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 the promoter activity, whereas phorbol 12-myristate 13-acetate increased the promoter activity. Forskolin resulted in a decreased reporter activity, whereas phorbol 12-myristate 13-acetate increased the promoter activity.

Introduction

CREB3L2 (BBF2H7) was first identified as the partner gene of FUS in a chimera found in two cases of low grade fibromyxoid sarcoma (LGFM S) (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-X8-C-X7-L-X7-L-X6-L-X5-L-X5-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 intramembranous 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 (CREB3L2ΔTM and FUS/CREB3L2ΔTM) 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 endoplasmatic 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 μl reaction volume containing 1X AccuPrime Pfx reaction mix, 1 unit AccuPrime Pfx DNA polymerase (Invitrogen, Carlsbad, CA), 0.3 μM of each of the forward and reverse primers (Table I) and 200 ng template DNA. The PCR was run on a
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 pFLhRL 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’-GAAAGGTCGACCTGTGGAATGTGTGTCAGT-3’ and the reverse primer 5’-CGCGGATCCCTTGGCTCTGTTCTTCAGC-3’, which contain *Sal*I and *Bam*HI restriction sites, respectively. The fragment was digested with *Sal*I and *Bam*HI restriction endonucleases and subcloned between the corresponding sites of the pGL4.10[luc2] vector, downstream of the firefly luciferase cDNA.

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 pFLhRL vector between *Kpn*I and *Bgl*II 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 CREB3L2ΔTM (amino acids 1-372; in this form the transmembrane domain and the C-terminal part have been deleted), and FUS/CREB3L2ΔTM (amino acids 1-366 of the FUS/CREB3L2 protein) were used. For the pCR3.1-CREB3L2ΔTM construction, a 1116-bp CREB3L2 cDNA fragment was amplified using the forward primer 5’-GGGATTCTAGAATGCGTGCACAGGACG and the reverse primer 5’-GGTACCATGCGGCCGCTTGGCTACG-3’, which contain *Sal*I and *Bam*HI restriction sites, respectively. The fragment was digested with *Sal*I and *Bam*HI restriction endonucleases and subcloned between the corresponding sites of the pGL4.10[luc2] vector, downstream of the firefly luciferase cDNA.
between the BamHI and EcoRI restriction sites of the pCR3.1 vector. For the construction of pCR3.1-ATF6ΔTM, the forward primer GTCTGGGAAGCTT ATG GGG GAG CCG GCT GGG which contained a HindIII site and the reverse primer GCCGGCTCAG TCT CGG CTT TGG ACT AGG GAC T with an XhoI site were used to amplify a 1313-bp ATTF6 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 CGG CTC GGC ACA GAC ACT CC was used together with 1) AAG CAG GAG GAC TTC CTC GGC ACA GAC ACT CC, 2) AAG CAG GAG GGG GAA CTC CTC CTG CTT GAG CGC GGC CCG GTG GTG ACA GAC ACT CC, and 4) AAG CAG GAG GAG GAG TTC CTC GGT GTT GTC GTC ACA ACT CC to introduce the ΔTGACGT, GTTGCT, GTTTGT and GTTTTT 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 μg/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, 5x10^4 cells were seeded in 96-well plates, and 24 h later they were transfected with the appropriate plasmid combinations using 1 μl 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 ATF6ΔTM, CREB3L2ΔTM and FUS/CREB3L2ΔTM, 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, tunicamicin (at concentration 0.5 μg/ml), thapsigargin (at concentration 500 nM) or brefeldin (at concentration 500 nM) 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 μM forskolin, or 100 nM PMA or 10 μM 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 μl 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 luminesometer (Turner Biosystems, Sunnyvale, CA) using 10 μl 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.statistixxl.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 search system (http://cgislands.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 cell lines and the dysgerminoma was extracted using the EASYspin RNA isolation kit (Promega), (diluted 1:100) and reverse transcribed with the AMV reverse transcriptase (Promega). PCR products were analyzed on 1% agarose gel electrophoresis and the DNA was extracted using the QIAquick gel extraction kit (Qiagen), directly sequenced and the products were submitted to the DNA sequencing core laboratory for sequence analysis. 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
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).

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 mammalians revealed high conservation of the CRE BS (Table III). Interestingly, 18 nucleotides (20 for opposum) downstream of the CRE BS an ACATTCC sequence motif was found to be conserved in all mammalians. 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 (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 (MDLSAASHRIPLSDGNSIPIIGLGTYSEPST) would be replaced by 42 amino acids (MLGSLAWDAILTAPNAWLNSSAALGLSSVNHSIASLYAGVWP), while the 566-bp fragment (GenBank accession no. FM160743)
Table III. Partial alignment in the promoter region of 17 mammals.\textsuperscript{a}

<table>
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<th>Species</th>
<th>Ensembl Gene ID</th>
<th>Position</th>
<th>Sequence</th>
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<tr>
<td>Human</td>
<td>ENST00000330387</td>
<td>-575 to -518</td>
<td>GGAGGAGTTC CTCGGTGACG TCAAC--GAC ACTCCCGGCG TACATTCCAA GGCCTGGGCG</td>
</tr>
<tr>
<td>Mouse lemur</td>
<td>ENSMICG00000003690</td>
<td>-583 to -526</td>
<td>GGAGGAGTTC CCGGGTGACG TCACAA--GAC ACTGCCCGCG TACATTCCAA AGCTGGGCG</td>
</tr>
<tr>
<td>Squirrel</td>
<td>ENSSTOT00000009816</td>
<td>-571 to -514</td>
<td>GGAGGAGTTC CCCGGTGACG TCAAG--GAC GCTCCCGGCG TACATTCCAA GGCATGGGCG</td>
</tr>
<tr>
<td>Cow</td>
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<td>-586 to -529</td>
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</tr>
<tr>
<td>Horse</td>
<td>ENSCEAG00000007609</td>
<td>-590 to -533</td>
<td>GGAGGAGTTC TCCGGTGACG TAAAC--GAC ACGCCCTGCC CACATTCCAA AGCTGGGCG</td>
</tr>
<tr>
<td>Cat</td>
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<td>-591 to -534</td>
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</tr>
<tr>
<td>Pika</td>
<td>ENSOPRG000000004323</td>
<td>-604 to -547</td>
<td>GGAGGAGTTC CCCGGTGACG TCAAA--GAC GACCCCGGCG TACATTCCAA AGACTAGGC</td>
</tr>
<tr>
<td>Armadillo</td>
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<td>-600 to -503</td>
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<tr>
<td>Tree shrew</td>
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<td>-569 to -512</td>
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<td>Mouse</td>
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<td>Rabbit</td>
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<tr>
<td>Guinea pig</td>
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<td>-514 to -457</td>
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<tr>
<td>Shrew</td>
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<tr>
<td>Opposum</td>
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</tr>
<tr>
<td>Elephant</td>
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<td>-480 to -423</td>
<td>GGAGGAGTTC GCTGGTGACG TCAAA--GGC GCGCCCGGCC CACATTCCA CAGCGGGGCG</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The 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.
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 ΔTGACGT, TGTCGT, TGTTGT and TGTTTT mutations, respectively. Compared to the p-328/+1 construct activity reduction in the activity by 10, 17, 30 and 65% 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.

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, tunicamicin 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 ΔTGACGT or TGTTGT there was not any statistically significant difference in the reporter activity between control and the treatment with tunicamicin, 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 ΔTGACGT (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
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
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 5β-reductase which catalyzes the reduction of the 4-ene of 3-ketosteroids, converting them into 5β-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 ACTACAnTTCC (12). GABPA, E2F1 and CCAAT

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 replicas and the results are presented as the median together with 25th and 75th percentiles.

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 ΔTGACGT, 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. Complete removal of the binding site resulted in the same reduction (33%) as the replacement of three nucleotides. (B) Effects of the ATF6ΔTM, CREB3L2ΔTM and FUS/CREB3L2ΔTM 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 ATF6ΔTM and CREB3L2ΔTM transcription factors, while FUS/CREB3L2ΔTM had virtually the same effect as the empty pCR3.1 vector on the promoter activity. (C) Effects of the ATF6ΔTM, CREB3L2ΔTM and FUS/CREB3L2ΔTM transcription factors on the p-328/+1 construct without CRE site (ΔTGACGT). Plasmid p-328/+1 (200 ng) were co-transfected with 50 ng of pCR3.1-based expression plasmids. Deletion of the CRE site resulted in a reduction of the promoter activity induced by CREB3L2ΔTM but did not abolish the reporter activity induced by ATF6ΔTM. 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 replicas and the results are presented as the median together with 25th and 75th percentiles.
motifs are present in the region between +1 of CRE binding site. In the cells treated simultaneously with both forskolin and PMA, forskolin + PMA or without them. Culture of HEK293 cells with 0.1% fetal bovine serum with 13-acetate (PMA). HEK293 cells transfected with p-328/+1, ΔTGACGT or TGTTGT were cultured in a medium with 0.1% serum. Effects of CRE related activators forskolin (For) and phorbol 12-myristate 13-acetate (PMA). HEK293 cells transfected with p-328/+1, ΔTGACGT 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 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.

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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References


