Alternative Exon Splicing Controls a Translational Switch from Activator to Repressor Isoforms of Transcription Factor CREB during Spermatogenesis*

William H. Walker‡, Christophe Girardet§, and Joel F. Habener¶

From the Laboratory of Molecular Endocrinology, Massachusetts General Hospital, Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts 02114 and the Department of Pathologie, Centre Medico Universitaire, CH1211 Geneve 4, Switzerland

The cAMP/protein kinase A signaling pathway activates the cAMP-responsive transcription factor CREB. Here we describe a unique alternative RNA splicing event that occurs during the development of germ cells in the testis, resulting in a translational switch from an mRNA encoding activator CREB to an mRNA encoding novel inhibitor CREB isoforms (I-CREBs). Alternative splicing of an additional exon into the CREB mRNA in mid to late pachytene spermatocytes results in the premature termination of translation and consequent downstream reinitiation of translation producing I-CREBs. The I-CREBs down-regulate cAMP-activated gene expression by inhibiting activator CREB from binding to cAMP response elements. Further, the developmental stage-specific expression of I-CREBs in germ cells of the seminiferous tubules correlates with the cyclical down-regulation of activator CREB, suggesting that I-CREBs repress expression of the cAMP-inducible CREB gene as well as other genes transiently induced by cAMP during the 12-day cycle of spermatogenesis.

CREB1 (cAMP response element-binding protein) is a member of a family of DNA-binding proteins known as bZIP proteins that consist of distinct DNA-binding and transactivation domains (1). In Sertoli cells of the rat testis, CREB mRNA is induced in a repeated cyclical pattern corresponding to the specific 12.5-day temporal and anatomical cell association stages of spermatogenesis (2). The levels of CREB mRNA increase in cell association stages II–VI following increases in FSH-induced cAMP levels in stages I–V. Levels of CREB mRNA then fall rapidly to near undetectable levels in stages VII–XIV as CREB concentrations decrease due to internalization of FSH receptors and the down-regulation of the FSH receptor gene (2, 3). Characterization of the CREB promoter identified three cAMP response elements (CREs) responsible for CREB induction of transcription (4). Phosphorylation by cAMP-dependent PKA activates CREB bound to the CREs of the CREB promoter, thereby stimulating transcription and the production of additional CREB, resulting in an autopositive feedback loop (4, 5). This autocatalytic regulation of CREB gene expression is proposed to account for the rapid and large stage-specific increase in CREB mRNA that accumulates in the nuclei of Sertoli cells during stages II–VI (2, 4, 5).

The CREB gene contains at least 12 exons, several of which are alternatively spliced, resulting in a variety of CREB isoforms. In the testis, alternatively spliced exons (exons W, Y, and Y') encode blocked translation reading frames so that translation is terminated prematurely (2, 6, 7). The resulting CREB isoforms lack the bZIP domain and the nuclear localization signal and therefore are unable to act as transcription activators during specific stages of spermatogenesis. In the rat testis, inclusion of exon W in the RNA and the synthesis of CREB-W precedes a pronounced fall in the levels of CREB mRNA, suggesting that CREB-W antagonizes the synthesis of CREB mRNA (2). The decreased production of full-length activator CREB incurred by the splicing of exon W is predicted to interrupt a positive feedback loop (2, 5).

Here we show that alternative splicing of exon W into CREB mRNA may interrupt a positive feedback loop by an unexpected mechanism. Termination of translation by stop codons within exon W permits translation to reinitiate in-frame at downstream initiation codons resulting in the production of inhibitor CREB isoforms (I-CREBs). The I-CREBs compete with CREB for binding to CREs (such as those located in the promoter of the CREB gene) and down-regulate cAMP-stimulated gene expression. I-CREBs are expressed at specific stages of spermatogenesis predominately in spermatocytes and may account for cell- and stage-specific repression of cAMP-regulated genes.

MATERIALS AND METHODS

Plasmid Constructs—For expression of CREB proteins in vitro, pCRII (Invitrogen) and pGEM (Promega) vectors were used to synthesize CREB mRNAs. The constructs are described relative to the CREB amino acids encoded (1–327) with or without insertion of exon W. pCRII CREB-W AUG → AUC was produced by site-directed mutagenesis using the sense oligomer 5′-GTAAAGCAGGATccCAGT-GAATTGA-3′. To create the pCRII CREB-W stem-loop construct, the hp7 stem-loop-forming sequence (8) was introduced between the stop codon located 15 nucleotides into exon W and the AUG codon 8 nucleotides further downstream. For expression of CREB proteins in COS-1 cells, the CREB cDNAs described above were inserted into the pCMV5 vector (9). Detailed explanations of plasmid constructs are available upon request.

Expression of CREB Proteins in Vitro and in Vivo—For in vitro uncoupled transcription-translation assays, 5 μg of linearized plasmid DNA were transcribed with Sp6 polymerase in the presence or absence of GppGp. Run-off RNA transcripts (~2 μg) were translated using rabbit reticulocytes (Promega) in the presence of [35S]methionine. For...
studies using coupled transcription-translation derived CREB, 1 µg of purified plasmid DNA was added to a reticulocyte lysate-coupled in vitro transcription and translation system (TNT T7/SP6 Coupled Reticulocyte Lysate System, Promega) in the presence of [35S]methionine. Resultant protein lysates were treated with RNase A and either precipitated with trichloroacetic acid or immunoprecipitated with CREB specific antiserum (αCREB) directed against amino acids 255–275 of CREB and fractionated on 16.5% T, 3% C Tricine SDS-polyacrylamide gels. For in vivo production of CREB proteins, COS-1 cells were transfected with CREB expression vectors, and whole cell lysates were prepared as described (10).

DNA-binding Studies—Electrophoretic mobility shift assays (EMSA) were performed with 32P-end-labeled duplex containing an optimized CRE (Col8) (11) or CREB promoter CREs (CRE1-2) (5). Either 1–5 µl of coupled transcription-translation extract or 7 µl of COS-1 whole cell extract was incubated with the probe. For competition EMSA studies, CREB and I-CREB(s) proteins were overexpressed in bacteria transformed with the plasmids pETCREB327 and pETCREB74, respectively.
In competition EMSA binding reactions with a consensus CRE probe (Col8), a 5- to 115-fold excess of I-CREB(s) relative to CREB was used.

Cell Transfections and Transactivation Assays—Human JEG-3 choriocarcinoma cells were transfected using the CaPO4 coprecipitation method (13). The 2278CREBCAT reporter plasmid (1 mg) containing CREB promoter sequences extending 278 bp upstream of the translation start site (including 100 bp upstream of the major transcription start site) (5) was transfected with or without the PKA catalytic subunit expression vector, RSVCAT-β (15) (1 µg), and either empty pCMV5 expression vector (EV) (1 µg) or pCMV5 containing I-CREB(s), I-CREB(l), CREB-W, or CREB. CAT activity of JEG-3 choriocarcinoma cells transfected with the CREBCRECAT (−278CREBCAT) (5) reporter plasmid (1 µg) containing CREB promoter sequences extending 278 bp upstream of the translation start site (including 100 bp upstream of the major transcription start site) (5) was transfected with or without the PKA catalytic subunit expression vector, RSVCAT-β (1 µg) (14). Either pCMV5 I-CREB(s), pCMV5 CREB-W, pCMV5CREB, or the empty pCMV5 expression vector (0.5 µg) and pBluescript SK(+) (Stratagene) were added to transfections to give a total of 5 µg of plasmid per 60-mm² plate. CAT activity was determined as described previously (15), except that fluorescent BODIPY chloramphenicol (Molecular Probes Inc.) was used in place of [14C]chloramphenicol. Enzyme activity was quantitated using ImageQuant software and a FluorImager 575 (Molecular Dynamics).

Western Immunoblots, Immunocytochemistry, and in Situ Histohybridization—For Western immunoblots, whole-cell extracts of adult rat (60-day) testis and germ cells from 17-day rat testis (60-day) testis and germ cells from 17-day rat testis were prepared by disruption and extraction of the tissue in radioluminoprecipitation (RIPA) buffer (5, 16). Extracts were fractionated by electrophoresis on Tricine SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and immunostained with rabbit antisera raised against the amino-terminal 16 amino acids of CREB(l) (SVT1NSQRQFYAAPGC) (αCREB(l) α340) followed by analysis using the ECL chemiluminescence system (Amersham). Immunocytochemistry was performed on frozen sections from adult rat testis. Sections were immunostained with antiserum α1-CREB(l) or antiserum directed against amino acids 255–270 of CREB (αCREB, α338) and Cy3 fluorescent secondary anti-rabbit serum (Jackson ImmunoResearch Laboratories Inc.).

RESULTS AND DISCUSSION

CREB-W mRNA Encodes Multiple Proteins—In certain circumstances, premature termination of the translation of mRNAs allows for the reinitiation of translation at internal AUG (methionine) codons (17–19). Inspection of the open trans- lational reading frames in the CREB-W mRNA revealed two potential initiator codons (AUG) in-frame with CREB: one located in exon W seven nucleotides downstream of the in-frame stop codon and the other at methionine codon 254 (Fig. 1A). Both of these potential initiator AUG codons reside in a context of flanking nucleotides favorable for the initiation of translation (20). Translation initiated at the two internal methionines in exon W and at codon 254 would encode small CREB proteins containing the carboxyl-terminal basic region (BR) and leucine zipper (ZIP) domain necessary for DNA binding, but would lack transactivation domains. Because these shortened isoforms of
CREB could serve as inhibitors of activator forms of CREB, they were named inhibitor CREBs long and short, I-CREB(l) and I-CREB(s), respectively.

To investigate the possibility that I-CREB proteins might be synthesized from CREB-W mRNA, CREB and CREB-W proteins were synthesized in an uncoupled transcription-translation system in vitro using capped mRNA (Fig. 1B). The CREB-W RNA produced the expected 29-kDa truncated CREB-W protein consisting of the amino-terminal 230 amino acids of CREB plus the five amino acids encoded within exon W before the termination of translation. However, additional proteins of 8 kDa and 16 kDa were synthesized that were not present after the translation of CREB RNA lacking exon W. The 8- and 16-kDa proteins were also produced by the amino-terminal truncated CREB169-327+W construct but not CREB169-327 which encoded a 20-kDa protein due to translation initiating in the CREB reading frame at position 195 (the 5′-most AUG codon). These findings were considered to be consistent with internal translation, dependent upon the presence of exon W in the mRNA.

Additional uncoupled in vitro transcription-translation reactions were performed followed by immunoprecipitation with an antisera directed against the carboxyl terminus of CREB (see Fig. 3A) to confirm that the 8- and 16-kDa I-CREB proteins were synthesized from CREB-W and contained the CREB bZIP domain (Fig. 1B). As expected, the 30-kDa CREB-W protein, lacking the basic region, was not immunoprecipitated by αCREB. The 20-kDa protein produced by the CREB169-327 was also immunoprecipitated by αCREB as well as a small amount of 8-kDa I-CREB(s) due to translation initiating internally at methionine 254. Utilization of the methionine 254 in the CREB-W and CREB169-327 vectors and not the longer CREB1-327 vector likely reflects a reduction of elongational occlusion by ribosomes due to translation initiating in exon W and less efficient translation initiation from methionine 195 compared to the relatively strong initiation site encoded at the 5′ end of the CREB-W mRNA. Insertion of exon W into the 169–327 vector results in a marked enhancement in the production of the 16-kDa and 8-kDa I-CREB proteins derived from the reinitiation of translation at the two AUG codons in exon W and the other in exon H (AUG254). It was also found that the presence of a GpppG cap at the 5′ end of CREB-W mRNA did not influence the production of I-CREBs.

I-CREBs Compete with CREB for Binding CREs and Down-regulate cAMP-stimulated Transcription—To examine the DNA-binding properties of the CREB proteins encoded by the CREB-W mRNA, electrophoretic mobility shift assays (EMSA)

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**Fig. 3.** I-CREB(l) is expressed in immature germ cells. A, left, immunoblot showing the I-CREB(l)-specific antisera (α340) detects I-CREB(l) but not I-CREB(s), CREB, or CREM. Middle, CREB-specific antisera (α338) recognizes I-CREB(s), I-CREB(l), and CREB, but not CREM. Right, as a control to ensure that CREM protein was present on the left and middle blots, P-box-specific antisera (αR1090) was used to detect CREB and CREM. B, Western immunoblot of whole adult rat testis and 17-day rat testis germ cells fractionated by Tricine-SDS-PAGE. CREB and I-CREB(l) produced in bacteria are provided as markers. Bacterially expressed I-CREB(l) is 4 kDa larger than normal, due to the addition of amino acids encoded by the parent pRSET A vector. Digestion of I-CREB(l) with enterokinase protease removes the vector-encoded amino acids resulting in the predicted 16-kDa I-CREB(l) protein. I-CREB(l) is detected in rat 17-day germ cell extracts as indicated. C and D, immunocytochemistry of rat testis. C, CREB-specific antisera α338 stains nuclei of spermatocytes, early and mid-stage round spermatids, and Sertoli cells. D, antisera α340 directed against the amino terminus of I-CREB(l) stains nuclei of late pachytene spermatocytes and, to a lesser extent, early round spermatids and Sertoli cell nuclei of rat seminiferous tubules. Stages of seminiferous tubules are shown (I–XIV). Magnification = 700×. P = pachytene spermatocytes, S = Sertoli cell nuclei, Sd = spermatids, Sz = spermatocytes.
were used to determine whether I-CREBs would bind to CRES and potentially compete with activator CREB isoforms. Binding reactions were performed with a synthetic DNA probe containing a consensus cAMP-response element (CRE) and different variants of CREB proteins translated both in vitro and in vivo from plasmid expression vectors encoding either CREB or CREB-W RNAs (Fig. 1C). In both expression systems, DNA-CRE complexes were formed corresponding to homodimers of the I-CREB(l) and I-CREB(s) as well as heterodimers among the two I-CREBs, full-length activator CREBs, truncated CREB translated from the CREB169-327 vector, and endogenous CREB-like proteins. Proteins of 8 kDa and 16 kDa from COS-1 extracts that formed complexes with the CRE probe were identified by UV cross-linking to a bromodeoxyuridine-substituted, 32P-labeled CRE probe and SDS-PAGE fractionation (data not shown). These observations confirm that the presence of exon W switches the translation of CREB mRNA from activator CREB to I-CREBs in vivo as well as in vitro, that the internally translated I-CREBs are capable of independently binding to a CRE and forming heterodimers with activator forms of CREB and could serve as transcriptional repressors.

To further establish that the 16-kDa protein arises from translational initiation within exon W, the AUG codon in exon W was mutated to AUC (Fig. 1D). EMSA studies employing in vitro translated proteins demonstrated that mutation of the I-CREB(l) initiator codon abolished the synthesis of the 16-kDa protein and enhanced production of the 8-kDa protein. The increased production of I-CREB(s) is likely a consequence of the loss of elongational occlusion by ribosomes that otherwise would initiate at the AUG in exon W. To test whether this AUG may act as an internal ribosomal entry site rather than a capture site for continued scanning of the 40 S ribosome, a DNA fragment that encodes an RNA stem-loop structure was inserted between the stop and start codons in exon W. Such a stable stem-loop (ΔG = -61 kcal) was previously shown to block further scanning of 40 S ribosomes on RNA and to suppress translation reinitiation by continued scanning at downstream AUG codons (8, 21). The presence of the stem-loop attenuated the synthesis of the 16-kDa protein and markedly enhanced the synthesis of the 8-kDa protein, suggesting that the AUG at position 254 is a bona fide internal ribosomal entry site (Fig. 1D). These findings are consistent with a role for exon W in switching CREB mRNA from a monocistronic to a polycistronic function.

The I-CREBs act as competitive inhibitors of transactivator CREB in competition EMSA DNA-binding assays in vitro, as well as in co-transfection/expression transactivation assays in vivo (Fig. 2). In competition EMSA studies, addition of increasing amounts of I-CREB(s) inhibited the binding of CREB to an oligonucleotide probe containing a CRE (Fig. 2A). A comparison of the relative binding affinities of CREB and I-CREB(s) for a consensus CRE (ColCRE) versus the non-consensus tandem CREs of the CREB promoter (CREBCRE) showed that I-CREB(s) binding was only slightly less efficient (2- to 3-fold reduced) with the CREB promoter CREs (Fig. 2B). In contrast, CREB binding affinity for the CREB promoter CREs was dramatically lower than that for the consensus CRE. These data suggest that I-CREBs may be important regulators of CREB gene expression because, even at low levels, I-CREBs may effectively out-compete CREB for non-consensus CRE-binding motifs, including those in the CREB promoter. I-CREBs were shown to be functional transcriptional repressors as expression plasmids encoding I-CREB(s) and CREB-W transfected into JEG-3 choriocarcinoma cells inhibited expression of cAMP-responsive reporters consisting of either a consensus CRE (data not shown) or the autoregulatable promoter of the CREB gene (CREBCRECAT) induced by co-expression of cAMP-dependent protein kinase A (5) (Fig. 2C).

I-CREB Expression Is Highest in Spermatocyte Germ Cells—To determine whether I-CREBs are present in vivo at levels that might be physiologically significant, Western immunoblot analysis of testis tissue was performed. Using the CREB-specific (αCREB) antisemum I-CREB(s) could not be detected in rat germ cell or whole testis tissue protein extracts. In contrast, I-CREB(l) was present in germ cell nuclear extracts from immature 17-day rats (Fig. 3A). The detection of I-CREB(l) but not I-CREB(s) is in agreement with CREB-W in vitro translation results (Fig. 1B) that showed I-CREB(l) is produced in preference to I-CREB(s). Also, because germ cells from immature 17-day rats have not matured past the spermatocyte stage (22), these data suggested that I-CREBs may be restricted to early stage germ cell types.

Using an antisemum raised against the unique amino-terminal region of I-CREB(l) encoded by exon W, I-CREB(l) protein was detected in mid to late premeiotic pachytenie spermatocytes, predominantly in stages V–XIV (Fig. 3, B and C). I-CREB(l) is also present, to a lesser extent, in Sertoli cells and early postmeiotic round spermatids. The restriction of much of I-CREB(l) to the spermatocyte subset of cell types may explain how the relatively low levels of I-CREB(s) detected in extracts of whole testis may result in changes in cell-specific transcriptional control. The temporal specificity of I-CREB expression suggests that I-CREBs may act at certain developmentally important checkpoints to alter gene programming.

The novel mechanism for the synthesis of negative acting I-CREBs may be responsible for the down-regulation of the expression of the CREB gene in germ cells. We have shown previously that CAMP-mediated regulation of the transcription of the CREB gene occurs by interactions of CREB itself with two CREs located in the promoter (4, 5). I-CREB(l) reaches the highest levels in spermatocytes during the stages of spermatogenic development when the levels of CAMP and CREB mRNA are declining (stages VIII–XIV), suggesting that the binding of I-CREBs to the CREs in the promoter of the CREB gene is responsible for the stage-specific decline in the levels of CREB. It is important to note that the majority of CAMP regulation in spermatocytes is most likely controlled by CREB:I-CREB ratios as activator forms of CREM are not detected until later stages of germ cell differentiation (23). During critical times of early germ cell development, alterations in splicing protocols for exon W, perhaps mediated by hormonal signals (CAMP), would dictate relative levels of repressor I-CREBs and activator CREBs. Competition between CREB and I-CREBs would then determine the rates of transcription of CAMP-regulated genes including the CREB gene.

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Note Added in Proof—While this manuscript was in press, work describing human I-CREB protein expression was published (24) that provides additional details of the methods used in this study and discussion of the biological significance of I-CREBs.

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