

Characterization of the human *CREB3L2* gene promoter

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Abstract. *CREB3L2* encodes a member of the CREB3 family of transcription factors. We characterized its promoter region, showing that it is asymmetrically bidirectional, also driving the expression of a variant of *AKR1D1*. It has a CRE binding site which is conserved among mammals; removal or alteration of it resulted in reduced promoter activity. When transiently transfecting the HEK293 cell line with constructs with partially deleted promoter regions, 5' deletions beyond 1058-bp upstream of the transcription starting site resulted in successive reduction of the activity. The inclusion of the untranslated part of *CREB3L2* exon 1 strongly inhibited the promoter activity. Forskolin resulted in a decreased reporter activity, whereas phorbol 12-myristate 13-acetate increased the promoter activity irrespective of the status of the CRE binding site. The presence of the CRE site indicates autoregulation of *CREB3L2* and/or regulation via other members of the CREB3 family or a variety of bZIP transcription factors.

Introduction

CREB3L2 (*BBF2H7*) was first identified as the partner gene of *FUS* in a chimera found in two cases of low grade fibromyxoid sarcoma (LGFMS) (1). Screening of a larger number of LGFMS demonstrated that virtually all are characterized by a chimeric *FUS/CREB3L2* gene, and that rare cases may display a variant *FUS/CREB3L1* fusion (2,3). Genomic characterization of *CREB3L2* showed that it spans >120-kbp genomic DNA, is composed of 12 exons and is expressed in most of the 24 human tissues examined (1). The *CREB3L2* protein is a member of the CREB3 family of transcription factors. Its B-ZIP domain is highly similar to that in the *CREB3L1* (OASIS), *CREB3L3* (CREB-H), *CREB3L4* (CREB4), *CREB3* and *Drosophila* BBF-2 transcription factors with 80, 60, 59, 56 and 71% identity, respectively and the leucine zipper motif of *CREB3L2* is similar to that in *CREB3L3* and *CREB3L4* (pattern L-X₆-C-X₆-L-X₆-L-X₆-L-X₆-L). Downstream of the B-ZIP domain, and similar to

CREB3L1, *CREB3L2* contains a hydrophobic region, which was predicted to be an α -helical transmembrane domain and is a typical structural feature for the transcription factors activated by regulated intramembrane proteolysis (1,4). These proteins can dimerize with each other, can bind CRE DNA sequences, can activate transcription through box-B and ATF6 elements, and contain a transcriptional activation domain at the N-terminal part. Although the exact cellular role of the *FUS/CREB3L2* fusion in the pathogenesis of LGFMS is still unclear, one can postulate that all the above mentioned functions may be influenced since the B-ZIP encoding domain of *CREB3L2* is fused to the N-terminal part of *FUS* and comes under the control of the *FUS* promoter. Thus, *FUS/CREB3L2* may act as an abnormal transcription factor that undergoes intramembraneous proteolysis, allowing the N-terminal *FUS* domain with the bZIP domain of *CREB3L2* to enter the nucleus and to activate/deregulate a number of target genes.

In a previous study we assessed various cellular outcomes after transfection of NIH3T3 and HEK-293 cells with constructs containing full-length and truncated (*CREB3L2 Δ TM* and *FUS/CREB3L2 Δ TM*) versions of *CREB3L2* and *FUS/CREB3L2* (5). The minimum region for transcriptional activation by *CREB3L2* was seen in constructs containing the first 120 amino acids (aa). Much stronger transcriptional activation was consistently seen for the *FUS/CREB3L2* constructs, in which the first 224 aa of *CREB3L2* are replaced by the first 212 aa of *FUS*, than for the corresponding *CREB3L2* constructs. Transcriptional activity was achieved through the box-B element, ATF6 and CRE binding sites, as well as the *GRP78* promoter. Proteins encoded by full-length *CREB3L2* and *FUS/CREB3L2* were localized to reticular structures of the cytoplasm, whereas the corresponding, truncated proteins lacking the transmembrane domain and the carboxy-terminal part of *CREB3L2* resided within the nucleus. Recently, *CREB3L2* was shown to be induced at the translational level during endoplasmic reticulum stress, suggesting that it might contribute to a late phase of unfolded protein response signalling (4).

In the present study, we characterized the promoter region of the *CREB3L2* gene.

Materials and methods

Vectors. All PCR amplifications were performed in a 50 μ l reaction volume containing 1X AccuPrime Pfx reaction mix, 1 unit AccuPrime Pfx DNA polymerase (Invitrogen, Carlsbad, CA), 0.3 μ M of each of the forward and reverse primers (Table I) and 200 ng template DNA. The PCR was run on a

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Table I. Plasmid name, primers used for the amplification of promoter region, size of the amplified products and the corresponding promoter region of *CREB3L2*.

Plasmid	Forward primer	Reverse primer	Size (bp)	Promoter region
p-1297/+396	CGGGGTACCGATAGGACGACAAATGCAATA	GGAAGATCTCGCTCTCCAGCACCTCCAT	1693	-1297/+396
p-1058/+396	CGGGGTACCTGTTCGGGGGAATTCCTTTAG	GGAAGATCTCGCTCTCCAGCACCTCCAT	1454	-1058/+396
p-831/+396	CGGGGTACCTTGACGCATCCCTGTTTCTGTC	GGAAGATCTCGCTCTCCAGCACCTCCAT	1227	-831/+396
p-580/+396	CGGGGTACCTGAAGAACAATGACAATTTCCT	GGAAGATCTCGCTCTCCAGCACCTCCAT	976	-580/+396
p-328/+396	CGGGGTACCGCTATTTCAATGGCTGTACCTT	GGAAGATCTCGCTCTCCAGCACCTCCAT	724	-328/+396
p-177/+396	CGGGGTACCAACAGACACTCCCGGCTACA	GGAAGATCTCGCTCTCCAGCACCTCCAT	573	-177/+396
p-1297/+1	CGGGGTACCGATAGGACGACAAATGCAATA	GGAAGATCTGCTCTGCTCCAGGACCCAGC	1297	-1297/+1
p-1058/+1	CGGGGTACCTGTTCGGGGGAATTCCTTTAG	GGAAGATCTGCTCTGCTCCAGGACCCAGC	1058	-1058/+1
p-831/+1	CGGGGTACCTTGACGCATCCCTGTTTCTGTC	GGAAGATCTGCTCTGCTCCAGGACCCAGC	831	-831/+1
p-580/+1	CGGGGTACCTGAAGAACAATGACAATTTCCT	GGAAGATCTGCTCTGCTCCAGGACCCAGC	580	-580/+1
p-328/+1	CGGGGTACCGCTATTTCAATGGCTGTACCTT	GGAAGATCTGCTCTGCTCCAGGACCCAGC	328	-328/+1
p-177/+1	CGGGGTACCAACAGACACTCCCGGCTACA	GGAAGATCTGCTCTGCTCCAGGACCCAGC	177	-177/+1

PCT-200 DNA Engine (MJ Research, Waltham, MA). The cycling included an initial denaturation at 95°C for 2 min, followed by 30 cycles of 15 sec at 95°C, 30 sec at 58°C, and 2 min at 68°C, and a final extension for 5 min at 72°C. All ligations were performed overnight at 16°C in a 10 µl reaction volume containing 1X Ligase reaction buffer, 5 units T4 DNA ligase (Invitrogen) and 1:3 vector to insert ratio. When purification was required, the DNA fragments were purified using either the QIAquick gel extraction kit or the QIAquick PCR purification kit (Qiagen, Hilden, Germany). For sequence confirmation, the ABI Prism BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) was used. Dye-incorporated DNA fragments were subsequently purified by centrifugation using DyeEX spin column (Qiagen) according to the manufacturer's instructions and were analyzed on an Applied Biosystems model 3100-Avant DNA sequencing system. All the plasmids were prepared using Qiagen's plasmid miniprep kit.

In order to measure simultaneously promoter activity and transfection efficiency, a report vector, pFhRL, was constructed to contain both *Photinus pyralis* (firefly) and *Renilla reniformis* luciferase genes (6). In the pFhRL vector, the luciferase genes are placed in opposite directions to avoid read-through, 'leaky' transcription and the expression of *Renilla* luciferase is driven by the SV40 early enhancer/promoter. For the construction of pFhRL, a 1327-bp fragment containing the SV40 early enhancer/promoter and *Renilla* luciferase reporter gene was amplified from the pGL4.73 [*hRluc*/SV40] plasmid (Promega) using the forward primer 5'-GAAAGGTCGACCTGTGGAATGTGTGTCACT-3' and the reverse primer 5'-CGCGGATCCAATTACTGCTCGTTCTTCAGC-3', which contain *Sall* and *Bam*HI restriction sites, respectively. The fragment was digested with *Sall* and *Bam*HI restriction endonucleases and subcloned between the corresponding sites of the pGL4.10 [*luc2*] vector, downstream of the firefly luciferase gene.

DNAs containing various lengths of the *CREB3L2* promoter region were PCR amplified using the BAC clone RP11-377B19 (AC009263) as template and various primer sets (Table I) and cloned in the pFhRL vector between *Kpn*I and *Bgl*III restriction sites, upstream of the firefly luciferase cDNA.

For experiments concerning transcription activation through various DNA-binding sites, pCR3.1 (Invitrogen)-based constructs containing the truncated *CREB3L2*ΔTM (amino acids 1-372; in this form the transmembrane domain and the C-terminal part have been deleted), and FUS/*CREB3L2*ΔTM (amino acids 1-366 of the FUS/*CREB3L2* protein), and ATF6ΔTM (amino acids 1-377) were used. For the pCR3.1-*CREB3L2*ΔTM construction, a 1116-bp *CREB3L2* cDNA fragment was amplified using the forward CGGGATCCGTATG GAG GTG CTG GAG AGC G and the reverse GTA GAATTCCTTA GCC AGC TAA CTT GCA GGT TCG primers with *Bam*HI and *Eco*RI restriction sites, respectively, and the pCR3.1-*CREB3L2* plasmid as template (5). For the construction of pCR3.1-FUS/*CREB3L2*ΔTM, a 1097-bp cDNA fragment of FUS/*CREB3L2* was amplified using the FUS forward primer GGCGGATCC ATG GCC TCA AAC GAT TAT ACC C, the above mentioned reverse primer and the pCR3.1-FUS/*CREB3L2* plasmid as template (5). Subsequently, the amplified cDNA fragments were digested and inserted



SPANDIDOS PUBLICATIONS The *Bam*HI and *Eco*RI restriction sites of the pCR3.1 or the construction of pCR3.1-ATF6 Δ TM, the forward primer GTCTGGAAGCTT ATG GGG GAG CCG GCT GGG which contained a *Hind*III site and the reverse primer GCGGCTCGAG TCT TCG CTT TGG ACT AGG GAC T with an *Xho*I site were used to amplify a 1131-bp *ATF6* cDNA fragment (amino acids 1-377) from a cDNA of the K562 cell line.

Site-directed mutagenesis was performed using the Gene Tailor site-directed mutagenesis system (Invitrogen) according to the manufacturer's instructions. The reverse primer: ACC GAG GAA CTC CTC CTG CTT GAG CGC GGC CCG CTG TC was used together with 1) AAG CAG GAG GAG TTC CTC GGC ACA GAC ACT CC, 2) AAG CAG GAG GAG TTC CTC GGT GTC GTC ACA GAC ACT CC, 3) AAG CAG GAG GAG TTC CTC GGT GTT GTC ACA GAC ACT CC, and 4) AAG CAG GAG GAG TTC CTC GGT GTT TTC ACA GAC ACT CC to introduce the Δ TGACGT, TGTCGT, TGTTGT and TGTTTT mutations respectively, at the CRE site of the promoter in the p-328/+1 plasmid (Table I).

Cell lines and transfection experiments. The cell line HEK293 (obtained from The Banca Cellule e Colture in GMP, Italy) was used for transfection experiments. The cells were cultured in RPMI-1640 medium, supplemented with fetal bovine serum (10%), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin. Transfections were performed with PolyFect transfection reagent (Qiagen) according to the manufacturer's recommendations (<http://www1.qiagen.com/literature/protocols/pdf/TFP03.pdf>). Briefly, 5×10^4 cells were seeded in 96-well plates, and 24 h later they were transfected with the appropriate plasmid combinations using 1 μ l PolyFect transfection reagent. For experiments concerning transcription potential of various parts of the *CREB3L2* promoter (deletion constructs) 500 ng of each vector was used. To study the effects of ATF6 Δ TM, CREB3L2 Δ TM and FUS/CREB3L2 Δ TM, 200 ng of the plasmid p-328/+1 were co-transfected together with 5, 50 and 500 ng of pCR3.1-based expression plasmids which contain the above mentioned transcription factors. To study the effects of unfolded protein response stress inducers, 28 h after transfection, tunicamycin (at concentration 2 μ g/ml), thapsigargin (at concentration 500 nM) or brefeldin (at concentration 0.5 μ g/ml) were added and the cells were further cultured for 16 h. To study the effects of CRE-related activators, forskolin and phorbol 12-myristate 13-acetate (PMA) were used. After 24 h transfection the culture medium was replaced with a new medium which contained 0.1% fetal bovine serum and 10 μ M forskolin, or 100 nM PMA or 10 μ M forskolin +100 nM PMA. All the above mentioned chemicals were purchased from Sigma-Aldrich.

Dual luciferase assay. Cells were lysed 48 h after transfection in 40 μ l of 1X passive lysis buffer (Promega) and assayed for the firefly and *Renilla* luciferase activities using the Dual Luciferase assay system (Promega), according to the manufacturer's instructions. The results were normalized against the *Renilla* luciferase activities. Measurements were performed with the Veritas 96-microplate luminometer (Turner Biosystems, Sunnyvale, CA) using 10 μ l cell lysate and 96-well medium binding Lumitrac 200 plates (Greiner bio-one,

Kremsmuenster, Austria). Each construct was measured in eight replicas. The results are presented as the median together with the 25th and 75th percentiles. The Mann-Whitney 2-tailed test was used for the statistical analysis using the statistiXL software (<http://www.statistixl.com>).

In silico analysis. The UCSC Genome Browser was used (<http://genome.ucsc.edu/index.html?org=Human>). The sequences of human *CREB3L2* (ENSG00000182158) and the orthologues from 16 mammals were obtained from ensembl (Ensembl release 49 - March 2008; <http://www.ensembl.org/index.html>). For genomic alignment the MultAlin software (7) was used (<http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html>). The Patch public 1.0 software was used to identify transcription factor binding sites (<http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi>). In addition, the pre-computed whole genome alignments of the Vista suite of programs and databases were used for comparative genomic sequences (<http://genome.lbl.gov/vista/index.shtml>): Vista browser for the pairwise alignment of the human and mouse sequence, and the Whole Genome rVista in order to evaluate conserved transcription factor binding sites (TFBS) in the upstream region of *CREB3L2*. The repeat-masker was used to identify sequence repeats (<http://www.repeatmasker.org/>). The sequence together with the first exon of *CREB3L2* was also screened for CpG islands using the CpG Island searcher (<http://cpgislands.usc.edu/>) (8). Default parameters for the identification of CpG islands included using the lower-limit values 500 bp for length, 55% for GC content, and 0.65 for Observed CpG/Expected CpG (8).

Expression analysis. Expression analyses (RT-PCR) were performed using testis, liver and colon ready cDNA obtained from human MTC panels I and II (Clontech, Mountain View, CA), the cell lines TERA-2 (HTB-106) and NCCIT (CRL-2073) obtained from LGC Promochem (<http://www.lgcpromochem-atcc.com/>) and an ovarian dysgerminoma from a 13-year-old girl. Total RNA from the two mentioned cells lines and the dysgerminoma was extracted using the TRIzol reagent (Invitrogen) and cDNA synthesis was done as described (1). The primer combination AKR1AK-1F (AAT AGA GGG CTT GGG TCC GGA AGT) and AKR1D1AK-396R (TTG GGT CCG GAA GTG ATG GAC TG) was used for the amplification. The 50 μ l reaction volume contained 20 mM Tris-HCl pH 8.4 (at 25°C), 50 mM KCl, 1.25 mM MgCl₂, 0.2 mM of each dNTP, 1 unit PlatinumTaq DNA polymerase (Invitrogen), 0.5 μ M of each of the forward AKR1AK-1F and reverse AKR1D1AK-396R primers, and 3 μ l of the ready cDNA or 2 μ l cDNA from the cell line. PCR was run using a PCT-200 DNA engine (MJ Research) and the cycling was: an initial cycle at 94°C for 2 min, 35 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, followed by a final extension for 10 min at 72°C. PCR products (15 μ l) were analyzed by electrophoresis through 1.5% agarose gels, stained with ethidium bromide, and photographed. The amplified DNA fragments were further purified using QIAquick gel extraction kit (Qiagen), directly sequenced using the ABI Prism BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems) and analyzed on an Applied Biosystems model 3100-Avant DNA sequencing system. The

Table II. Summary of all conserved transcription factor binding sites located in a 2000-bp upstream region of *CREB3L2* (locus link 64764) identified with the engine Whole Genome rVista.

Number of hits	Transcription factor
1	AREB6, ATF3, BARBIE, CAAT, CDPCR3HD, CREBATF, CREBP1CJUN, DR3, E4F1, ER, FOXM1, FOXO3A, GATA3, HAND1E47, HFH3, HNF4ALPHA, IK3, IRF1, LEF1TCF1, MMEF2, NKX3A, NKX61, NRF2, PAX6, PITX2, POU1F1, PPARG, RBPJK, RFX1, SOX9, TEF1, XBP1
2	AP1, ATF, ATF1, ATF4, BACH2, CEBPDELTA, CEBPGAMMA, CETS1P54, CREBP1, E2F1DP1, E2F1DP2, E2F4DP2, ELK1, FOXJ2, FREAC7, GATA4, HELIOSA, HNF3B, HOXA4, KROX, LDSPOLYA, MAZ, MAZR, MINI19, MYCMAX, NFAT, PBX, POU6F1, SZF11, TBX5, TFIII, XPF1
3	CHOP, HLF, NFY, SF1, SOX5, TFIIA
4	ETS, IRF7, STAT, VMAF
5	MRF2, MTATA
6	E2F1, FOX, IRF
7	CDX2, CP2, LEF1
8	CDC5
9	CREB, EFC, SRF
10	MEF2
11	CDX, CMAF, EGR, PIT1
12	HNF3ALPHA
13	XVENT1
15	CREL
16	FOXD3, PAX3
17	HNF3, NFKB, TST1
19	CACD, TATA
21	GC
24	OCT4, PAX
27	OCT1
31	E2F, PAX4
33	LYF1
36	EVI1
37	VDR
46	DR4, HNF1, HNF4
59	PPAR
61	AP2
82	DR1
83	SP1

BLAT (<http://genome.ucsc.edu/cgi-bin/hgBlat?command=start>) and BLAST (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) programs were used for computer analyses of sequence data.

Results

In silico and expression analyses. A 2000-bp DNA fragment upstream from the first exon of *CREB3L2* was chosen arbitrarily for promoter analysis. The DNA sequence was first screened for interspersed repeats and low complexity DNA sequences using the RepeatMasker program. The analysis identified a 305-bp ALU repeat, a 259-bp LINE2 fragment, and a 209-bp MER2 DNA element positioned 1296-1600 bp, 686-975 bp and 1791-2000 bp upstream of *CREB3L2* exon 1, respectively (Fig. 1). In addition, the sequence together with exon 1 of *CREB3L2* has one 974-bp CpG-island with 60% CG content (from position -477 through entire exon 1) and ObservedCpG/ExpectedCpG ratio 0.785. Using the Patch public 1.0 software and the Whole Genome rVista 140 conserved transcription factor binding site (TFBS) were identified (Table II). Furthermore, the Whole Genome rVista program showed that CRE binding site (BS), GTGACGTCAC, was highly conserved (Fig. 1). Two other conserved BS were also identified with TFBS conserved track in UCSC genome browser: a TGIF site within exon 1 of *CREB3L2* and an NKX6-1 site, 139-bp upstream of the CRE site (Fig. 1).

Alignment of the human (ENSG00000182158) and the orthologues sequences from 16 mammals revealed high conservation of the CRE BS (Table III). Interestingly, 18 nucleotides (20 for opossum) downstream of the CRE BS an ACATTCC sequence motif was found to be conserved in all mammals. The ACATTCCA sequence was reported to be a binding site of miR-206, miR-1 and miR-122a (9). No TATA-box was found but at position -97 to -90 a palindromic CCAATTGG was observed.

According to the UCSC genome browser, 283-bp upstream of the +1 transcription site and in opposite direction to *CREB3L2* a cDNA, AK058142, has been mapped. The sequence is specific for testis, is supported by four other spliced EST sequences (BU561106, AI198697, AI656277 and AI652102) and seems to be an alternative variant of *AKR1D1* which codes for aldo-keto reductase family 1, member D1 (δ 4-3-ketosteroid-5- β -reductase). RT-PCR with the AKR1AK-1F forward primer and the AKR1D1AK-396R reverse primer amplified three fragments from the testis cDNA but none from liver, colon, TERA-2, NCCIT or the dysgerminoma (Fig. 2). Direct sequencing of the amplified fragments showed that they were alternative transcript variants of the *AKR1D1* gene and contained parts of AK058142, *AKR1D1* (NM_005989) and an additional sequence presumably represented by not previously defined exons specifically expressed in testis (Fig. 2). From the long 1092-bp transcript (GenBank accession no. FM160741) the entire AKR1D1 (NP_005980) would be translated. The 930 bp (GenBank accession no. FM160742) would result in an AKR1D1 protein with an alternative N-terminal; the first 32 amino acids (MDLSAASHRIPLSDGNSIPIIIGLGTYPSEP KST) would be replaced by 42 amino acids (MLGSLAWDAISLTPNAWLNSSAALGLSSVNHSIASLYAGVWP), while the 566-bp fragment (GenBank accession no. FM160743)

Table III. Partial alignment in the promoter region of 17 mammals.^a

Species	Ensembl Gene ID	Position	Sequence			
Human	ENST00000330387	-575 to -518	CTCGGTGACG	TCACA--GAC	ACTCCCCGGC	TACATTCCAA
Mouse lemur	ENSMICG00000003960	-583 to -526	CCGGGTGACG	TCAAA--GGC	ACTGCCCGCC	CACATTCCAA
Squirrel	ENSSTOT00000009816	-571 to -514	CCCGGTGACG	TCAAG--GGC	GCTCCCCGGC	CACATTCCAA
Cow	ENSBTAG00000015802	-586 to -529	CCCGGTGACG	TCAAA--GGC	GCTGCCTGCC	CACATTCCAA
Horse	ENSECAG00000007609	-590 to -533	TCCGGTGACG	TAAAC--GGC	ACGCCCTGCC	CACATTCCAA
Cat	ENSFCAG00000000648	-591 to -534	CCCGGTGACG	TCAAA--GGC	GCTCCCCGGC	CACATTCCAA
Pika	ENSOPRG00000004323	-604 to -547	CCCGGTGACG	TCAAA--GGC	GCACCCGGCC	CACATTCCAA
Armadillo	ENSNDNOG00000004102	-560 to -503	CACGGTGACG	TCAAA--GAA	GCGCCCTCC	CACATTCCAA
Tree shrew	ENSTBET00000004884	-569 to -512	CTCAGTGACG	TCAGA--GGC	ACTCCCCGGC	CACATTCCAA
Dog	ENSACAFG00000013481	-563 to -506	CCCGGTGACG	TCAGA--GGC	GCTCCCCGGC	CACATTCCAA
Bushbaby	ENSOGAT00000010848	-540 to -483	CCCGGTGACG	TCAAA--GTC	ACGCCTCGCC	TACATTCCAA
Mouse	ENSMUSG000000038648	-559 to -502	CCCAGTGACG	TAAA--GAC	CCTCTCCGCC	CACATTCCGA
Rabbit	ENSOCUT00000007313	-505 to -448	CTGTTGACG	TCCAG--GGC	GCTCCCCGGC	CACATTCCAA
Guinea pig	ENSCPOG00000008872	-514 to -457	CCCGGTGACG	TCAAA--GGT	GCTCCCAGCC	CACATTCCAA
Shrew	ENSSART00000000499	-564 to -508	CTCGGTGACG	TCAAA--G-C	GCTCCCCGGC	TACATTCCAC
Opposum	ENSMODG00000013876	-650 to -591	CCCGGTGACG	TCAAACCGGG	ACTCCCCTCC	CACATTCCCTA
Elephant	ENSLAFG00000008469	-480 to -423	GCTGTTGACG	TCAAA--GGC	GCGCCCGGCC	CACATTCCAC
Consensus			cccGTGACG	TcAaa Ggc	gCtCCCCgCC	cACATTCCaa
			GgAGGaGTTc			aGcctgGGCg

^aThe sequences are extracted from Ensembl release 49-Mar 2008. Position is calculated upstream from the first ATG codon. The consensus GTGACGT-18N-ACATTCC is found in all species but opposum and shrew.

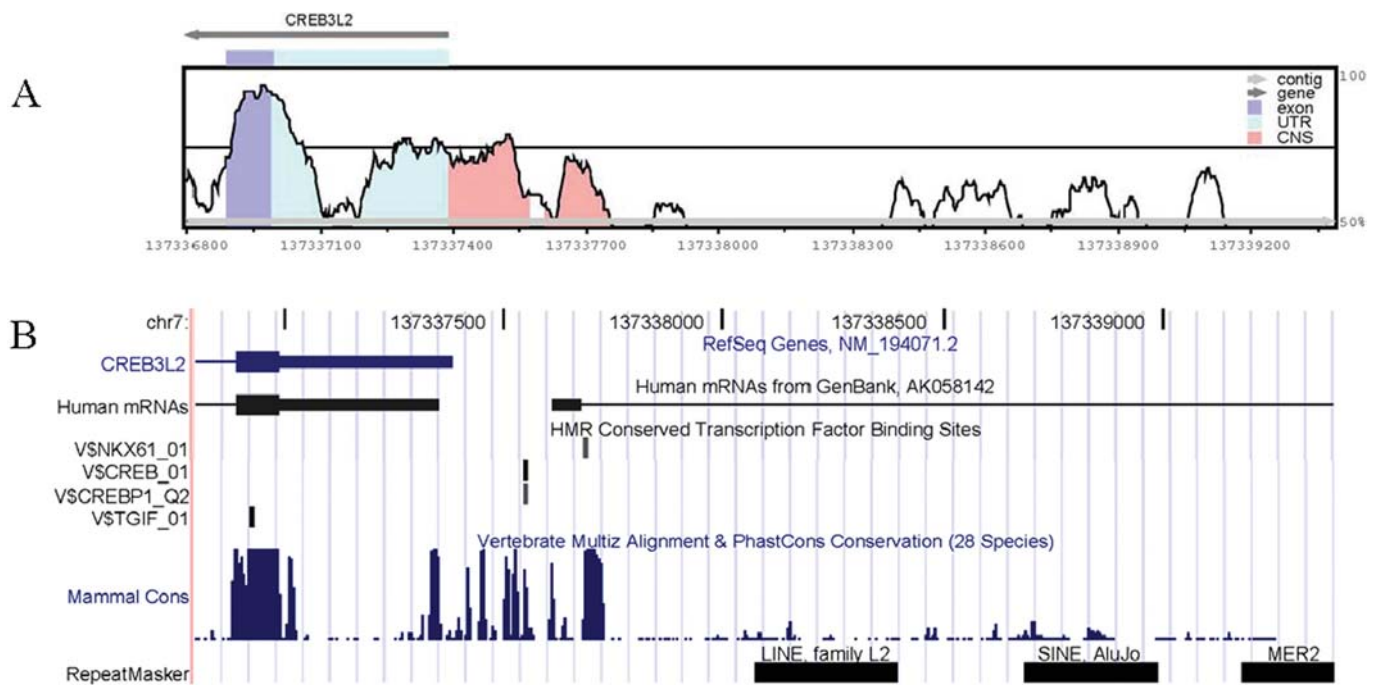


Figure 1. The promoter region of *CREB3L2*. (A) Alignment of mouse chr6 (+) 37371228-37373645 with human chr7:137336800-137339386 (X-axis). The pairwise alignment of Human March 2006 genome with the Mouse February 2006 genome was performed with Vista browser. (B) Diagram, obtained from the UCSC genome browser on Human March 2006 assembly, showing *CREB3L2*, AK058142, conserved transcription factor binding sites, evolutionary conserved DNA regions and interspersed DNA repeats. CNS indicates conserved non-coding sequence.

would correspond to a shorter form of AKR1D1 lacking the first 153 amino acids.

Deletion analysis of the *CREB3L2* promoter. To obtain experimental evidence and identify regions that are important for promoter activity of the 5'-flanking sequences, a series of partial deletion promoter constructs was used to transiently transfect HEK293 cell line. The highest activity was obtained with the constructs p-1297/+1 and p-1058/+1 (Fig. 3). Further 5' deletions resulted in successive reduction of the promoter activity. Compared to the p-1297/+1 construct activity reduction in the activity by 10, 17, 30 and 60% was observed for p-831/+1, p-580/+1, p-328/+1 and p-177/+1, respectively. The inclusion of the untranslated part of *CREB3L2* exon 1 strongly inhibited the promoter activity (Fig. 3), suggesting the presence of negative regulatory elements.

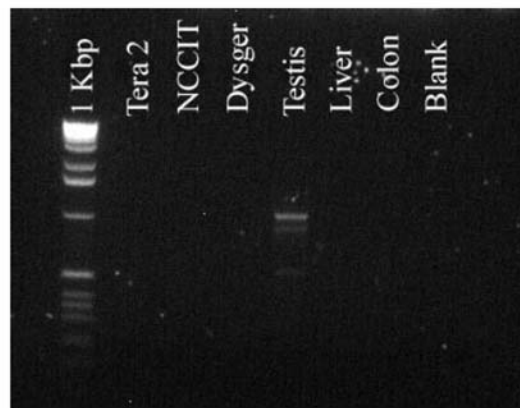
Analysis of CRE binding site. To test the functional importance of the CRE BS, GTGACGTCAC, directed mutagenesis was used to selectively disrupt the core of the site. Thus, the CRE BS of the promoter in the p-328/+1 plasmid was completely removed or 1, 2 or 3 nucleotides were altered generating Δ TGACGT, TGTCGT, TGTTGT and TGTTTT mutations, respectively. Compared to the p-328/+1 construct, a progressive reduction of the promoter activity by 16, 27 and 35% was obtained with substitution of one, two or three nucleotides, respectively (Fig. 4A). Complete removal of the binding site resulted in the same reduction (33%) as the replacement of three nucleotides (Fig. 4A).

To study the effects of the ATF6 Δ TM, CREB3L2 Δ TM and FUS/CREB3L2 Δ TM transcription factors on the *CREB3L2*

promoter, 200 ng of the plasmid p-328/+1 was co-transfected together with 5, 50 and 500 ng of pCR3.1-based expression plasmids which contain the above mentioned transcription factors. The analysis showed that there was an increase in the promoter activity with increased amount of the co-transfected ATF6 Δ TM and CREB3L2 Δ TM transcription factors (Fig. 4B), while FUS/CREB3L2 Δ TM had virtually the same effect as the empty pCR3.1 vector on the promoter activity. Deletion of the CRE BS resulted in a 30% reduction of the promoter activity induced by CREB3L2 Δ TM but only in 10% reduction of the reporter activity induced by ATF6 Δ TM (Fig. 4C).

Effects of ER stress inducers brefeldin, thapsigargin and tunicamycin and the CRE-related activators forskolin and phorbol 12-myristate 13-acetate (PMA) on the promoter activity of *CREB3L2*. In cells transfected with p-328/+1 plasmid, tunicamycin increased the reporter activity by 11% ($p=0.05$), thapsigargin by 14% ($p=0.05$) and brefeldin by 31% ($p=0.0001$) compared to the control (Fig. 5A). However, in cells transfected with Δ TGACGT or TGTTGT there was not any statistically significant difference in the reporter activity between control and the treatment with tunicamycin, thapsigargin or brefeldin (Fig. 5A).

Culture of HEK293 cells with 0.1% serum increased the reporter activity for all constructs compared to culture with 10% serum. Thus, there was a 30% increase in the activity of p-328/+1 ($p=0.0003$) and Δ TGACGT ($p=0.05$) and a 12% increase in the activity of TGTTGT ($p=0.028$) (Fig. 5B). Treatment of cells with forskolin resulted in a decreased reporter activity, whereas treatment with PMA increased the promoter activity irrespectively of the presence, mutation or

**B**

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1  AATAGAGGGC TTGGGTCCGG AAGTGATGGA CTGGAGGCTT GACTAATGTG AAAGGGGCGT
61 CTGAAAGAGA AGCCAGCTGC CATGCCATGA ACTGCAATGT GAAAAGGCCA ATGTAGCAAG
121 GATATGTCCA GCCAACAGCC AGTGAGGATC TGAGGCCCTC AGACCAGCAG CCCATGAGCA
181 ACTGAATTTT GCTAATAACC ATGTAAGTGA ACACAGAAGT GGATCCTTCC CCAGGCGAGC
241 C TTCATAAGT CCACAGACCC AGTCAAGACT TTGACTGCAG CCTTGTGAGA GCGGTTCAATC
301 CAGAGGACCC AGCTAAGCCA CGTTCAACTT CCTGACCTGT CAGATCTTGG CCGTTC AATC
361 TGGAACCTTG GTGTGCTGCG TGTGAATACA GATGCTGGGA AGCTTAGCCT GGGATGCCAT
421 CTCTCTGACC CCTAATGCTT GGCTGAACTC TTCTGCTGCA CTCGGTCTTT CCTCTGTAAA
481 TCACAGCATT GCATCACTAT ACGCTGGAGT CTGGCCCTAG GACACCTTTC TAAAAAGACT
541 CCCTGTGGTG TTCAGAATCA CTCCTACAGT CAGGTTCTCC ACAATGGATC TCAGTGCTGC
601 AAGTCACCGC ATACCTCTAA GTGATGGAAA CAGCATTTCC ATCATCGGAC TTGGTACCTA
661 CTCAGAACCT AAATCGACC CTAAGGGAGC CTGTGCAACA TCGGTGAAGG TTGCTATTGA
721 CACAGGGTAC CGACATATTG ATGGGGCCTA CATCTACCAA AATGAACACG AAGTTGGGGA
781 GGCCATCAGG GAGAAGATAG CAGAAGGAAA GGTGCGGAGG GAAGATATCT TCTACTGTGG
841 AAAGGCATGG GCTACAAATC ATGTCCAGAG GATGGTCCGC CCAACCCTGG AGAGGACACT
901 CAGGGTCCTC CAGCTAGATT ATGTGGATCT TTACATCATT GAAGTACCCA TGGCCTTTAA
961 GCCAGGAGAT GAAATATACC CTAGAGATGA GAATGGCAA TGGTTATATC ACAAGTCAAA
1021 TCTGTGTGCC ACTTGGGGG CGATGGAAGC TTGCAAAGAC GCTGGCTTGG TGAAATCCCT
1081 GGGAGTGTCC AA

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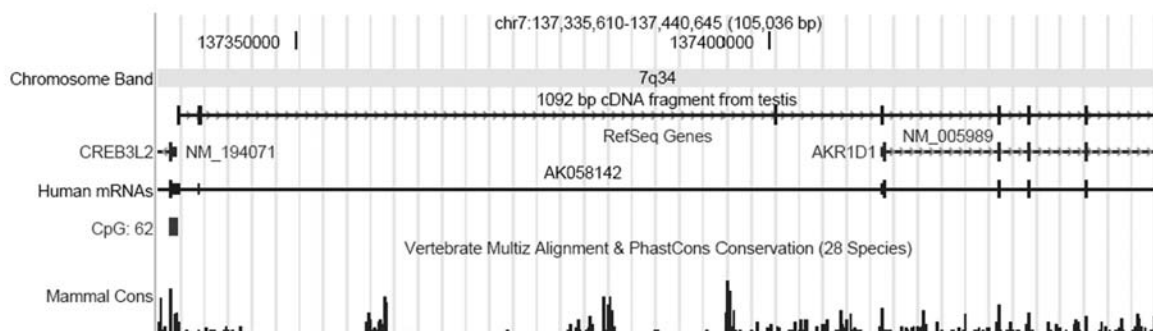
C

Figure 2. The transcript variants of *AKR1D1* found in testis. (A) Gel electrophoresis showing that cDNA fragments are amplified only from testis. (B) Sequence of the long 1092-bp fragment. The sequences from 415 to 670 and 415 to 1030 are absent in the middle and short amplified fragments, respectively. The three ATG starting codons are in box. (C) Diagram showing the position of the long 1092-bp cDNA fragment, the AK058142 mRNA and the reference sequences of *CREB3L2* and *AKR1D1*. GenBank accession nos. FM160741, FM160742, FM160743.

absence of the CRE binding site (Fig. 5B). In the cells treated simultaneously with both forskolin and PMA the promoter activity was similar to that observed in cells cultured with 0.1% serum (Fig. 5B).

Discussion

In the present study, the promoter region of *CREB3L2*, a gene widely expressed in many tissues, was characterized. The

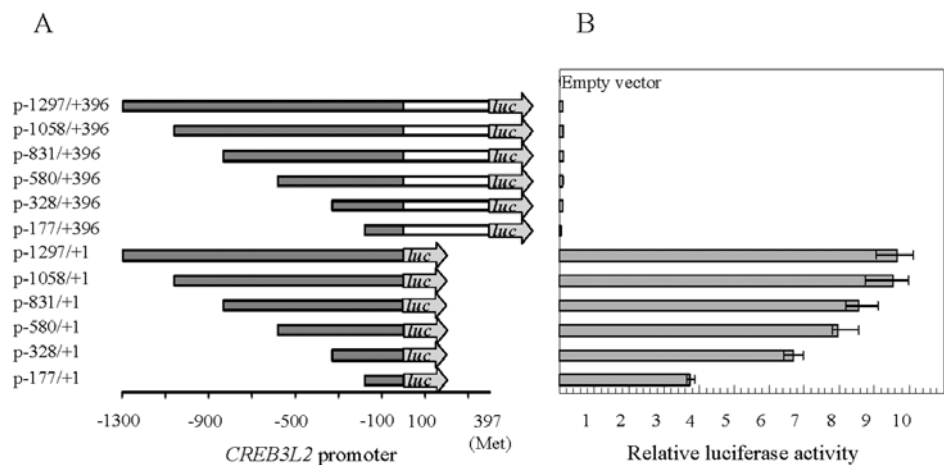


Figure 3. Constructs and functional analysis of the *CREB3L2* promoter. (A) Schematic representation of the various deletion fragments cloned upstream of the luciferase coding sequence (B). Transcriptional activities measured as relative luciferase activity. HEK293 cells were transiently transfected with the reporter plasmids and 48 h after transfection luciferase activity was quantified and normalized against *Renilla* luciferase activities. Each construct was measured in eight replicates and the results are presented as the median together with 25th and 75th percentiles.

sequence CCAATTGG found a few nucleotides upstream of the +1 transcription site of *CREB3L2* suggested that the CCAAT-box could be used bidirectionally. Information obtained from the database and supported by our RT-PCR analysis (Fig. 2) indicated that the promoter might be asymmetrically bidirectional, exhibiting strong directionality towards the *CREB3L2* transcript initiation, with expression of

AKR1D1 alternative transcripts only in testis. *AKR1D1* codes for the enzyme 5 β -reductase which catalyzes the reduction of the 4-ene of 3-ketosteroids, converting them into 5 β -dihydro-3-ketosteroids (10) and is highly expressed in liver and weakly in testis and colon (10).

Bidirectional promoters have been reported for >10% of the genes in the human genome, with the majority of them showing activity in both directions. About 10% seem to function only in one direction suggesting that these promoters drive the expression of one gene while inhibiting expression in the other direction (11). The impact, if any, of expression of *AKR1D1* on *CREB3L2* is unknown. In general, bidirectional promoters lack TATA boxes, are both GC-rich and enriched in CpG islands, and have an overrepresented set of motifs, including GABPA, MYC, E2F1, E2F4, NRF-1, CCAAT, YY1, and ACTACAnnTCC (12). GABPA, E2F1 and CCAAT

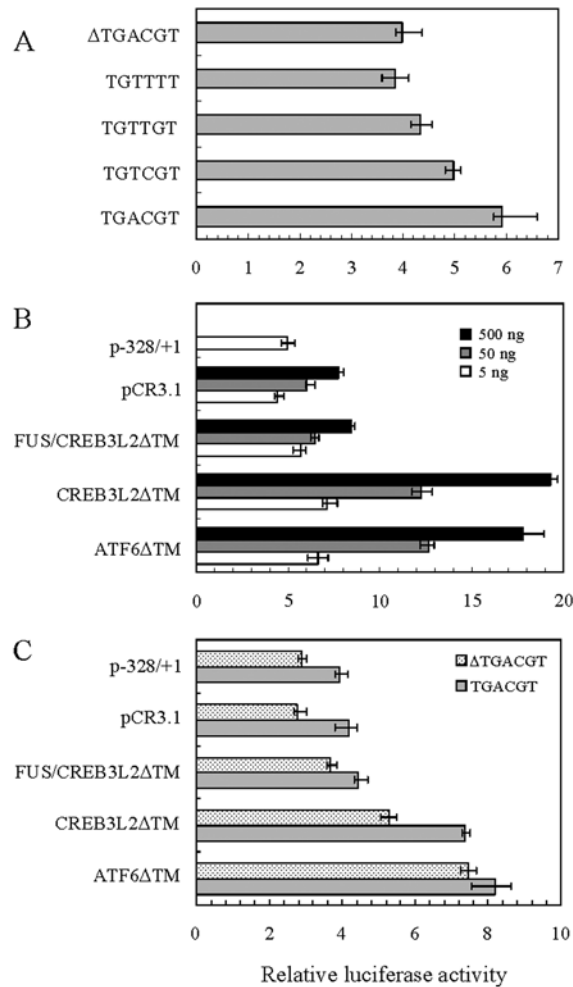


Figure 4. Analysis of the CRE binding site. (A) Site-directed mutagenesis was performed on the p-328/+1 plasmid to completely remove the CRE binding site of the promoter or to alter 1, 2 and 3 nucleotides generating Δ TGACGT, TGT TTT, TGT TGT and TGT CGT mutations, respectively. Compared to the p-328/+1 construct, a progressive reduction of the promoter activity by 16, 27 and 35% was obtained with substitution of one, two or three nucleotides, respectively. Complete removal of the binding site resulted in the same reduction (33%) as the replacement of three nucleotides. (B) Effects of the ATF6 Δ TM, CREB3L2 Δ TM and FUS/CREB3L2 Δ TM transcription factors on the *CREB3L2* promoter. Plasmid p-328/+1 (200 ng) were co-transfected with 5, 50 and 500 ng of pCR3.1-based expression plasmids which contain the above mentioned transcription factors. There was an increase in the reporter activity with increased amount of the co-transfected ATF6 Δ TM and CREB3L2 Δ TM transcription factors, while FUS/CREB3L2 Δ TM had virtually the same effect as the empty pCR3.1 vector on the promoter activity. (C) Effects of the ATF6 Δ TM, CREB3L2 Δ TM and FUS/CREB3L2 Δ TM transcription factors on the p-328/+1 construct without CRE site (Δ TGACGT). Plasmid p-328/+1 (200 ng) were co-transfected with 50 of pCR3.1-based expression plasmids. Deletion of the CRE site resulted in a reduction of the promoter activity induced by CREB3L2 Δ TM but did not abolish the reporter activity induced by ATF6 Δ TM. HEK293 cells were transiently transfected with the reporter plasmids and 48 h after transfection luciferase activity was quantified and normalized against *Renilla* luciferase activities. Each construct was measured in eight replicates and the results are presented as the median together with 25th and 75th percentiles.

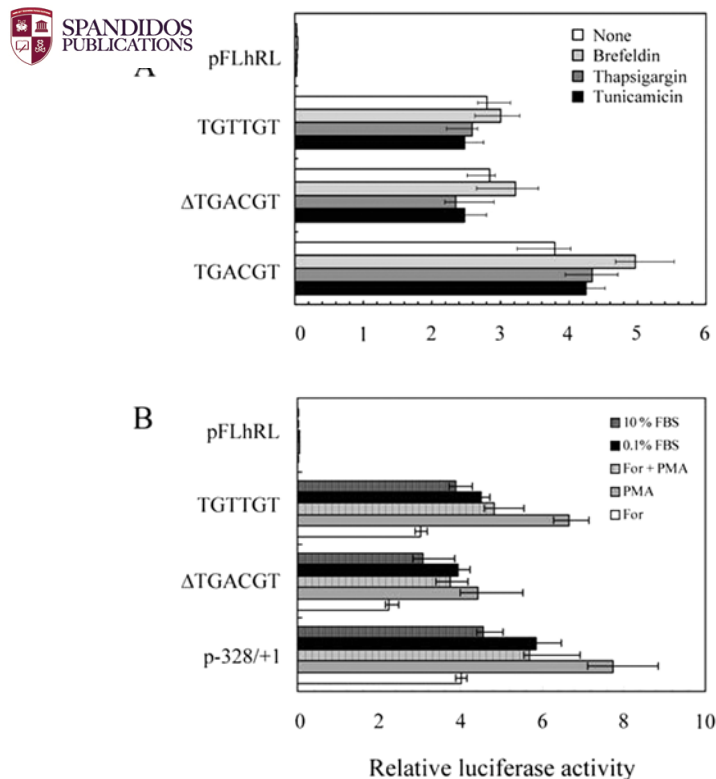


Figure 5. (A) Effects of ER stress inducers brefeldin, thapsigargin and tunicamycin on the promoter activity of CREB3L2. Apart from the p-328/+1 plasmid with the wild-type CRE (TGACGT) binding site, plasmids with Δ TGACGT and TGTTGT were studied. In cells transfected with p-328/+1 plasmid and compared to control, tunicamycin increased the reporter activity 11% ($p=0.05$), thapsigargin by 14% ($p=0.05$) and brefeldin by 31% ($p=0.0001$). In cells transfected with Δ TGACGT and TGTTGT there was not any statistical significant difference between the reporter activity of the control and cells treated with tunicamycin, thapsigargin or brefeldin. B. Effects of CRE related activators forskolin (For) and phorbol 12-myristate 13-acetate (PMA). HEK293 cells transfected with p-328/+1, Δ TGACGT or TGTTGT were cultured in a medium with 0.1% fetal bovine serum with forskolin, PMA, forskolin + PMA or without them. Culture of HEK293 cells with 0.1% serum (FBS) increased the reporter activity for all constructs compared to culture with 10% serum. Treatment of cells with forskolin resulted in a decreased reporter activity, whereas treatment PMA increased the promoter activity irrespective of the presence, mutation or absence of the CRE binding site. In the cells treated simultaneously with both forskolin and PMA the promoter activity was similar to that observed in cells cultured with 0.1% serum.

motifs are present in the region between +1 of *CREB3L2* and +1 of the *AK058142* transcript. Moreover, the 10-mer palindromic, GTGACGTCAC, CRE BS found in this region can regulate the transcription in both directions. Interestingly, of the mammals presented in Table III only humans have the 10-mer palindromic sequence. The presence of the CRE BS suggests that *CREB3L2* is a member of the 'CREB regulon' (13).

The CRE BS is known as a cAMP responsive element and is bound by a variety of bZIP transcription factors including CREB, ATF1, ATF6, ATF2/JUN heterodimers and all members of the CREB3 family of the transcription factors (4,14,15). Thus, autoregulation of *CREB3L2* might be suggested as well as regulation via the widely expressed *CREB3L1*, the liver-specific *CREB3L3* and testis- and prostate-specific *CREB3L4*. Since FUS/*CREB3L2* Δ TM had virtually no effect on the promoter of *CREB3L2* a function of

the chimera could be a disruption of *CREB3L2* autoregulation. To study the effects of CRE-related activators, forskolin, which activates adenylate cyclase and results in an increase of intracellular cyclic AMP, and PMA which activates protein kinase C, were used.

The negative effect of forskolin, irrespective of the presence or absence of TGACGT, suggested that other putative binding sites for the CRE family might be present and that cAMP may serve as a negative regulator of the *CREB3L2* transcript. Negative effects of forskolin have been reported for other gene promoters such as *GYS1*, *CD38*, and the insulin promoter (16-18). Similarly to the *CREB3L2* promoter, Ding *et al* (18) showed that mutagenesis of the CRE-like element in the insulin promoter had no effect on the forskolin-induced suppression. PMA induced promoter activity of *CREB3L2* irrespective of the presence or absence of the CRE site. Indeed, PMA function might involve Egr1, AP1 and Sp1 transcription factor sites found in the promoter of *CREB3L2* (19). Interestingly, forskolin and PMA have opposite effects on the *CREB3L2* promoter. ER stress was shown to increase the amount of total *CREB3L2* protein by more than ten-fold and the mRNA level by two-fold (4). To study the effects of ER stress on the promoter activity of *CREB3L2*, tunicamycin (an inhibitor of N-linked glycosylation), thapsigargin (which blocks the ER calcium ATPase pump, leading to depletion of ER calcium stores) and brefeldin (which specifically blocks protein transport from ER to the Golgi apparatus) were used. All three ER stress inducers increased promoter activity but not to the same magnitude as reported for the protein level. Our data indirectly support the conclusion drawn by Kondo *et al* that the *CREB3L2* protein is up-regulated at the translational level during ER stress (4).

In conclusion, our data show that the expression driven by the promoter of *CREB3L2* is, at least in part, regulated by CRE binding transcription factors. Consequently, this regulation would be disrupted in low grade fibromyxoid sarcoma with the FUS/*CREB3L2* fusion, which in turn might be significant for tumor development.

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