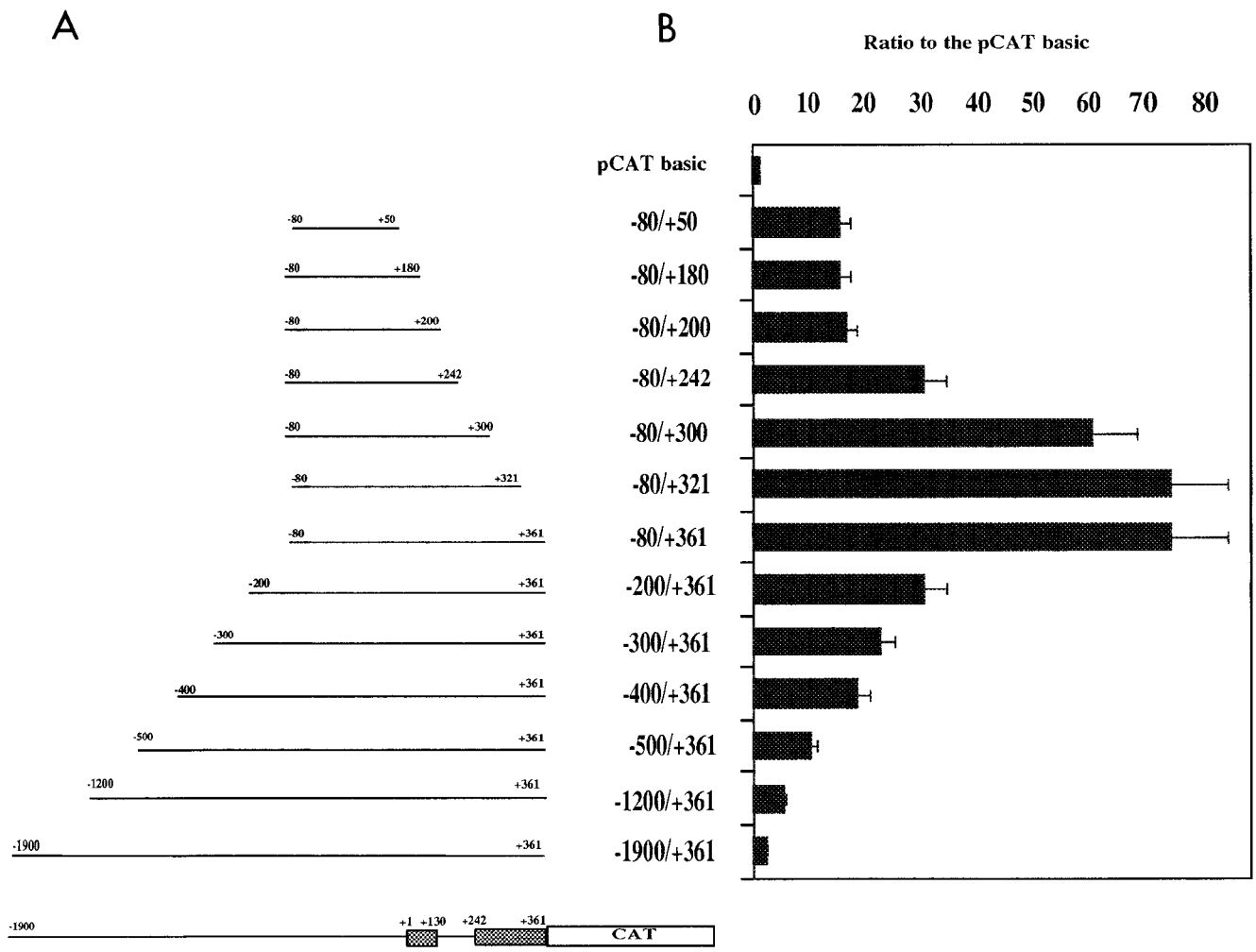

FIG. 2. Calspermin promoter activity as a function of DNA length. NIH 3T3 cells were transfected with 10 μg of DNA containing different portions of the calspermin promoter ligated to the CAT reporter gene. (A) The structure of the parental plasmid is shown at the bottom. The promoter region extends from −1900 to +361 and contains the calspermin-specific exon (+1 to +130, first shaded box), the first intron (+130 to +241), and part of the calspermin second exon (+242 to +361, second shaded box). The 5′- and 3′-terminal nucleotides of each promoter construct are shown. (B) CAT activity is calculated as the ratio of the activity obtained for the construct to be tested to that obtained for pCAT basic, which is a promoterless CAT construct. The results shown are the mean values of at least three independent experiments ± SEMs. The value for pCAT basic is 50 cpm ± 15 (n = 50).

different segments of DNA upstream of the calspermin gene were amplified by PCR and linked to a CAT reporter gene. The structure of the parental construct is shown in Fig. 2A. The transcriptional initiation site of the calspermin gene is designated +1. The calspermin exon sequences include the calspermin-specific exon IV from +1 to +130 and a portion of common exon V from +242 to +361. The sequence from +130 to +241 is the first calspermin intron. The DNA segment from −1900 to +1 contains about 1.8 kb of the kinase intron sequence which is located between exon III and exon IV. Figure 2A shows the extent of each DNA fragment linked to the CAT gene. The ability of each construct to direct expression of the CAT gene was analyzed following transfection into NIH 3T3 cells by the $CaPO_4$ method. The results shown in Fig. 2B 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 construct), were included.

The CAT construct containing the DNA fragment from −80 to +361 (the −80/+361 construct) produced the highest level of CAT activity, which is the same level of activity produced by the simian virus 40 promoter plus an enhancer (i.e., about 100 times that of pCAT basic). Similar results were obtained from preliminary studies using other cell lines, including HeLa and NS20 (data not shown). This result suggests that the upstream region of the calspermin gene harbors a transcriptionally active promoter element. Several deletion mutations were made from +361 to +50. Although the first two deletions to +321 and +300 had almost no effect on CAT activity, the deletions close to (the sequence spanning −80 to +242) or into (−80 to +200 and −80 to +180) the intron sequence greatly reduced CAT activity, suggesting that the intron sequence is important for promoter activity. Since a glucocorticoid receptor binding site was identified by computer analysis, an expression vector encoding a glucocorticoid receptor was cotransfected with the −80/+361 construct and dexmethasone was added to cells to serve as a ligand for the receptor. The presence of a hormone-bound receptor had no effect on promoter activity (data not shown). Extensions to the promoter from −80 to −1900 pro-

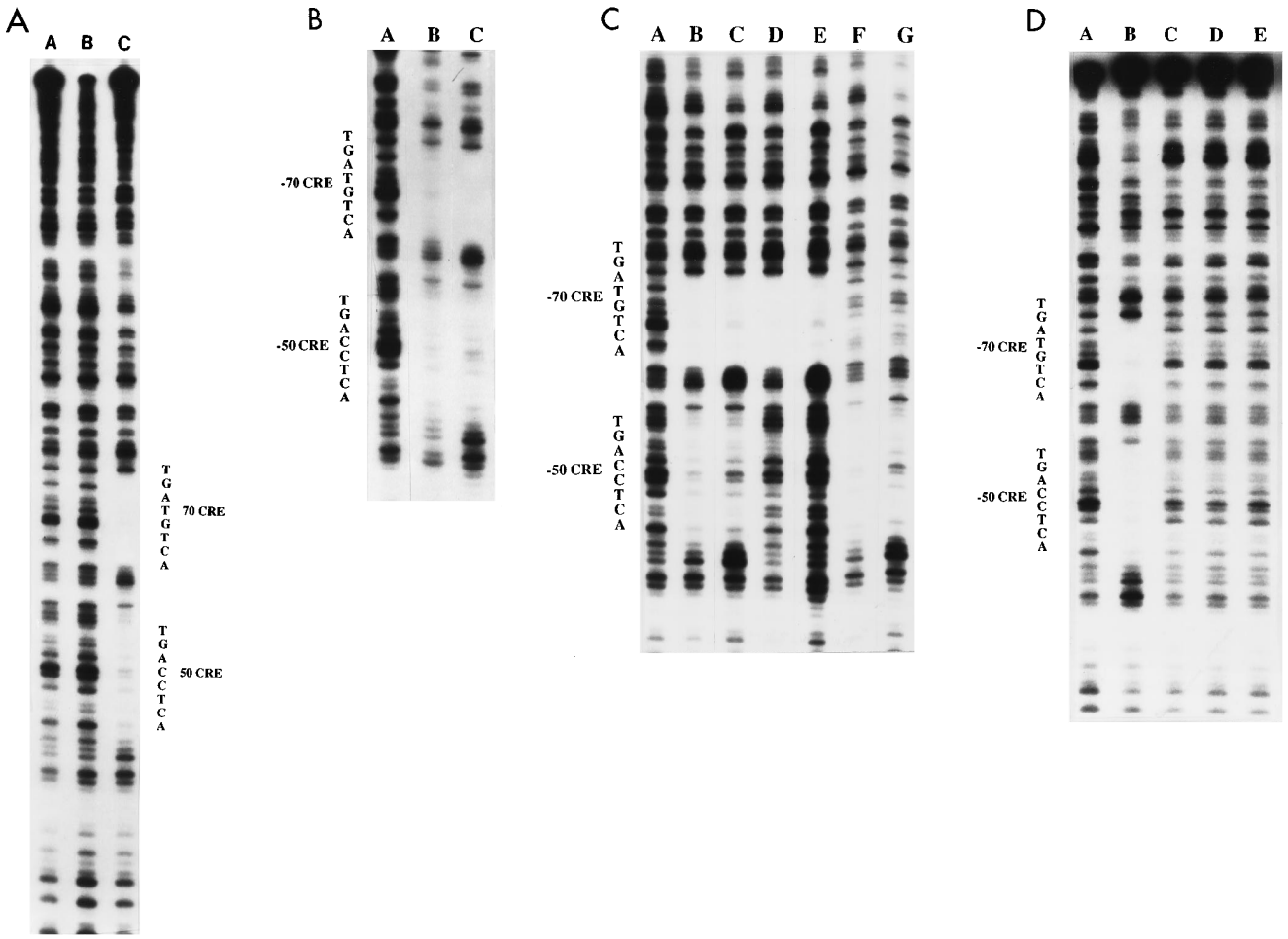

FIG. 3. (A) Identification of two CRE binding sites of the core calspermin promoter by DNase I footprinting with NIH 3T3 cell nuclear extract. The probe sequence spanned −500 to +50. Lanes A and B, BSA (control); lane C, NIH 3T3 cell nuclear extract. (B) Protection of the two CREs by purified CREB. The probe sequence spanned −500 to +50. Lane A, BSA (control); lane B, testis nuclear extract; lane C, purified CREB. (C) Mutation of the two CREs prevents protection by CREMτ and testis nuclear extract. The probe sequence spanned −500 to +50. Lane A, BSA (control); lane B, wild-type probe and testis nuclear extract; lane C, wild-type probe and purified CREMτ; lane D, probe containing mutated −50 CRE and testis nuclear extract; lane E, probe containing mutated −50 CRE and purified CREMτ; lane F, probe containing mutated −70 CRE and testis nuclear extract; lane G, probe containing mutated −70 CRE and purified CREMτ. (D) Two CREs are specifically footprinted by testis nuclear extract. Testis nuclear extract was used to footprint a probe (sequence, −500 to +50). Lane A, BSA (control); lane B, testis nuclear extract; lane C, competition assay with 20 ng of cold −50 CRE oligonucleotide; lane D, competition assay with 20 ng of cold −70 CRE oligonucleotide; lane E, competition assay with 20 ng (each) of cold −50 and cold −70 CRE oligonucleotides.

gressively reduced CAT activity, and the −1900/+361 construct had no activity in NIH 3T3 cells. This result indicates that there are negative regulatory elements between −80 and −1900 that contribute to the reduced CAT activity in NIH 3T3 cells.

Identification of two CREs by DNase I footprinting. In order to determine whether there are regulatory elements within the promoter region that function as active binding sites for transcription factors, we carried out DNase I footprint analysis. The probe containing the sequence from −500 to +50 was labeled at the +50 end. NIH 3T3 cell nuclear extract (50 μg) was incubated with this probe for 20 min prior to partial digestion with DNase I. The digested products were separated on a 6% sequencing gel, and footprint results were observed by autoradiography (Fig. 3A). Two clearly protected regions were detected (Fig. 3A, lane C) and mapped to the positions of the two putative CREs around −50 and −70. Both CRE-like motifs were footprinted by NIH 3T3 cell nuclear extract but not by bovine serum albumin (BSA) (Fig. 3A, lanes A and B).

It has been shown that CREB and CREMτ bind to CREs and stimulate transcription from promoters that contain these elements (9, 25, 30, 47). In order to determine whether both CREs can directly interact with CREB or CREMτ, we performed footprint assays with these purified transcription factors. The results show that both CRE domains were protected by CREB (Fig. 3B, lane C) purified from rat brains (24) and CREMτ (Fig. 3C, lane C) expressed in and purified from bacteria (6), revealing that CREB and CREMτ can directly bind to both elements. Interestingly, the −70 CRE is much better protected than the −50 CRE by both CREB and CREMτ, which may reflect a differential affinity of the DNA-binding proteins for the different CRE sequences.

In order to determine whether the consensus sequences of both CREs are important for CREMτ binding to the calspermin promoter, point mutations were made to disrupt the inverted repeat structure of each element. The −50 CRE was changed from TGACCTCA to AGTCCTGA, and the −70 CRE was changed from TGATGTCA to TCATGACT. Probes containing the mutated CREs were subjected to footprint assays as shown in Fig. 3C. Mutation of either the −50 CRE (Fig.

3C, lane E) or the −70 CRE (Fig. 3C, lane G) prevented protection by CREMτ, whereas both sequences were protected in the wild-type construct (Fig. 3C, lane C). This result indicates that the binding of CREMτ to both CREs is sequence dependent.

Physiologically, calspermin mRNA is detected only in the mature testis. In order to address the question of whether elements in the promoter could also be utilized by the testis, we carried out DNase I footprint assays with testis nuclear extract (Fig. 3D). The same two CREs which were protected by NIH 3T3 cell nuclear extract were also protected by testis nuclear extract (Fig. 3D, lane B). A competition assay was performed to determine the specificity of these interactions in the testis. Oligonucleotides based on either the −50 CRE (Fig. 3D, lane C), the −70 CRE (Fig. 3D, lane D), or both CREs (Fig. 3D, lane E) effectively competed for the protection of both CREs in the context of the promoter. Mutation of either the −50 CRE or the −70 CRE prevented protection by both CREMτ (Fig. 3C, lanes E for the mutated −50 CRE [CREm] and G for the −70 CREm) and testis nuclear extract (Fig. 3C, lanes D for the −50 CREm and F for the −70 CREm).

A DNA bandshift experiment was performed to determine whether the CREMτ present in adult testis nuclear extract could bind both CREs. The results are shown in Fig. 4A. Purified CREMτ shifted both the −50 CRE (Fig. 4A, lane 1) and the −70 CRE (Fig. 4A, lane 6). In both cases the shifted band was specifically inhibited by competition with corresponding oligonucleotides containing the CRE. Bandshift experiments with testis nuclear extract yielded three bands for both CREs. The second band comigrated with CREMτ (Fig. 4B, lane 2, and 4C, lane 2) and it was also effectively inhibited by competition with the corresponding oligonucleotides (Fig. 4B and C). Since CREMτ antibody recognizes the DNA binding domain, a supershift assay could not be performed. As an alternative approach, CREMτ in testis nuclear extract was depleted by preincubating with CREMτ antibody for 30 min before the probes were added. This treatment greatly reduced the signal of the band that comigrated with CREMτ (Fig. 4B, last lane, and 4C, last lane). The above-described results reveal that both CREs specifically bind to purified CREMτ as well as to CREMτ present in adult testis nuclear extract.

The two CREs influence activity of the core promoter. To test whether the two CREs have an effect on the promoter activity, CAT assays with the mutated DNA sequences that did not bind CREMτ were performed (Fig. 3C). The mutant constructs were transfected into NIH 3T3 cells, and CAT assay results are presented in Fig. 5. Figure 5A shows the structure of the wild-type construct, which consists of the −80/+361 promoter containing the −50 CRE and the −70 CRE. The structures of the mutant constructs are also shown (Fig. 5A). Mutation of the −50 CRE had a small but significant effect. Mutation of the −70 CRE reduced CAT activity by about 60%, suggesting that although both CREs contribute to promoter activity, the −70 CRE contributes more than the −50 CRE. These results agree with the footprint assay results, which indicated that the −70 CRE was much better protected than the −50 CRE. Mutation of both CREs resulted in about the same reduction of CAT activity as that observed with the −70 CRE mutation alone. In addition, deletion of the −70 CRE alone had an effect on CAT activity that was similar to the effect of mutating the −70 CRE alone and also to that for the promoter with both CREs mutated. We conclude that although both CREs have a positive role in regulation of the calspermin gene promoter activity, the role played by the −70 CRE seems more important.

Transcriptional activation of the calspermin promoter by

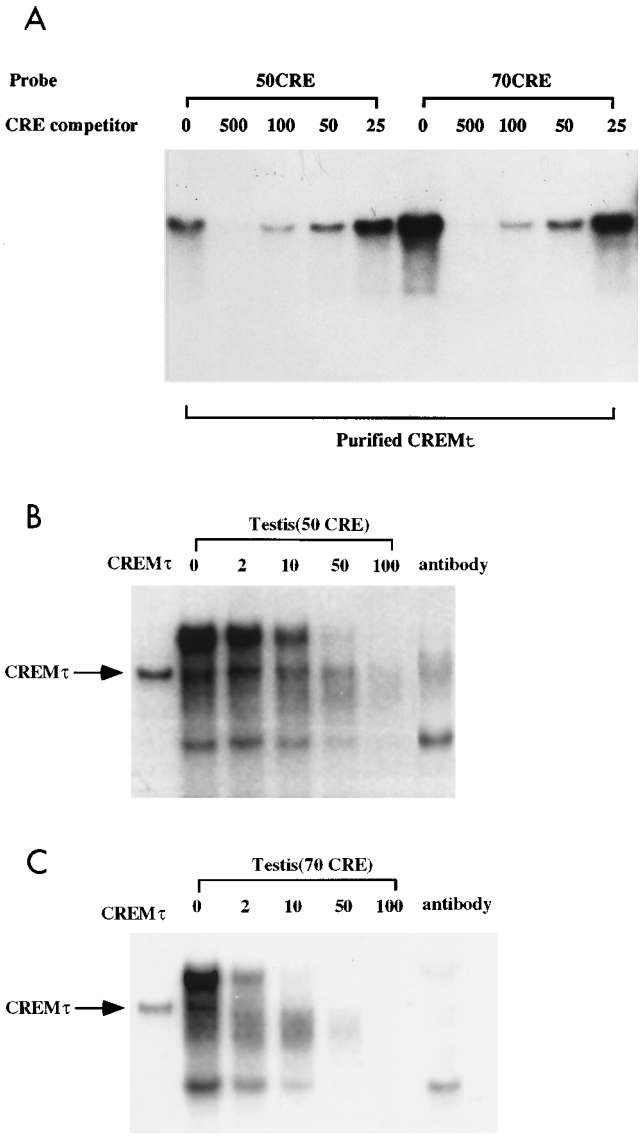

FIG. 4. (A) Two CREs are bandshifted by authentic CREMτ. Synthesized double-stranded DNA oligonucleotides representing either the −50 CRE or the −70 CRE were used as probes to perform bandshift assays with CREMτ. The amount of cold competitor oligonucleotide added is indicated, in nanograms. (B) The −50 CRE is bandshifted by CREMτ present in testis nuclear extract. The −50 CRE probe was used to perform a bandshift assay with testis nuclear extract. The amount of cold competitor oligonucleotide added is indicated, in nanograms. Last lane, a bandshift assay with testis nuclear extract pretreated by incubation with CREMτ antibody. (C) The −70 CRE is bandshifted by CREMτ present in testis nuclear extract. The −70 CRE probe was used to perform a bandshift assay with testis nuclear extract. The amount of cold competitor oligonucleotide added is indicated, in nanograms. Last lane, a bandshift assay with testis nuclear extract pretreated by incubation with CREMτ antibody.

CREMτ and PKA. It has been shown that CREMτ binds to CREs and stimulates transcription (9). CREMτ functions as a transcriptional activator only after it is phosphorylated by PKA. In order to determine whether CREMτ has an effect on the activity of the calspermin core promoter (the −80/+361 construct), which contains two CREs, NIH 3T3 cells were cotransfected with calspermin promoter CAT constructs and expression vectors of CREMτ or the catalytic subunit of PKA. The CAT assay results are shown in Fig. 6A. CAT activity generated by the promoter was increased eightfold by cotrans-

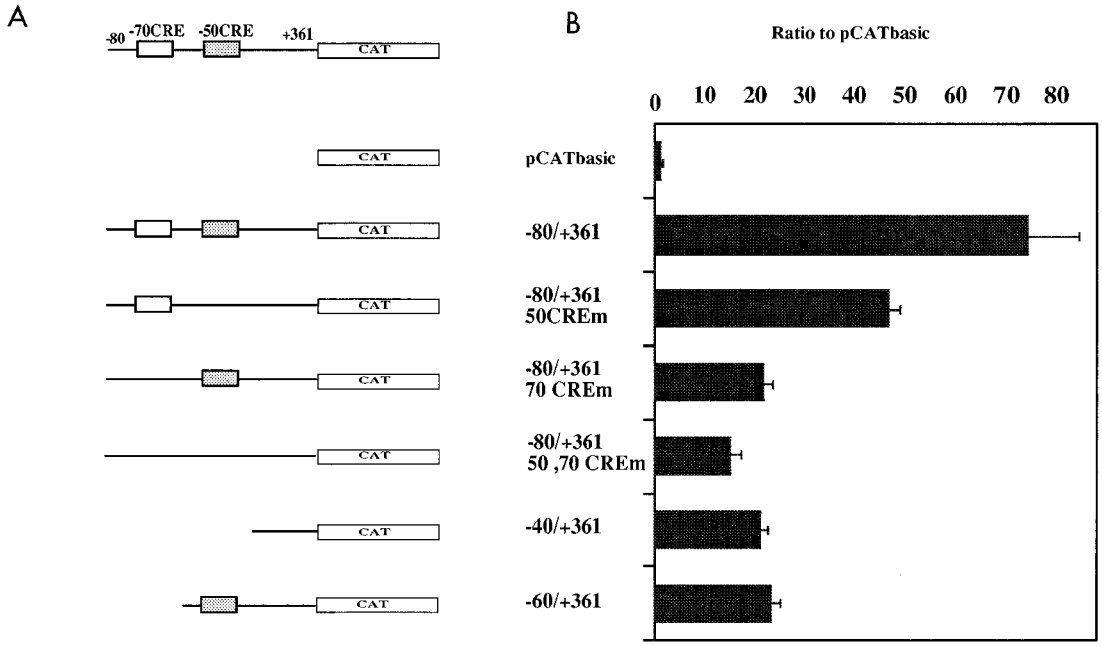

FIG. 5. Reduction of core promoter activity by mutation of the two CREs. (A) The parental plasmid is shown at the top and contains a core promoter plus the wild-type −50 and −70 CREs. Also shown are diagrams of the mutant constructs. The −50 CRE and the −70 CRE were mutated separately or together. Deletion of the −70 CRE or both CREs were done in the bottom two constructs. Ten micrograms of plasmid DNA was used in each transfection assay. (B) CAT activity is calculated as the ratio of the activity obtained from the construct being tested to the activity of pCAT basic. The results shown are the means of at least three separate experiments ± SEMs.

fection of CREMτ and PKA, whereas CREMτ alone did not result in stimulation. Similar levels of activation were achieved by cotransfection of the CREB and PKA expression vectors. PKA alone can induce some activity, a result that was predictable because NIH 3T3 cells contain endogenous CREB. These results suggest that CREMτ and CREB can greatly enhance transcription from the calspermin promoter and that this enhancement requires phosphorylation by PKA.

In order to determine whether the two CREs present in the core promoter directly mediated activation by CREMτ, cotransfection experiments which were similar but which used the deleted CRE constructs illustrated in Fig. 5 were performed. In our original experiment 5 μg of the CREMτ expression vector was used in each transfection. We were surprised to find that under these conditions, CAT activity of the −40/+361 construct, from which the two CREs had been deleted, was stimulated to the same extent by CREMτ as was found with the wild-type −80/+361 construct (compare the second set of bars in Fig. 6B). This suggested the possibility that a large excess of the transcription factor might be nonspecifically increasing transcription. To test this idea, we performed a series of transfections in which the amount of the CREMτ expression plasmid was decreased. As can be seen in Fig. 6B, 0.3 and 0.5 μg of CREMτ stimulated the activity of the wild-type construct (−80/+361) to a level similar to that produced by 5 μg of CREMτ. However, decreasing the amount of CREMτ progressively reduced the activity produced from the −40/+361 construct and at the 0.3- and 0.5-μg amounts, CAT activity was not significantly greater than that observed in the absence of CREMτ. These results suggest that (i) nonspecific effects can result from high levels of CREMτ and (ii) both CREs are directly involved in activation of the calspermin promoter by CREMτ.

Restoration of calspermin promoter activity by CREMτ and PKA. Whereas the calspermin promoter (−80/+361) exhibits very high levels of activity in NIH 3T3 cells, it is totally inhibited when the length of the DNA 5′ of the promoter is extended to 2.1 kb (the −1.9 kb/+361-CAT construct). Since we have shown that CREMτ stimulates promoter activity, we wanted to determine whether CREMτ could restore activity of the extended DNA fragment in NIH 3T3 cells. The results shown in Fig. 7 confirm that the 2.1-kb promoter-CAT construct alone produced no CAT activity but that cotransfection of CREMτ and PKA resulted in a robust stimulation of CAT activity. Transfection of CREMτ alone did not restore activity. Similar reactivation of the promoter activity of the 2.1-kb promoter was achieved by cotransfection of CREB and PKA but not by cotransfection of PKA and CREMα, which is a transcriptional repressor of the CREM family. Similar cotransfection experiments were performed with the mutated CRE constructs previously shown not to be protected by CREMτ or testis nuclear extract (Fig. 3C). The CREMτ- or CREB-mediated restoration of activity of the full-length calspermin promoter was greatly reduced. These results indicate that CREMτ and PKA are sufficient to activate the full-length calspermin promoter in NIH 3T3 cells and that both CREs are involved in this restoration of transcription.

Transcriptional activation of the calspermin promoter by CaM kinase IV and CREMτ. In vitro studies have shown that CREB can be phosphorylated by CaM kinase II (4, 35) and CaM kinase IV (3) and that CREMτ can be phosphorylated by CaM kinase II (5). We wanted to determine whether either CaM kinase IV or CaM kinase II could substitute for PKA to phosphorylate CREMτ and activate the calspermin promoter in cotransfection experiments. A plasmid that expressed a constitutively active truncated form of CaM kinase IV (aa 1 to 314) (pCMV-KIVa) was constructed. A plasmid expressing an inactive form of this CaM kinase IV (pCMV-KIVi) was created by mutating Lys-71 of the ATP binding site to Met (K71M). Two similar constructs that express constitutively ac-

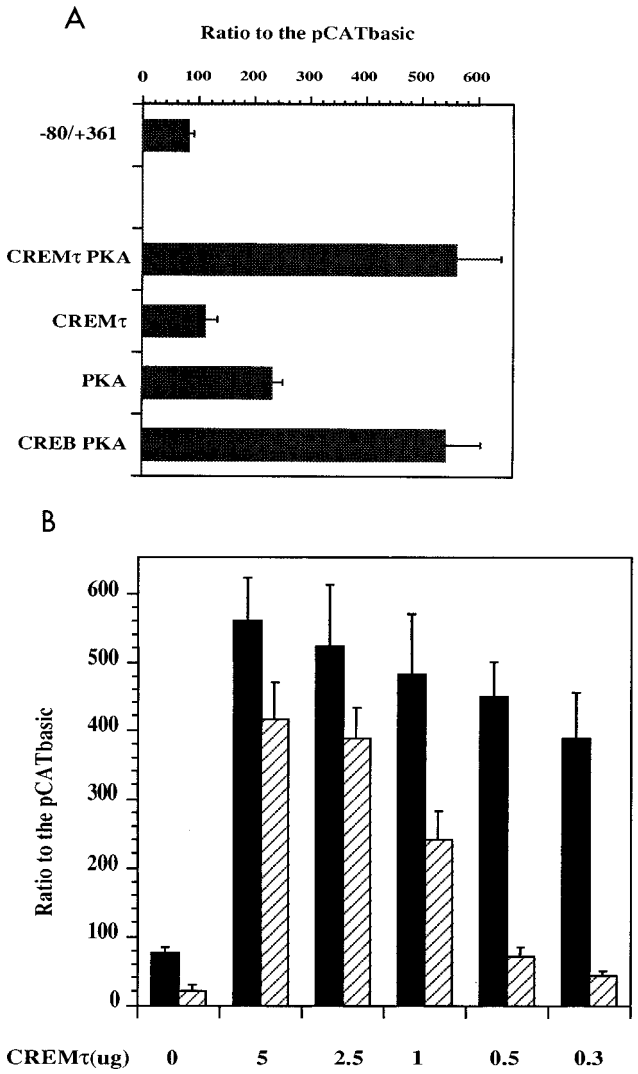

FIG. 6. Stimulation of calspermin promoter activity by CREMτ and PKA. Ten micrograms of wild-type or mutated promoter constructs was cotransfected with 0.5 μg of the CREMτ and 1 μg of the PKA expression vectors. For controls, the CAT constructs were transfected alone or cotransfected with CREMτ or PKA only. The mutant promoter-CAT constructs are the same as the ones shown in Fig. 4. The CAT activity is calculated as the ratio of the activity obtained from the tested construct to the activity of pCAT basic, and the results are the mean values of at least three independent experiments ± SEMs. (A) Wild-type −80/+361 CAT constructs were cotransfected with CREMτ and PKA. (B) Different amounts of CREMτ were cotransfected with either the wild-type −80/+361 CAT construct (black bars) or the deletion mutation CAT construct −40/+361 (hatched bars).

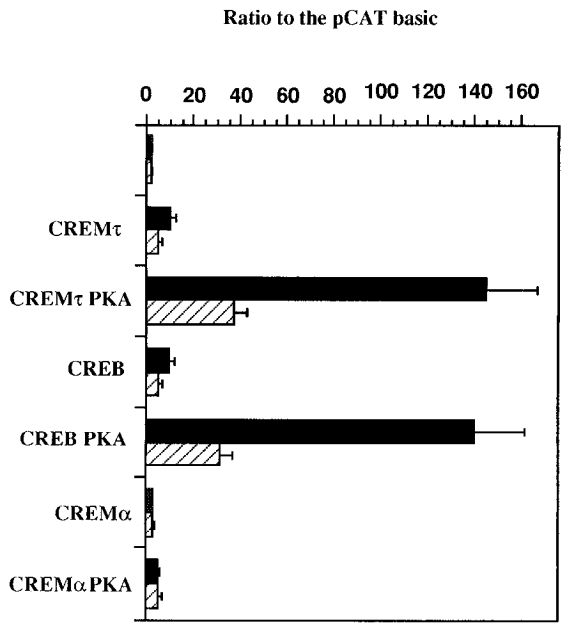

FIG. 7. Restoration of inactive calspermin promoter activity by CREMτ and PKA. Ten micrograms of either the −1.9 kb/+361-CAT construct (black bars) or the −1.9 kb/+361-CAT construct with both CREs mutated (hatched bars) were cotransfected with 0.5 μg of the CREMτ and 1 μg of the PKA expression vectors. The CAT activity is calculated as the ratio of the activity obtained from the tested construct to that of the pCAT basic. The results shown are the mean values of at least three independent experiments ± SEMs.

tive CaM kinase IIα (pRSV40-KIIa) and inactive CaM kinase IIα (aa 1 to 290) (pRSV40-KIIi) were also prepared. The assay results are shown in Fig. 8A. Cotransfection of CaM kinase IV and CREMτ resulted in an eightfold stimulation of core promoter activity, which is the same level of stimulation achieved by cotransfection of PKA and CREMτ. There was no stimulation of CAT activity if the plasmid encoding inactive CaM kinase IV was used instead of the plasmid encoding the active kinase, demonstrating that kinase activity is required for this stimulatory effect. Cotransfection of CaM kinase IV and CREB resulted in a level of activation similar to that generated by CaM kinase IV and CREMτ. Similar experiments were performed with CaM kinase IIα expression plasmids. In contrast to CaM kinase IV, neither the active nor inactive CaM kinase IIα resulted in stimulation of calspermin promoter activity. The results show that CaM kinase IV but not CaM kinase IIα can replace PKA to phosphorylate CREMτ and transcriptionally activate the calspermin promoter.

In order to determine whether CaM kinase IV can also replace PKA in the restoration of activity of the 2.1-kb calspermin promoter, we performed cotransfection experiments whose results are shown in Fig. 8B. Transcription from the inactive 2.1-kb calspermin promoter was restored by cotransfection of PKA and CREMτ as well as by cotransfection of CaM kinase IV and CREMτ but not by either inactive kinase alone or CaM kinase IIα. Similar restoration of transcription could also be accomplished by CaM kinase IV and CREB. These results reveal that like PKA, CaM kinase IV can phosphorylate CREMτ and restore activity to an otherwise inactive calspermin promoter in NIH 3T3 cells.

DISCUSSION

The calspermin transcript is initiated in an intron of the CaM kinase IV gene. This fact had been recognized by three laboratories working on both rat and mouse genes (16, 22, 26). Our results provide strong evidence that the novel transcript arises by differential transcriptional initiation relative to the CaM kinase IV gene and that *cis*-acting DNA sequences around the calspermin transcriptional initiation site function as a promoter. The optimal sequence required for basal promoter activity in several heterologous cell lines was found to be −80 to +361. At least four motifs are involved in transcription. Two of these are the CRE elements at −70 and −50. Mutation or deletion of both elements greatly reduces transcription. The calspermin promoter also contains an initiator sequence around the transcriptional initiation site. Studies by Smale et

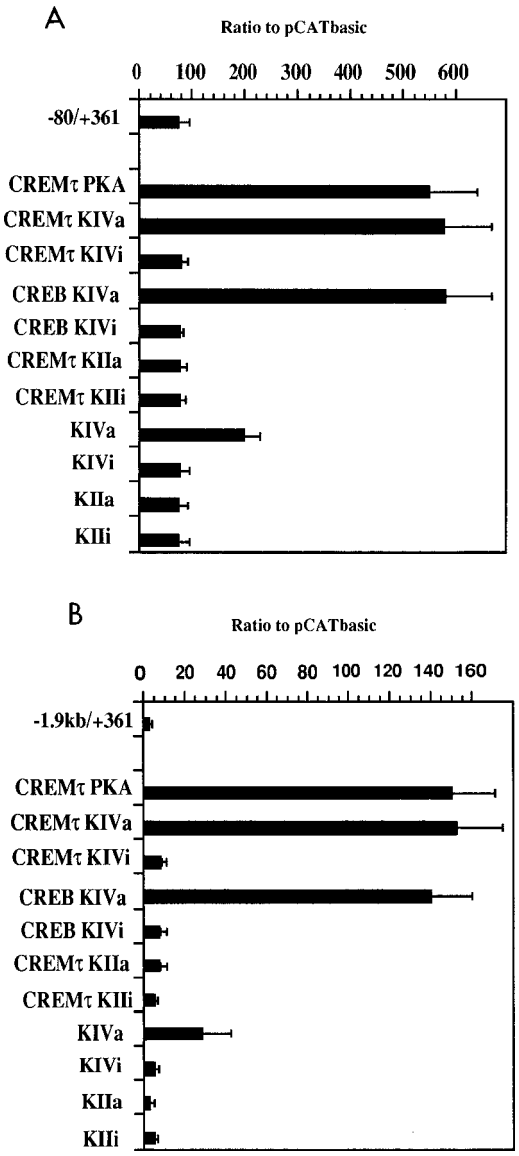

FIG. 8. Activation of calspermin promoter activity by CREMτ and CaM kinase IV. KIVa is a constitutively active truncated form of CaM kinase IV (aa 1 to 314). KIVi is the inactive CaM kinase IV (aa 1 to 314; K71M). KIIa is a constitutively active truncated form of CaM kinase IIα (aa 1 to 290). KIIi is the inactive form of CaM kinase IIα (aa 1 to 290; K42M). (A) Ten micrograms (each) of the −80/+361-CAT constructs was cotransfected with 1 μg of the indicated expression vectors. (B) Ten micrograms (each) of the −1.9 kb/+361-CAT constructs was cotransfected with 0.5 μg of the CREMτ and 1 μg of the indicated kinase expression vectors. The CAT activity is calculated as the ratio of the activity of the tested constructs to that of pCAT basic, and the results are the mean values from at least three independent experiments ± SEMs.

al. (37, 38) have shown that in several other genes this motif can direct RNA polymerase II to initiate transcription independent of a TATA sequence and that the presence of an upstream activator element results in high levels of accurately initiated transcripts. The fourth and perhaps most surprising element is the 111-bp intron that separates the unique 5′ end of the calspermin transcript from the sequences common to calspermin and the kinase. Deletion or inversion of this intron also abolishes transcription, although it contains no obvious transcription factor binding sites. Finally, our preliminary experiments measuring in vitro transcription in testis nuclear extract also reveal that optimal promoter activity requires the −80/+361 construct and confirm the importance of the intron as well as the CREs (data not shown).

Utilization of an alternative promoter in a gene to regulate expression of a transcript produced only in postmeiotic germ cells is not unique to the calspermin gene. Langford et al. (18) revealed that this was the mechanism used to generate the testis-specific form of the ACE from the gene that encodes the larger form of the enzyme expressed in somatic tissues. In this instance transcription begins 7 kb downstream from the promoter utilized to initiate the somatic transcript. Subsequently Howard et al. (14) revealed that the 91 bp of DNA immediately upstream of the testis ACE transcriptional initiation site was sufficient to target expression of a β-galactosidase fusion gene to postmeiotic germ cells in transgenic mice. A CRE motif is located at position −52 of this 91-bp promoter. This motif served as a positive transcriptional element in in vitro transcription assays and was protected by a protein present in the testis nuclear extract utilized to support transcription, as analyzed by DNase I footprinting. In addition, the region between −44 and −9 contained a cryptic TATA-like sequence, TCTTAT, that also supported strong transcriptional activity even in the absence of the CRE-like element.

The protamine genes are expressed at a time during spermatogenesis similar to that for the ACE and calspermin genes. However, these genes are expressed exclusively in the testis and are not parts of sequences also transcribed in somatic tissues (13, 26). As was shown for ACE, a 113-bp fragment of the mouse protamine 1 gene was demonstrated to target expression of a β-galactosidase fusion gene to postmeiotic germ cells in transgenic mice (48). This promoter fragment also contained a CRE-like element at about −60, the sequence of which is TGACTTCA. This sequence and the CRE-like motif in the ACE promoter have been suggested to bind a testis-specific factor. Such a factor, called Tet-1, has been reported by Tamura et al. (40). However, Zambrowicz and Palmiter (49) have utilized mutagenesis to identify residues 5′ of the CRE-like element that are required for binding of the factor present in testis nuclear extract but are not involved in Tet-1 binding. These authors conclude that Tet-1 probably is not involved in transcription of the protamine 1 gene mediated by the CRE motif and suggest that CREMτ might represent such a factor, although this was not tested directly.

Like the ACE and protamine 1 genes, the calspermin promoter contains a CRE-like sequence at about −50 (21). This sequence, TGACCTCA, and a second motif around −70, TGATGTCA, are important for promoter function in NIH 3T3 cells. They bind a protein present in testis nuclear extract and also bind either CREB or CREMτ. Our evidence suggests that the two motifs bind the same factor in testis nuclear extract even though the middle 2 nt differ from each other and from the consensus CREB-binding inverted repeat TGACG TCA, as well as from the CRE-like motifs present in the ACE and protamine 1 promoters. Either CREB or CREMτ will also markedly stimulate transcription from the −80/+361 calspermin basal promoter construct and restore activity to a longer DNA fragment when cotransfected with expression vectors for PKA or a constitutively active form of CaM kinase IV. The questions are which of these transcription factors operates in vivo and whether the same factor is responsible for transcriptional activation of all genes that contain a CRE in the promoter and are expressed at the same time during spermatogenesis.

Both CREB and CREMτ can function as transcriptional activators and are present in male germ cells, albeit at different stages. Whereas CREB predominates in premeiotic cells,

CREMτ predominates in postmeiotic cells. Premeiotic cells also contain the transcriptional repressor isoforms of CREM, namely, α, β, and γ. These isoforms can heterodimerize with CREB and inhibit its transactivation function (19). CREMτ transcripts first appear in late meiosis and arise from an alternative splicing event of the CREM gene (10). The generation of CREMτ can be enhanced by treatment with follicle-stimulating hormone, and the mRNA appears at a time during spermatogenesis similar to that for the calspermin transcript (11). The CREMτ protein accumulates to very high levels in postmeiotic cells. Whereas CREB also exists in the adult testis, the concentration is very much lower than that of CREMτ in postmeiotic cells (7). In addition, the CREB produced also arises by an alternative splicing mechanism, but in this case, truncated forms are generated that contain neither nuclear translocation signals nor DNA binding domains (44, 45). So the only transactivation-competent form of CRE-binding protein in spermatids is CREMτ, and the levels of this protein are much higher than that of CREB in most other tissues (7, 8, 14, 19, 23). We find by bandshift analysis that a protein present in testis nuclear extract comigrates with CREMτ and can be inhibited by competition with synthetic oligonucleotides representing either the −50 or −70 CREs present in the calspermin gene promoter. Furthermore, the signal of the band comigrating with CREMτ is greatly reduced by pretreating the testis nuclear extract with CREMτ antibody. Together these data provide the initial proof that CREMτ can physically interact with and transcriptionally regulate a haploid germ cell-specific gene. On the basis of the available evidence we suggest that CREMτ is the relevant transcriptionally active CRE-binding protein in postmeiotic germ cells. The abundance of this protein and rearrangement of DNA that occurs in meiosis may expose CRE-containing promoters that are normally inaccessible in somatic cells.

The transcriptional activation activity of CREMτ requires phosphorylation on Ser-117 in the kinase interaction domain (5). Phosphorylation of this residue in vitro can be catalyzed by PKA or CaM kinase II. Delmas et al. (7) show that phosphorylation of CREMτ on Ser-117 is also catalyzed by PKA present in an extract of postmeiotic germ cells. Since the addition of Ca^{2+}/calmodulin failed to result in a similar phosphorylation, the authors concluded that the physiologically important second messenger in haploid germ cells is cyclic AMP rather than Ca^{2+}. Whereas this is certainly plausible, it should be pointed out that haploid male germ cells contain high levels of calspermin, which binds calmodulin with a 1 nM K_d (17, 28). This affinity is at least 100 times higher than the calmodulin activation constant of CaM kinase II (i.e., K_{CaM}, the calmodulin concentration required for half-maximal enzymatic activity). So a great excess of calmodulin would be required to overcome the calspermin concentration. Furthermore, such an extract also contains both CaM kinase II and CaM kinase IV (our unpublished observation). Whereas PKA and CaM kinase IV specifically phosphorylated Ser-117 of CREMτ (and Ser-133 of CREB), CaM kinase II also stoichiometrically phosphorylates Ser-142 of CREB (39). This phosphorylation prevents CREB from serving as a transcriptional activator and is dominant. When CREB is activated by PKA or CaM kinase IV and then phosphorylated by CaM kinase II, it no longer functions as a transcriptional activator. Mutation of Ser-142 to Ala produces a protein that can be activated by PKA, CaM kinase IV, and CaM kinase II since the only site phosphorylated is Ser-133. CREMτ has a Ser at position 126 which is in exactly the same context as Ser-142 in CREB, and de Groot et al. (5) present evidence that Ser-126 of CREMτ is phosphorylated in vivo. Therefore, we predict a similar inhibitory phosphorylation of CREMτ to be catalyzed by CaM kinase II.

The CaM kinase IV mRNA is first detected in preleptotene primary spermatocytes at the very beginning of meiosis (22). The highest levels are found in midmeiosis (in pachytene spermatocytes). This is a pattern very similar to that reported for CREMτ (10) but quite different from the patterns of calspermin and calspermin mRNA, which is initially detected in the very late stages of meiosis and then continues to increase during spermiogenesis (22). Indeed, calspermin is one of the most abundant soluble proteins in sperm where CaM kinase IV activity cannot be detected. Calmodulin levels are also very high in pachytene spermatocytes and subsequently decrease as the spermatids differentiate (36). On the basis of all the available data, we suggest that CaM kinase IV initially phosphorylates CREMτ, which then participates in the activation of the calspermin gene (as well as other genes expressed at the same time that contain CREs). When calspermin is produced and accumulates, it would bind calmodulin which would eventually result in the inactivation of CaM kinase IV. As spermiogenesis continues, other genes that contain a regulatory CRE are activated by CREMτ. Since much of the calmodulin would be predicted to be bound to calspermin, the cyclic AMP pathway could then assume the role of regulating CREMτ phosphorylation. Such a mechanism could protect the cell from an excess of calmodulin, which is generally inhibitory to differentiation at high concentrations (8), but allow CREMτ-mediated gene expression to continue. Following differentiation the calmodulin could be released from calspermin, possibly during the acquisition of sperm motility, as calmodulin is required for this process (1, 41).

ACKNOWLEDGMENTS

We thank Richard A. Maurer and Stanley L. McKnight for many helpful discussions as well as for sharing unpublished results. Richard A. Maurer also generously provided us with expression vectors for PKA and CREB. CREB protein was obtained from Marc R. Montminy. We thank Deepak Lala for technical help in DNase I footprinting and Qihui Huang for preparing the CaM kinase IIα expression vector. We also thank Libby MacDougall for preparing and maintaining cells.

This research was supported by NIH grant HD-07503 to A. R. Means.

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