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An isoform of transcription factor CREM expressed during spermatogenesis lacks the phosphorylation domain and represses cAMP-induced transcription

(alternative exon splicing/protein kinase A/spermatids/seminiferous tubule)

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ABSTRACT **cAMP response element-binding protein (CREB) and modulator protein (CREM) regulate the transcription of cAMP-responsive genes via phosphorylation by cAMP-dependent protein kinase A. Reverse transcription and polymerase chain amplification of RNA from male germ cells identify an alternatively spliced CREM isoform, CREMΔC-G, lacking four exons including those encoding the protein kinase A-regulated phosphorylation domain and the flanking glutamine-rich transcriptional activation domains. CREMΔC-G retains exons that encode the basic–leucine zipper (bZIP) DNA-binding domain, binds to cAMP response elements (CREs), and competitively inhibits binding of CREB and CREM to CREs. Expression of CREMΔC-G inhibits transcription of a CRE-containing chloramphenicol acetyltransferase reporter plasmid induced by endogenous CREB. Antiserum to CREM detects CREMΔC-G in elongated spermatids from rat testis. These observations indicate that CREMΔC-G is a unique form of a competitive negative regulator of CREB-mediated gene transcription expressed in a maturation-dependent manner in haploid germ cells. The developmental specificity of CREMΔC-G suggests that it may play a role in transcriptional regulation during spermatogenesis.**

One mechanism for the mediation of the cAMP-dependent regulation of gene transcription is via phosphorylation by protein kinase A (PKA) of a single serine in the structurally similar transcription factors CREB, CREM, and ATF-1 (1–6). CREB and CREM in particular share several structural motifs, including the carboxyl-terminal DNA-binding bZIP domain, which consists of a positively charged DNA-binding region (b) and an adjacent leucine zipper (ZIP) dimerization region. In addition, CREB and CREM contain a unique amino-terminal phosphorylation domain (P-box, kinase-inducible domain) flanked by two glutamine-rich transcription activation domains. Alternative exon splicing of CREB and CREM RNA transcripts and alternative translational start sites for CREM account for proteins ranging from full-length transcriptional activators to truncated transcriptional repressors. Repressor forms of CREM are generated in the testis by alternative deletion of the glutamine-rich (Q-rich) exons that mediate transcriptional activation (7, 8), in the brain by internal translation that deletes one of the Q-rich exons and the P-box (9), and in neuroendocrine cells by alternative utilization of an internal promoter and translational start site between the Q-rich/P-box domains and the DNA-binding (bZIP) domain (10, 11).

In the rat and mouse testis, alternatively spliced forms of CREB and CREM are expressed in stage- and cell-specific patterns during spermatogenesis, the generation of spermatozoa from germ cells. In the process of spermatogenesis, stem cells give rise to spermatogonia that further develop into diploid primary spermatocytes. As a result of two meiotic divisions, spermatocytes give rise to haploid round spermatids which shed their cytoplasm and elongate to become spermatozoa (12, 13). In germ cells and Sertoli cells that surround and support maturing germ cells (14), full-length and truncated cytoplasmic CREB proteins are alternatively expressed in 12-day cycles (15–17). For CREM a switch from expression of repressor isotypes to full-length activators occurs during meiosis, with full-length CREM being expressed in round spermatids (18). We report here that maturing spermatids express unique alternatively spliced CREM variants lacking internal exons C–F, including the P-box and one or both Q-rich transcriptional activation domains (CREMΔC-F and CREMΔC-G, respectively).§ We show that one of these CREM variants binds a cAMP response element (CRE) as a homodimer, heterodimerizes with exon-replete CREB and CREM activators, and acts as an inhibitor of CRE-mediated transcription induced by PKA. This exon-deleted repressor form of CREM is expressed in elongated spermatids, suggesting a role for the CREM repressor in spermatid maturation.

MATERIALS AND METHODS

RNA Isolation and Southern Analysis of cDNA. Germ cells were isolated from rat testes (19) and purified by centrifugal elutriation (20). RNA was extracted from germ cells or total testis (21) and reverse transcription–polymerase chain reaction (RT–PCR) was performed (22). PCR primers for rat CREM cDNAs (primers contain flanking restriction sites to facilitate subcloning) were 5′-TCAAGCTTGGATCCGTATGACCATGGAAACAGTTGA-3′ (nt 1–20) and 5′-GAGCTCGAATTCCCAATTCACACTCTACAGCAG-3′ (nt 1145–1171) (nucleotide positions are relative to the CREM translation start site). Southern analysis of amplified whole-testis and germ-cell cDNA was performed with CREM exon-specific oligonucleotide probes.

Expression of CREMΔC-G and Electrophoretic Mobility-Shift Assays (EMSAs). Bacterial CREM (CREM$\tau\alpha$) and CREMΔC-G expression vectors were constructed by insertion of each cDNA as a *Bam*HI–*Eco*RI fragment into Reset C (Invitrogen). The CREB-327 expression vector and the isolation of bacterial fusion proteins have been described (23). The ^{32}P-radiolabeled Col8 double-stranded CRE oligonucleotide (2 fmol) (5′-GATCCGGCTGACGTCATCAA-

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Abbreviations: CRE, cAMP response element; CAT, chloramphenicol acetyltransferase; PKA, protein kinase A; EMSA, electrophoretic mobility-shift assay; RT, reverse transcription.
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§The CREMΔC-G sequence reported in this paper has been deposited in the GenBank data base (accession no. U04835).

GCTA-3′) was incubated with bacterially expressed CREM or CREB proteins (24). Resultant DNA–protein complexes were resolved by electrophoresis in nondenaturing 5% polyacrylamide gels and analyzed by autoradiography.

Cell Transfections and Chloramphenicol Acetyltransferase (CAT) Assays. JEG-3 cells were transfected (25) with the CREα100CAT reporter (1 μg) containing a palindromic CRE inserted adjacent to the human chorionic gonadotropin α-subunit promoter (26). In addition, pCMV5CREMΔC-G (0.5–2.0 μg), constructed by incorporation of CREMΔC-G into the EcoRI site of pCMV5 (27) downstream of the human cytomegalovirus (CMV) promoter; PKA catalytic-subunit expression vector, RSVCat-β (28) (1 μg); and pBluescript SK(+) (Stratagene) were cotransfected with CREα100CAT in a total of 10 μg of plasmid per 60-mm plate. CAT activity was determined after 48 hr (29). COS-7 cells were transfected with pCMV5 and pCMV5CREMΔC-G expression vectors (10 μg) (30).

Protein Extracts. Seminiferous tubules were isolated from decapsulated rat testes by collagenase treatment (31), pelleted by unit-gravity sedimentation, and washed three times with phosphate-buffered saline, and tubule whole-cell extracts were prepared by the addition of radioimmunoprecipitation assay (RIPA) buffer (32). Whole-cell protein extracts were prepared by addition of boiling Laemmli buffer (33) containing 8 M urea and 0.7 M 2-mercaptoethanol to purified germ-cell fractions and transfected COS-7 cells.

Immunoblotting. Whole-cell lysates (50 μg) from seminiferous tubules of 7- to 60-day-old rats, purified germ cells, and COS-7 cells were fractionated by SDS/12.5% PAGE, transferred to Problott poly(vinylidene difluoride) membranes (Applied Biosystems), and probed with CREM- or CREB-specific antiserum. CREM-B72 and CREB/CREM R1090 antisera (15) were raised against residues 21–34 within rat CREM exon B and residues 92–124 within exon E of the CREB-327 protein, respectively. Immunoadsorption competition immunoblots were performed with CREB-B72 antiserum preincubated (8 hr, 4°C, constant rocking) with lysates from bacteria expressing CREM or nonexpressing bacteria. Western immunoblot analysis was performed with the ECL system (Amersham).

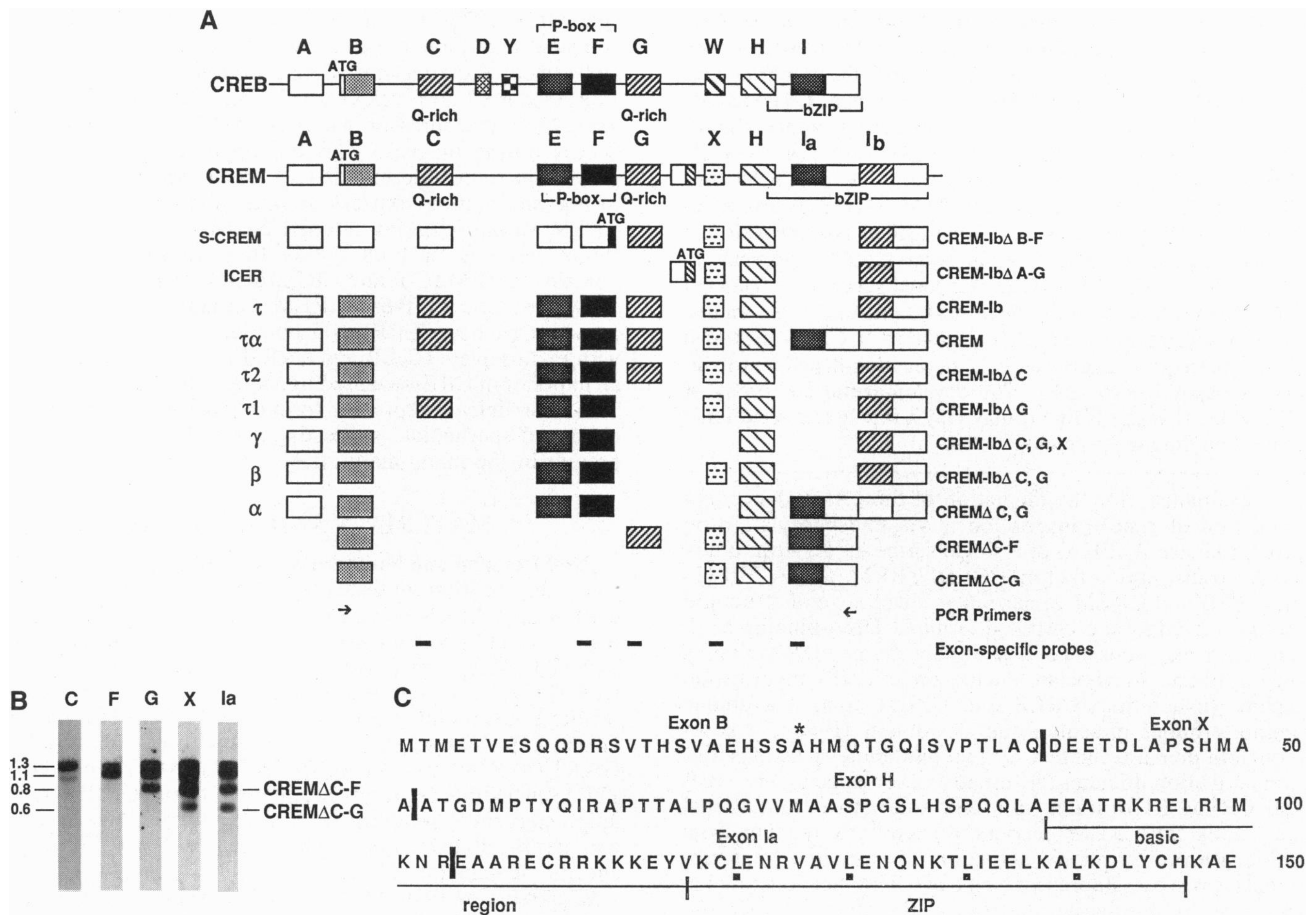

FIG. 1. Alternative exon splicing results in multiple CREM isoforms. (*A*) The exon structure of CREB and CREM genes is shown with nine previously described (2, 10) and two new CREM isoforms (CREMΔC-F and CREMΔC-G) resulting from alternative splicing. Q-rich transcription activation domains are located in exons C and G. The P-box site of phosphorylation by PKA is located in exon E. The carboxyl-terminal half of the bZIP domain is encoded alternatively by either exon Ia or Ib. Previously published nomenclature appears on the left (2, 10). The designations on the right describe alternatively spliced CREMIa and CREMIb isoforms. Protein-encoding sequences are shown by shaded boxes. Untranslated regions are shown in open boxes. The initiator codon is found in exon B except in cases of internal translation (S-CREM/CREM-IbΔB-F and ICER/CREM-IbΔA-G). Relative locations of primers used to amplify CREMΔC-G and probes used in Southern analysis are indicated. (*B*) Characterization of CREMΔC-G cDNA. Primers located within exons B and Ia were used to amplify cDNA derived from total testis germ cells. Amplified cDNA was resolved by agarose electrophoresis, transferred to nylon membranes, and probed with ^{32}P-labeled oligomers specific for CREM exons C, F, G, X, and Ia. (*C*) Amino acid sequence of CREMΔC-G derived from cDNA sequence. Exons are divided by bars. A deviation from the previously published rat CREM sequence (1) is noted (*) at position 24 (Leu → Ala). In addition, the residue at position 83 is assigned as histidine (H) because of its conservation in rat, mouse, and human CREMs, although the codon sequenced was TAC (tyrosine) rather than CAC (histidine). We attribute this discrepancy to an error in the RT–PCR cloning procedure. Squares (■) indicate heptad repeat of leucines in the leucine zipper (ZIP) region.

RESULTS

Testicular Germ-Cell RNA Expresses Alternatively Spliced Forms of CREM Lacking the Phosphorylation Domain. The CREB and CREM genes consist of multiple exons (Fig. 1*A*). Alternative splicing of these exons generates several CREB and CREM isoforms. The exon structures of CREM and CREB are highly homologous; however, one notable difference is the presence of a second alternatively spliced bZIP domain exon, Ib, in CREM. To date, nine CREM isoforms have been characterized (2, 10). Forms containing at least one of the Q-rich-domain exons C and G act as CRE-dependent transactivators, whereas isoforms lacking these exons act as antagonists of cAMP-induced transcription (4, 7).

RNA from adult rat germ cells was analyzed by RT–PCR for the presence of alternative splice variants of CREM with the bZIP-Ia motif. Oligonucleotide primers corresponding to exons C, F, G, X, and Ia were used as probes in Southern analysis of amplified CREM cDNAs (Fig. 1*A*). A 1.3-kb CREM form was visualized with all probes and probably represents full-length CREM cDNA (Fig. 1*B*). Smaller CREM cDNAs of 1.1, 0.84, and 0.65 kb lacking specific CREM exons were also seen. The 1.1-kb cDNA was recognized by all probes except that containing exon C sequences, whereas the 0.84-kb and 0.65-kb forms appeared to lack exons C and F and exons C, F, and G, respectively. Similar results were observed when total testis RNA was used as template for RT–PCR (data not shown). The 0.65-kb cDNA was isolated and subcloned. Sequence analysis confirmed a spliced isoform of CREM lacking exons C, E, F, and G (Fig. 1*C*). This 150-residue germ cell-specific CREM containing only exons B and X and the bZIP domain encoded by exons H and Ia was termed CREMΔC-G.

CREMΔC-G Protein Binds to a CRE. CREMΔC-G retains an intact DNA-binding and dimerization domain and therefore is predicted to form homodimers, dimerize with other bZIP proteins, and bind to CRE elements. The binding of bacterially expressed CREMΔC-G and full-length CREB and CREM to a ^{32}P-labeled consensus CRE was compared by EMSA. CREMΔC-G bound efficiently to the CRE as shown by a prominent DNA–protein complex that migrated much faster than the CREB–CRE complex (Fig. 2*A*, lanes 1 and 2). Mixing of lysates containing CREB and CREMΔC-G resulted in an intermediate complex not detected with addition of control lysate from bacteria containing empty Reset C expression vector (lanes 3 and 4). The formation of an intermediate complex shows that CREMΔC-G readily heterodimerizes with CREB. Coincubation of full-length CREM and CREMΔC-G resulted in heterodimer formation (lanes 10 and 11). CREMΔC-G also competed with CREB for binding to the CRE, as increasing concentrations of CREMΔC-G incubated with a fixed amount of CREB caused increased CREMΔC-G binding, formation of heterodimers, and corresponding inhibition of CREB homodimer binding to the CRE (Fig. 2*A*, lanes 5–9).

CREMΔC-G Inhibits PKA-Stimulated Gene Transcription. Because CREMΔC-G lacks transcriptional activator domains but retains dimerization and DNA-binding domains, it is a potential competitive repressor of CRE-activated gene expression. To test its functional activities, CREMΔC-G was subcloned into the mammalian expression vector pCMV5 (pCMV5CREMΔC-G). Repressor activity of CREMΔC-G was measured in transient-transfection assays of JEG-3 cells employing the CREα100CAT reporter plasmid. Cotransfection of a plasmid that expresses the catalytic subunit of PKA (RSVCat-*β*) caused a 25-fold increase in CAT activity from CREα100CAT, due to phosphorylation and activation of endogenous CREB (Fig. 2*B*). Expression of increasing amounts of pCMV5CREMΔC-G reduced the RSVCat-*β*-stimulated CAT activity by >80%, whereas the control

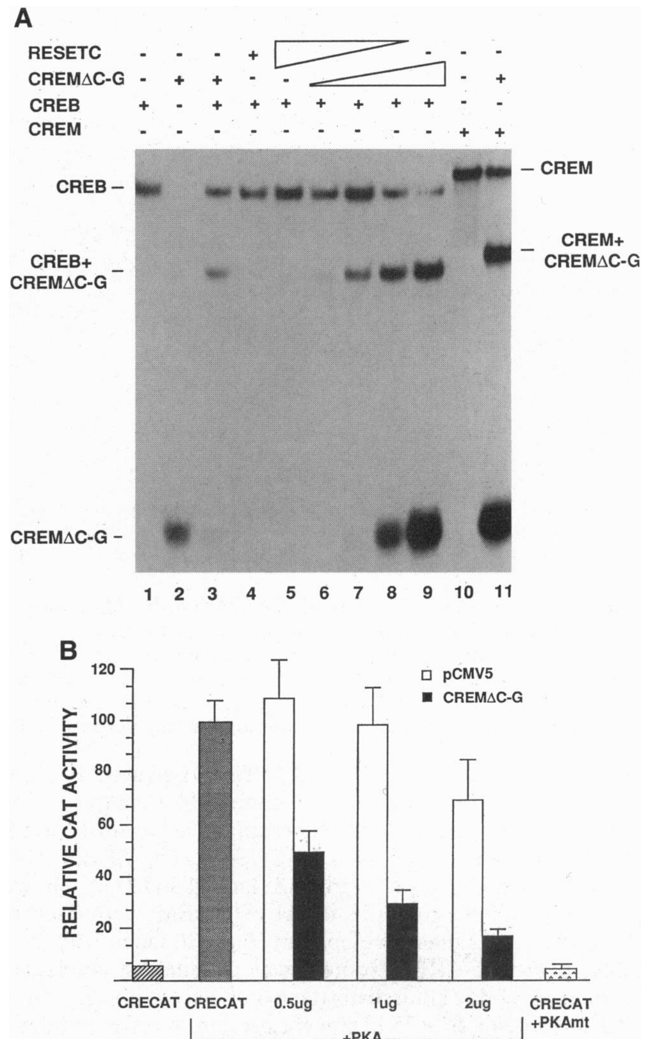

FIG. 2. CREMΔC-G represses PKA-induced transcription by binding to CRE motifs. (*A*) CREMΔC-G expressed in bacteria binds to a consensus CRE, heterodimerizes with full-length CREB and CREM, and competes for binding to CRE sites. In EMSAs, extracts from bacteria containing expression vectors encoding CREB, CREM, or CREMΔC-G or no recombinant protein (Reset C) were incubated alone or in combinations with a ^{32}P-labeled Col8 CRE probe. Bacterially expressed protein extracts were added as follows: lane 1, 1 μl of CREB; lane 2, 0.5 μl of CREMΔC-G; lane 3, 1 μl of CREB, 0.5 μl of CREMΔC-G; lane 4, 1 μl of CREB and 1 μl of Reset C; lanes 5–9, 1 μl of CREB with 8 μl of Reset C (lane 5), 7.5 μl of Reset C and 0.5 μl CREMΔC-G (lane 6), 6 μl of Reset C and 2 μl of CREMΔC-G (lane 7), 4 μl of Reset C and 4 μl of CREMΔC-G (lane 8), or 8 μl of CREMΔC-G (lane 9); lane 10, 1 μl of CREM; lane 11, 1 μl of CREM and 8 μl of CREMΔC-G. The various DNA–protein complexes formed are indicated. (*B*) CREMΔC-G represses CRE-regulated, PKA-induced transcription. JEG-3 cells were transfected with the CREα100CAT reporter plasmid (1 μg), with or without wild-type or mutant RSVCat-*β* (PKA catalytic-subunit expression vector) (1 μg). Results were normalized for protein concentration and are expressed as a percentage of CREα100CAT activity when cotransfected with RSVCat-*β* (21.4% conversion/μg of extract). Data shown represent the mean ± SE of six transfections from three independent experiments.

pCMV5 vector had no effect on CAT expression. As a control, cotransfection of a mutant RSVCat-*β* expression vector did not induce CREα100CAT. Furthermore, addition of pCMV5CREMΔC-G did not alter uninduced levels of CREα100CAT activity (data not shown). These observations indicate that CREMΔC-G can repress expression of a cAMP-inducible gene.

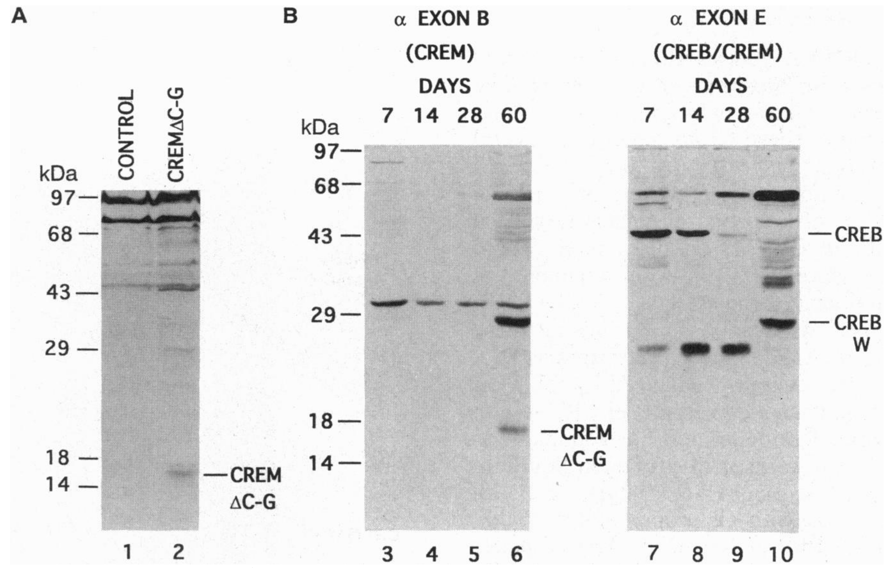

FIG. 3. Expression of CREB and CREM in testis. (*A*) Expression of CREMΔC-G in COS-7 cells. Whole-cell extracts (3×10^7 cells) from COS-7 cells transfected with pCMV5 or pCMV5CREMΔC-G were subjected to immunoblot analysis with CREM-B72 antiserum. The position of CREMΔC-G protein is indicated. (*B*) Immunoblot analysis of CREM and CREB proteins during testis development. Whole-cell extracts (50 μg) of seminiferous tubules from 7-, 14-, 28-, and 60-day (adult) rats were analyzed. The blot was probed with CREM-B72 antiserum (exon B), stripped, and reprobed with R1090 antiserum (exon E). Relative positions of CREMΔC-G, testis-specific truncated CREM-W isoforms, and full-length CREB(s) are indicated. Additional uncharacterized CREB and CREM antigens migrate between full-length CREB and CREB-W.

CREMΔC-G Protein Is Present in Testis Extracts. To identify CREMΔC-G in rat testis, immunoblot analysis was performed with CREM-B72 antiserum, raised against exon B of CREM. When expressed in COS-7 cells, CREMΔC-G coded for a 16-kDa protein (Fig. 3*A*, lanes 1 and 2). As shown previously (8, 9), multiple forms of CREM were present throughout testicular development (Fig. 3*B*, lanes 3–6). Notably, a 17-kDa CREM protein was detected in whole-cell extracts of 60-day adult testis (lane 6), whereas no detectable CREM proteins of <25 kDa were present in testis of immature 7- to 28-day-old rats (lanes 3–5). These findings suggest that CREMΔC-G is expressed late in germ-cell development in elongated spermatids and spermatozoa, because these germ cells do not appear until after 30 days of age. As expected, the 17-kDa protein CREMΔC-G was not recognized by R1090 antiserum, directed against exon E (Fig. 3*B*, lane 10). However, multiple CREB and CREM isoforms containing exon E were present in adult testis, including an abundant 29-kDa truncated protein, CREB-W, resulting from insertion of an alternatively spliced exon W encoding an in-frame stop codon (15).

To further define the developmental expression of CREMΔC-G, whole-cell extracts from purified round and elongated spermatids were subjected to immunoblot analysis using CREM and CREB antisera. With CREM antiserum, the 17-kDa CREMΔC-G was found to be more abundant in elongated spermatids than in round spermatids (Fig. 4*A*, lanes 1 and 2). These findings localize CREMΔC-G to more mature spermatogenic cells. Interestingly, CREB antigen appeared to be abundant in round spermatids, as CREB

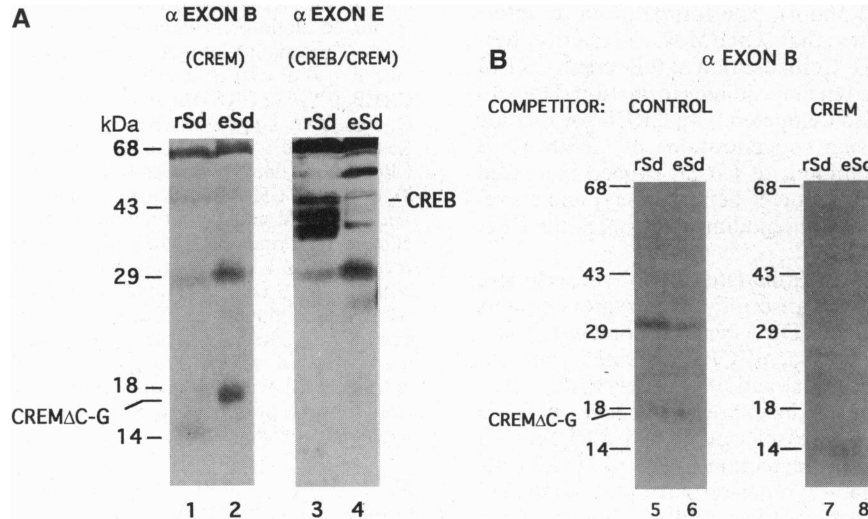

FIG. 4. CREMΔC-G is detected in spermatids. (*A*) Immunoblot analysis of whole-cell protein extracts from purified rat round spermatids (rSd) and elongated spermatids (eSd). Blots were probed with CREM-B72 antiserum (lanes 1 and 2), stripped, and reprobed with R1090 CREB/CREM antiserum (lanes 3 and 4). (*B*) Specificity of CREM antiserum. For immunoadsorption/competition experiments, extracts of round and elongated spermatids were probed with CREM-B72 antiserum that was preincubated with control bacterial lysate (lanes 5 and 6) or bacterial lysates containing CREM (lanes 7 and 8). The position of CREMΔC-G is indicated.

antiserum recognized antigens of 35–43 kDa not detected with CREM antiserum (Fig. 4*A*, lanes 1 and 3). In contrast to CREMΔC-G, CREB protein levels appeared to decrease during germ-cell maturation, as less CREB antigen was present in elongated spermatids (Fig. 4*A*, lanes 3 and 4). To examine the specificity of the CREM-B72 antiserum to recognize CREM, immunoadsorption of the antiserum was performed with CREM expressed in bacterial host cells. Preincubation of antiserum with control lysates of bacteria did not alter detection of CREM proteins on Western immunoblots of spermatid extracts (Fig. 4*B*, lanes 5 and 6), whereas preadsorption of the antiserum with bacterial lysate that contained CREM greatly abolished recognition of CREMΔC-G and another alternatively spliced form of CREM (8) (Fig. 4*B*, lanes 7 and 8).

DISCUSSION

These findings indicate that an alternatively spliced isoform of CREM (CREMΔC-G) lacking exons which code for the Q-rich transcriptional activation domains (exons C and G) and phosphorylation regulatory domain (exons E and F) is expressed in elongated spermatids. Because CREMΔC-G retains an intact DNA-binding domain and lacks transcriptional transactivation domains, CREMΔC-G represses CRE-mediated transcription by CREB and CREM. Although several alternative splice variants of CREM have been found in the testis, a testicular form of CREM lacking exons that encode the phosphorylation domain has not been described (2, 7–9, 18).

A recent report (4) indicates that the bZIP-1 domain (exon Ia), as contained within CREMΔC-G, has a higher dimerization affinity for CREB than does the bZIP-2 (exon Ib) domain. This difference in affinity may enhance the effectiveness of CREM variants containing bZIP-1 in the formation of heterodimers with CREB. The presence of the phosphorylation domain (P-box) encoded in exons E and F appears to decrease the inhibitory activity of experimentally truncated CREM forms lacking the C and G exons (4). In addition, the P-box domain and the Q-rich domains can act independently (34, 35) or in trans (35) with other transcription factors to stimulate gene expression. Thus, the absence of the P-box and Q-rich domains in CREMΔC-G may enhance its inhibitory potential.

At critical stages of spermatogenesis there are coincident changes in the CREB and CREM isoforms that are expressed. For example, during the development of spermatocytes, expression of alternatively spliced CREB RNAs encoding truncated, non-DNA-binding CREB isoforms increases to levels comparable to those of the intact transactivator CREB (17). Also, prepachytene germ cells express only transcriptional repressor CREM isoforms, whereas spermatids switch to expression of activator CREM isoforms (8). After meiosis, developing haploid spermatids may require altered signals for morphological changes associated with maturation of spermatozoa. Because many germ-cell transcription factors are expressed only in the postmeiotic phases of spermatogenesis (36), the CREMΔC-G repressor may be a part of the new programming necessary for spermatid–spermatozoon differentiation.

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