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Functional Characterization of Male Germ Cell-Specific CREM Isoforms

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Due to alternative promoter usage, splicing, and translational initiation, expression of the cAMP-responsive element modulator (CREM) gene results in the production of functionally different CREM proteins with either activating or repressing potential on target gene expression. Recently, we demonstrated 2 novel isoforms (CREM-θ2-F-G-H-Ib and CREM-θ2-G-H-Ib) in various germ cell types during normal and impaired human spermatogenesis. In contrast to known isoforms, these exhibit a transactivation domain but lack a kinase-inducible domain (KID) domain resulting in a disruption of the open reading frame. In the present study, we functionally analyzed these isoforms. Investigation of both in vitro and in vivo expressed proteins from human testis RNA suggests that a novel upstream open reading frame in exon θ2 is translated from isoform CREM-θ2-F-G-H-Ib, giving rise to a full-length protein. Furthermore, in both isoforms, usage of downstream adeninethymine-guanines (ATGs) for translation initiation could be observed. Sequence-specific DNA binding of CREM isoforms was confirmed by electrophoretic mobility shift assays. Luciferase reporter gene assays in cells transfected with novel CREM cDNAs demonstrated that protein kinase A dependent stimulation was inhibited by coexpression of CREM-θ2-F-G-H-Ib but not of CREM-θ2-G-H-Ib.

Key words: Alternative splicing, spermatogenesis

Promoters of many postmeiotic genes, such as protamines that are known to play a vital role for male fertility (Steger, 2003), contain a cAMP-responsive element (CRE) (Oliva and Dixon, 1991) serving as binding site for the transcription factor cAMP-responsive element modulator (CREM) (Sassone-Corsi, 1995). The *CREM* gene consists of 14 exons (Daniel et al, 2000; Gellersen et al, 2002) (Figure 1). Due to alternative promoter usage, alternative splicing, and alternative translation initiation, *CREM* gene expression results in the production of functionally different CREM proteins with either activating or repressing potential on target gene expression (Delmas et al, 1992; Walker and Habener, 1996; Gellersen et al, 1997; Behr and Weinbauer, 2000; De Cesare et al, 2000; Gellersen et al, 2002). To date, only a few CREM isoforms have been functionally characterized. This studies concluded that CREM activator transcripts, such as CREM-τ, contain the kinase-inducible domain (KID), at least 1 of 2 glutamine-rich transactivation domains (τ1, τ2), and 1 of 2 alternative DNA-binding domains (DBDI, DBDII), whereas CREM repressor transcripts consist of the KID domain and 1 of the DNA-binding domains but lack a transactivation domain (Foulkes et al, 1992; Walker et al, 1994). The KID is the target for phosphorylation by various protein kinases, including protein kinase A (PKA), resulting in recruitment of the coactivator CREB binding protein, while the C-terminus of CREM encodes the bZIP region, consisting of the basic region as the sequence-specific DNA-binding interface and the leucine zipper, which accommodates dimerization of members of the bZIP family of proteins (Montminy, 1997; Vinson et al, 2002).

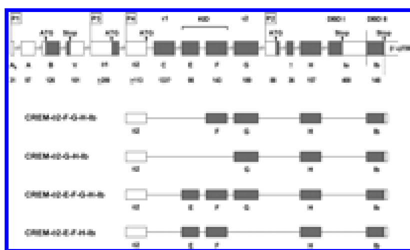

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Figure 1. Composition of novel CREM transcripts from human testis. A map of the human *CREM* gene according to Gellersen et al (2002) is shown on top. Four alternative promoters (P1–P4), sizes of the exons (number of nucleotides), and start and stop codons for known ORFs (shaded boxes) are indicated. Untranslated regions are represented by open boxes. Introns (thin lines) are not shown to scale. The novel P4-derived CREM isoforms CREM-θ2-F-G-H-Ib and CREM-θ2-G-H-Ib analyzed in this study are shown below. For comparison, previously characterized isoforms CREM-θ2-E-F-G-H-Ib and CREM-θ2-E-F-H-Ib (also designated CREM-θ2-τ2-ß and CREM-θ2-ß) (Gellersen et al, 2002) were included in the study and are depicted at the bottom.

A functional *CREM* gene is essential for male fertility, as male CREM-deficient mice have been demonstrated to be infertile due to round spermatid maturation arrest (Blendy et al, 1996; Nantel et al, 1996) and infertile men with round spermatid maturation arrest have been reported to exhibit substantially reduced or totally absent CREM-τ expression (Weinbauer et al, 1998; Steger et al, 1999) and inaccurately spliced CREM transcripts (Behr and Weinbauer, 2000; Blöcher et al, 2005). In a recent study, we demonstrated expression of 2 novel CREM isoforms, characterized by exons θ2-F-G-H and θ2-G-H, in various germ cell types during normal and impaired spermatogenesis (Blöcher et al, 2003; Blöcher et al, 2005). The aim of the present study was to functionally analyze these isoforms.

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Human testis total RNA served as starting material for identification and isolation of isoforms CREM-θ2-F-G-H-Ib and CREM-θ2-G-H-Ib. Isoforms CREM-θ2-E-F-G-H-Ib and CREM-θ2-E-F-H-Ib, also designated CREM-θ2-τ2-ß and CREM-θ2-ß, have been characterized previously (Gellersen et al, 2002) and were included in the experiments for comparison. First strand cDNA synthesis was performed using 2 µg of human testis total RNA (BD Biosciences, Heidelberg, Germany) and oligo $d(T)_{16}$ primers (Applied Biosystems, Weiterstadt, Germany) with the Omniscript RT Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. In addition, RNase inhibitor (Roche, Mannheim, Germany) was added. The polymerase chain reaction (PCR) amplification contained 5 µL *Pfu* DNA polymerase 10x buffer with $MgSO_4$, 1 µL 25 mmol $MgCl_2$, 1 µL dNTP mix (10 mmol each), 1.25 U *Pfu* DNA polymerase (all from Promega, Heidelberg, Germany), 1 µL sense and antisense primers (10 pmol) and 2 µL cDNA in a final volume of 50 µL. The primers used for PCR were: 5'-ACCGAAGTATGGGCACCA-3' (sense, localized in CREM exon θ2) (Gellersen et al, 2002) and 5'-CTAGTAATC(A/T)GTTTTGGGAGA-3' (antisense, localized in CREM exon Ib, according to the human cDNA sequence) (Gellersen et al, 2002; Masquilier et al, 1993). The thermal cycling conditions were: initial denaturation for 1 minute at 95° C, followed by 35 cycles for 45 seconds at 95° C, 30 seconds at 51° C, 2 minutes at 72° C, and 1 cycle for 5 minutes at 72° C. PCR products were separated on a 2% agarose gel, purified with the QIAEX II gel extraction kit (Qiagen) and cloned into pCR-Blunt II— TOPO using the Zero Blunt TOPO PCR Cloning Kit and verified by sequencing (Qiagen). PCR on the CREM cDNAs in pCR-Blunt II— TOPO was then performed with primers 5'-ACCGAAGTATGGGCACCA-3' (sense, localized in exon θ2) (Gellersen et al, 2002) and 5'-TGTATTCTAGATAGTAATC(A/T)GTTTTGGGAGA-3' (antisense, localized in exon Ib, eliminating the stop codon and adding a Xba I site to the 3'-end of the DBD II) and *Pfu* DNA polymerase. PCR conditions were as described above, but no additional $MgCl_2$ was used, and initial denaturation was extended to 2 minutes. PCR products were restricted with XbaI and inserted into the EcoRV/XbaI sites of the eukaryotic expression vector pcDNA/FLAG-His. This vector adds 3'-terminal FLAG- and 6xHis-epitopes to the inserted cDNAs and was generated as follows: from pcDNA3.1(+) (Invitrogen, Karlsruhe, Germany), the NheI-AflII fragment was excised from the polylinker to remove the 5' PmeI site and render the 3' PmeI site unique. Into the XbaI/PmeI sites of this modified vector, pcDNA3.1(P+), a fragment encoding the FLAG- and 6xHis-epitopes was inserted which had been retrieved from pVP22/FLAG-His by XbaI/PmeI digestion. The vector pVP22/FLAG-His is a modification of pVP22/myc-His (Invitrogen), from which the myc epitope had been removed by BstBI/AgeI digestion and replaced by a double-stranded oligonucleotide which encodes the FLAG epitope (DYKDDDDK) and destroyed the flanking BstBI/AgeI sites. All CREM inserts were verified by sequencing (Qiagen).

Protein Preparation

CREM isoforms in pcDNA/FLAG-His (500 ng) were expressed in vitro using the TNT T7 quick coupled transcription/translation system (Promega) in a final volume of 25 µL. For in vivo expression, the human uterine myosarcoma cell line SKUT-1B (HTB 115; American Type Culture Collection, Rockville, Md), was transiently transfected with CREM expression vectors using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Cells were maintained in DMEM/Hams F-12 (1:1; Sigma, Deisenhofen, Germany) supplemented with 10% FCS, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 mmol L-glutamine (PAA Laboratories, GmbH, Cölbe, Germany). For transfection, 2.5×10^5 cells/well were plated in 6-well tissue culture plates (BD Biosciences) coated with poly-L-lysine (Sigma). A mixture of 2.5 µg CREM/FLAG constructs and 10 µL Lipofectamine 2000 in OptiMEM I (Invitrogen) was added per well. Medium was changed 16 hours later, and whole cell protein was harvested 48 hours after transfection in 150 µL radioimmunoprecipitation buffer (PBS pH 7.4, 1% Igepal [Sigma], 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate and protease inhibitor cocktail [Complete; Roche]). Protein concentration was determined with a kit from BioRad (Munich, Germany).

Western Blot Analysis

In vitro translation products (5 µL) were mixed with 5 µL gel loading buffer (10 mmol Tris pH 7.2, 10% SDS, 25% 2-mercaptoethanol, 25% glycerol, 0.01% bromophenol blue). Whole cell extract (20 µg) was diluted in Laemmli gel loading buffer (50 mmol Tris pH 6.8, 100 mmol dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), and 2-mercaptoethanol was added to 10%. After denaturation at 95° C, proteins were separated on NuPAGE 4%– 12% Bis-Tris gels (Invitrogen) using NuPAGE MES SDS running buffer with antioxidant (Invitrogen) and subsequently transferred to polyvinylidene fluoride membrane (Millipore, Schwalbach, Germany) in NuPAGE transfer buffer (Invitrogen). Immunodetection was performed by enhanced chemiluminescence (Super Signal West Pico Chemiluminescent Substrate; Pierce, Bonn, Germany) with monoclonal anti-FLAG M2 antibody (1:1000; Stratagene, Amsterdam, The Netherlands) and horseradish peroxidase-conjugated goat anti-mouse IgG $F(ab')_2$-specific secondary antibody (1:5000; Jackson ImmunoResearch, Hamburg, Germany).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) was performed using 4 µL in vitro translation product. A Cy5-labeled double-stranded oligonucleotide with a consensus CRE (5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3') served as CRE probe. Proteins were incubated in bandshift buffer (20 mmol Tris pH 8.0, 1 mmol EDTA, 2.5 mmol DTT, 10% glycerol, 2 mmol $MgCl_2$, 0.1% Igepal, 50 ng/µL BSA) with 1.6 µg poly(deoxyinosinic acid-deoxycytidylic acid) for 15 minutes on ice. For competition reactions, 300-fold molar excess of unlabeled CRE or a mutated CRE sequence (5'-AGAGATTGCCTGtgGTCAGAGAGCTAG-3') was added. After addition of 100 fmol CRE probe, incubation was continued on ice for 30 minutes. For supershift studies, 2 µL anti-FLAG M2 antibody (Stratagene) were added and incubated at room temperature for 30 minutes. Reactions were resolved on 4% polyacrylamide gels in 0.25x Tris-borate-EDTA buffer (Invitrogen).

Reporter Gene Assays

Expression vectors for the catalytic (C) subunit of PKA (pcDNA-Cß), and an inactive mutant thereof (pcDNA-Cßmut), were generated by cloning the HindIII-XhoI full-length inserts from pRSV-Cß and pRSV-Cßmut, respectively (kindly provided by R. Maurer, Oregon Health & Science University, Portland, Ore) (Maurer, 1989), into the same sites of pcDNA3.1(+). For luciferase reporter gene assays, SKUT-1B cells (0.5×10^5 cells/well) were plated in 24-well plates coated with poly-L-lysine. Transient transfections were carried out in triplicates using Lipofectamine 2000 with 0.04 µg of CREM expression vectors, 0.5 µg of the cAMP-responsive reporter construct pCRE/-36rPRL/luc3 (Gellersen et al, 1997), and 0.01 µg of pcDNA-Cß or pcDNA-Cßmut. To keep the total amount of DNA constant, irrelevant plasmid was added to the reaction. The medium was replaced 16 hours later, and cells were incubated for an additional 24 hours before cell extracts were harvested for chemiluminescent luciferase assay (Promega). Statistical analysis was performed by one-way ANOVA followed by Bonferroni's post hoc test.

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Cloning of Novel CREM Isoforms

Using human testis RNA as the template, we obtained the cDNAs CREM-θ2-F-G-H-Ib and CREM-θ2-G-H-Ib (Figure 1), which were cloned into pcDNA/FLAG-His for expression of proteins with a 3' FLAG tag. For comparison, the known isoforms CREM-θ2-E-F-G-H-Ib and CREM-θ2-E-F-H-Ib (also designated CREM-θ2-τ2-ß and

CREM-θ2-ß, respectively) (Gellersen et al, 2002; Figure 1) were also inserted into the same expression vector.

In the new isoforms, omission of exon E or exons E and F disrupts the known ORF of CREM with translation initiation at the most 3' triplet in exon θ2 (Figure 2). Instead, an upstream ATG codon in exon θ2 might serve as initiator codon in CREM-θ2-F-G-H-Ib, resulting in a novel N-terminal sequence of 22 amino acids, which would then continue with the regular ORF in exon F (Figure 2). In the second isoform, CREM-θ2-G-H-Ib, however, both of the start codons in exon θ2 can only lead to translation of very short peptides terminating in exon G. The potential protein products and their predicted molecular masses (including the 3'-tag provided by the expression vector) are shown in Figure 2.

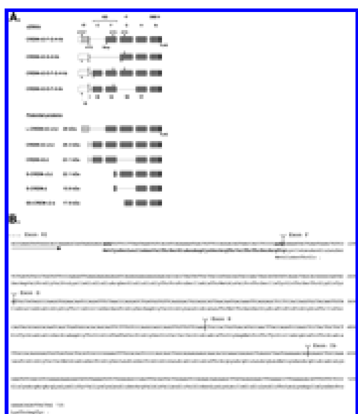

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Figure 2. Alternative translation initiation within P4-derived CREM isoforms. **(A)** *Top panel:* The CREM transcripts shown in Figure 1 were cloned into the expression vector pcDNA/FLAG-His so as to express a C-terminal FLAG epitope (black box). Putative ATG start codons in the cDNAs are indicated by arrowheads above the exons. The established ORF (shaded boxes) has been derived from isoforms CREM-θ2-E-F-G-H-Ib and CREM-θ2-E-F-H-Ib and is initiated at the ATG codon at the very 3'-end of exon θ2. Splicing of exons E or E and F, as in novel CREM isoforms CREM-θ2-F-G-H-Ib or CREM-θ2-G-H-Ib, respectively, disrupts this reading frame. Translation initiation at an upstream ATG in exon θ2 (labeled ATG?) would generate an alternative ORF in this exon (hatched box; 65 nucleotides), which then restores the downstream readingframe. Translation initiation which would result in early termination due to an altered reading frame is indicated by arrow heads below the exons; the respective stop codons are indicated by vertical lines. *Bottom panel:* The predicted proteins and their theoretical molecular masses (including the 3'-tag), resulting from alternative translation initiation at the start codons indicated in the top panel, are shown. **(B)** The cDNA sequence of CREM-θ2-F-G-H-Ib is shown from the sense primer used for amplification (arrow at the 5'-end) to the stop codon in exon Ib. Exon boundaries are indicated by Y-shaped symbols. Translation initiation at the regular ATG start codon located at the 3'-end of exon θ2 (italics, bold, underlined) results in an ORF of only 5 amino acids (italics). Utilization of the upstream ATG (bold, underlined), however, generates a novel alternative ORF of 22 amino acids (bold) that restores the regular ORF of CREM from exon F on.

Western Blot Analysis of CREM Isoforms

First, the CREM cDNAs θ2-F-G-H-Ib and θ2-G-H-Ib in the expression vector pcDNA/FLAG-His were subjected to in vitro transcription/translation in order to analyze potential resulting proteins in Western blotting with anti-FLAG M2 antibody. Isoforms CREM-θ2-E-F-G-H-Ib and CREM-θ2-E-F-H-Ib were included for comparison. As shown in Figure 3A, the CREM-θ2-F-G-H-Ib cDNA gives rise to proteins of 28 and 22.1 kd apparent molecular weight. This indicates that the novel upstream ORF in exon θ2 is translated to give rise to a full-length protein including part of the KID, the transactivation domain τ2, and the bZIP DBDII. The downstream ATG in exon G is not utilized, as it would have resulted in the SS-CREM-τ2-ß isoform of 17.9 kd (Figure 2). This isoform, however, is the only one detected as translation product from θ2-G-H-Ib. The cDNAs θ2-E-F-G-H-Ib and θ2-E-F-H-Ib give rise predominantly to products initiated in exon F (S-CREM-τ2-ß, 22.1 kd; S-CREM-ß, 15.9 kd). The 3' ATG in exon θ2 is used very inefficiently, as only a faint product of 29.3 kd and none of 23.1 kd are detected (Figures 2 and 3).

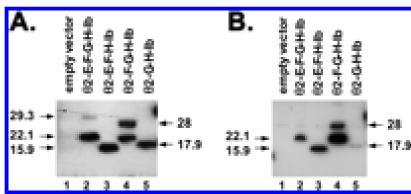

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Figure 3. Western blot analysis of CREM isoforms. **(A)** FLAG-tagged cDNAs for novel CREM isoforms CREM-θ2-F-G-H-Ib and CREM-θ2-G-H-Ib (lanes 4, 5) and known isoforms CREM-θ2-E-F-G-H-Ib and CREM-θ2-E-F-H-Ib (lanes 2, 3), cloned into pcDNA/FLAG-His, were subjected to in vitro transcription/translation. Products were detected by immunoblotting with anti-FLAG antibody. Sizes of isoforms are shown in kd. No signal was obtained with a transcription/translation reaction primed with the empty expression vector (lane 1). **(B)** The same constructs as in (A) were transfected into SKUT-1B cells. Whole cells extracts (20 µg) were subjected to Western blot analysis with anti-FLAG antibody.

As a next step, to ascertain generation of CREM isoforms in vivo, SKUT-1B cells were transfected with the CREM constructs and resulting proteins were extracted and immunodetected with anti-FLAG M2 antibody (Figure 3B). Essentially the same pattern of expression as described above for in vitro transcribed cDNAs was observed. It should be noted, however, that expression of the 17.9 kd product from CREM-θ2-G-H-Ib cDNA was much weaker in vivo than in vitro. No signals were detected when analyzing the empty expression vector pcDNA/FLAG-His.

Verification of Sequence-Specific DNA-Binding of CREM Isoforms

To investigate whether the proteins visualized in Western blotting are able to specifically bind to DNA, we performed EMSAs on in vitro translation products of the CREM isoforms (Figure 4). A Cy5-labeled oligonucleotide carrying a CRE consensus (TGACGTCA) served as the probe. Migration of free probe is seen in lane 1. Using transcription/translation mix primed with empty expression vector pcDNA/FLAG-His, 2 DNA/protein adducts were formed. The more slowly migrating complex (*), which cannot be competed by a 300-fold excess of unlabeled CRE, was also detectable with in vitro translation products of all CREM-isoforms. It is nonspecific and formed by a component of the reaction mix. A faster migrating complex (**) was also observed with all samples, including that primed with empty vector. It was competed not only by an excess of consensus CRE but also by the oligonucleotide carrying a mutated CRE. It can therefore be concluded that it is formed by proteins from the reaction mixture which do not bind to the core CRE sequence of the probe but to sequences flanking the palindromic octamer.

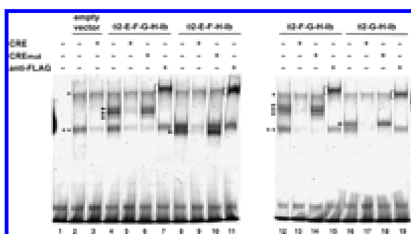

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Figure 4. Gelshift analysis of CREM isoforms. The same in vitro translation products as shown in Figure 3A were used for EMSA on a Cy5-labeled CRE consensus probe. Binding reactions were competed with an excess of unlabeled CRE consensus oligonucleotide (CRE; lanes 3, 5, 9, 13, 17) or a mutated CRE oligonuleotide (CREmut; lanes 6, 10, 14, 18). Supershift analysis was performed by addition of anti-FLAG antibody (lanes 7, 11, 15, 19). Specific shifted DNA-protein complexes are labeled with arrowheads, supershifted complexes by brackets. The asterisk (*) denotes a nonspecific complex. Two asterisks (**) mark a complex that is competed both by CRE and CREmut but is not supershifted.

Specific CRE binding was obtained with all samples primed with CREM expression constructs. θ2-E-F-G-H-Ib produced 3 complexes which were supershifted by FLAG antibody, competed by CRE but not by CREmut, and likely represent homo- and heterodimers of the 29.3 and 22.1 kd proteins (Figure 4,

lanes 4–7). The 15.9 kd product of the θ2-E-F-H-Ib cDNA resulted in 1 specific faster migrating complex (lanes 8–11). The novel CREM cDNA θ2-F-G-H-Ib produced 3 more slowly migrating products, likely representing homo- and heterodimers of the 28 and 22.1 kd isoforms (lanes 12–15), and the 17.9 kd isoform translated from the novel cDNA θ2-G-H-Ib formed 1 specific complex of intermediate mobility (lanes 16–19).

Verification of Functional Activity of CREM Isoforms In Vivo

Having demonstrated sequence-specific DNA binding ability of CREM isoforms, we subsequently determined their functional activity in vivo and transfected SKUT-1B cells with a luciferase reporter construct pCRE/-36rPRL/luc3, an expression vector for the catalytic subunit of PKA (pcDNA/Cß) or an inactive mutant (pcDNA/Cßmut), and the CREM cDNAs or empty expression vector (Figure 5). The active PKA subunit Cß, but not the mutated form, strongly stimulated reporter gene expression, due to phosphorylation of endogenous CRE binding proteins in SKUT-1B cells. This Cß-dependent stimulation was significantly inhibited by coexpression of CREM-θ2-E-F-G-H-Ib, θ2-E-F-H-Ib or novel θ2-F-G-H-Ib, but not by the novel θ2-G-H-Ib. This indicates that protein isoforms translated from the first 3 cDNAs are predominantly repressors. The 17.9-kd protein translated from the θ2-G-H-Ib cDNA is also predicted to be a repressor, as it lacks the KID, but the level of expression in SKUT-1B cells is probably too low to produce a functional response (compare Figure 3B, lane 5).

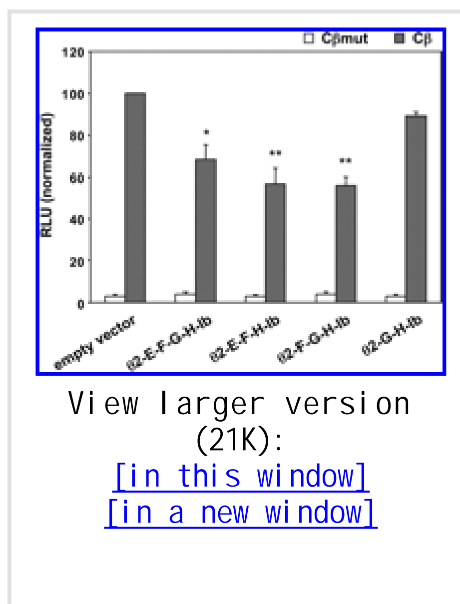

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Figure 5. Transcriptional activity of CREM isoforms. For functional studies, SKUT-1B cells were transfected with the reporter construct pCRE/-36rPRL/luc3, an expression vector for the catalytic subunit of PKA (Cß; shaded bars) or an inactive mutant thereof (Cßmut; open bars), and the indicated CREM-expression vectors. Controls received the empty expression vector pcDNA/FLAG-His. Luciferase activities were measured as relative light units (RLU) and normalized to the value obtained with empty vector plus Cß (set to 100). Results represent the means ± SEM of 3 independent experiments. Asterisks indicate statistically significant difference from the activity obtained with empty vector plus Cß (*$P < .05$; **$P < .001$).

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In the present study, we isolated the full-length cDNAs for 2 novel CREM splice variants which had previously been detected in human male germ cells (Blöcher et al, 2003; Blöcher et al, 2005), CREM-θ2-F-G-H-Ib and CREM-θ2-G-H-Ib, and subjected them to functional analyses.

Western blot analysis of the CREM-θ2-F-G-H-Ib translation product indicates that the novel upstream ORF in exon θ2 is translated to give rise to a full-length protein of 28 kd. Other proteins detected correspond to previously described truncated S- or SS-CREM isoforms resulting from downstream translation initiation (Gellersen et al, 2002). It has to be noted that predicted sizes of CREM protein isoforms in this study compared to a previous report (Gellersen et al, 2002) are

approximately 2 kd larger due to a longer 3' tag provided by the expression vector employed in the present study.

All alternative translation products potentially produced from the 4 CREM cDNAs included in this study are predicted to have sequence-specific DNA binding ability. This was confirmed in EMSA on a CRE consensus probe. Remarkably, the relative mobility and the number of DNA/protein complexes formed with the products of a particular cDNA reflected the relative mass and number of protein isoforms detected by Western blot analysis. CREMs, like all bZIP proteins, are known to bind to their palindromic target sequence as homo- or heterodimers with other bZIP proteins, due to interaction via the leucine zipper region (Walker and Habener, 1996). When a single product was detected by Western blot analysis, a single homodimeric complex was formed in EMSA, for example by S-CREM-ß (15.9 kd) or SS-CREM-τ2-ß (17.9 kd) translated from θ2-E-F-H-Ib or θ2-G-H-Ib, respectively. When 2 products were translated from a single cDNA, as was the case for θ2-E-F-G-H-Ib and θ2-F-G-H-Ib, 3 complexes were observed in EMSA, likely due to formation of 2 different homodimers and 1 heterodimer. From our observations in the gel shift study and the Western blot analyses of both in vitro translated and in vivo expressed CREM-θ2-F-G-H-Ib, we conclude that the proposed alternative longer ORF in exon θ2 is indeed expressed. It adds a novel N-terminal sequence of 22 amino acids to the C-terminal part of the KID, followed by the transactivation domain τ2 and the bZIP/DBDII. The resultant longer (L) protein of 28 kd (Figures 2A and 3) is readily detectable via the 3' FLAG epitope, proving maintenance of the regular ORF downstream of exon θ2. This protein, which we designated L-CREM-θ2-τ2-ß, engages in homo- and heterodimerization and binds to a CRE.

It remains to be established if this novel protein is a transcriptional activator or repressor. Activators are characterized by the presence of the KID, 1 or both of the transactivation domains (τ1, τ2), and 1 of the 2 DBDs, whereas repressors lack a transactivation domain (Walker et al, 1994; Behr and Weinbauer, 2001). While L-CREM-θ2-τ2-ß contains the τ2 as a prerequisite to be a transcriptional activator, it is not known at present if the truncated KID (encoded by exon F only) suffices for PKA-mediated phosphorylation and subsequent activation. The luciferase reporter gene assay does not allow unambiguous conclusions, because transfection of the CREM-θ2-F-G-H-Ib cDNA results in simultaneous production of L-CREM-θ2-τ2-ß (28 kd) and S-CREM-τ2-ß (22.1 kd), as shown by Western blot analysis of SKUT-1B cell extracts. The latter isoform is more abundantly expressed in transfected cells and may antagonize activation potential of the former. This phenomenon was observed for transfected CREM-θ2-E-F-G-H-Ib cDNA, included as a control in this experiment. This cDNA gives rise to a small amount of the established transcriptional activator CREM-θ2-τ2-ß (29.3 kd) and, more abundantly, repressor S-CREM-τ2-ß (22.1 kd). The net result was inhibition of PKA-Cß-stimulated CRE-driven reporter gene expression, indicating that protein isoforms translated from the 3 cDNAs CREM-θ2-E-F-G-H-Ib, CREM-θ2-E-F-H-Ib, and the novel CREM-θ2-F-G-H-Ib are predominantly repressors. The transcriptional activity of the 17.9-kd protein translated from the CREM-θ2-G-H-Ib cDNA, predicted to be a repressor because it lacks the KID, could not be determined due to the low level of expression in transfected cells.

In conclusion, we functionally characterized 2 novel CREM isoforms, θ2-F-G-H and θ2-G-H, which in contrast to known isoforms exhibit a transactivation domain (encoded by exon G) but lack a kinase-inducible domain (encoded by exons E and F), resulting in a disruption of the open reading frame. Transcriptional activity of these isoforms was investigated in transfected cells applying a CRE-driven luciferase reporter gene. Although data do not allow us to clearly decide whether these isoforms represent activators or repressors, electrophoretic mobility shift assays demonstrated that translation products of both θ2-F-G-H-Ib and θ2-G-H-Ib cDNAs are able to specifically bind to DNA. Both in vitro and in vivo protein analyses, in addition, indicated that a novel upstream open reading frame in exon θ2 is translated from θ2-F-G-H-Ib, giving rise to a full-length protein.

Finally, usage of downstream ATGs for translation initiation could be observed in both isoforms. Although our data contribute to shedding more light on the regulation of both CREM gene transcription and CREM transcript translation, further studies will be necessary to unravel the complex role of the variety of CREM activator and repressor isoforms for male (in)fertility.

References

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