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Activation of *TM7SF2* promoter by SREBP-2 depends on a new sterol regulatory element, a GC-box, and an inverted CCAAT-box

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ABSTRACT

TM7SF2 gene encodes 3β-hydroxysterol Δ^{14}-reductase, responsible for the reduction of C14-unsaturated sterols in cholesterol biosynthesis. *TM7SF2* gene expression is controlled by cell sterol levels through the SREBP-2. The motifs of *TM7SF2* promoter responsible for activation by SREBP-2 have not been characterized. Using electrophoretic mobility shift assays and mutation analysis, we identified a new SRE motif, 60% identical to an inverted SRE-3, able to bind SREBP-2 in vitro and in vivo. Co-transfection of promoter–luciferase reporter constructs in HepG2 cells showed that the binding of SREBP-2 to SRE produced approximately 26-fold promoter activation, whereas mutation of the SRE motif caused a dramatic decrease of transactivation by SREBP-2. The function of additional motifs that bind transcription factors cooperating with SREBP-2 was investigated. An inverted CCAAT-box, that binds nuclear factor Y (NF-Y), cooperates with SREBP-2 in *TM7SF2* promoter activation. Deletion of this motif resulted in the loss of promoter induction by sterol starvation in HepG2 cells, as well as a decrease in fold activation by SREBP-2 in co-transfection experiments. Moreover, co-transfection of the promoter with a plasmid expressing dominant negative NF-YA did not permit full activation by SREBP-2. Three GC-boxes (1, 2, 3), known to bind Sp1 transcription factor, were also investigated. The mutagenesis of each of them produced a decrease in SREBP-2-dependent activation, the most powerful being GC-box2. A triple mutagenized promoter construct did not have an additive effect. We conclude that, besides the SRE motif, both the inverted CCAAT-box and GC-box2 are essential for full promoter activation by SREBP-2.

1. Introduction

TM7SF2 gene encodes 3β-hydroxysterol Δ^{14}-reductase, a protein of the endoplasmic reticulum catalyzing the reduction of C14-unsaturated sterol intermediates during the conversion of lanosterol to cholesterol [1]. Disruption of *Tm7sf2* gene in mice did not result in impairment of cholesterol biosynthesis, due to recovery of the enzymatic activity by lamin B receptor, a protein of the inner nuclear membrane [2,3]. Despite this evidence, unlike lamin B receptor gene, *TM7SF2* gene appears to play a crucial role in cholesterol biosynthesis, its expression being controlled by cell sterol levels [4]. Microarray analysis of transgenic mice indicated that this control is exerted through Sterol Regulatory Element-Binding Protein-2 (SREBP-2) [5]. Our previous studies demonstrated SREBP-2-dependent transactivation of *TM7SF2* promoter and defined the minimal region for transactivation to occur [4]. However, the Sterol Regulatory Element (SRE) sequence retrieved by TRANSFAC analysis of this region was characterized by low score and exhibited very low homology with described SREs.

Ever since SRE sequences were described in the promoters of low density lipoprotein receptor (LDLR), HMG-CoA synthase, and HMG-CoA reductase [6–8], the number of genes involved in cholesterol homeostasis regulated through SREs present in their promoters has increased continuously. Concomitantly, it became evident that SREs are characterized by quite variable sequences. Although most of them exhibit significant homology with the well characterized SRE-1 of LDLR promoter, SRE-2 and SRE-3 sequences have also been reported [7,9].

SREBPs are weak activators and require additional transcription factors to achieve optimal regulation of sterol sensitive genes. Sp1 and the trimeric nuclear factor Y (NF-Y) are transcription factors acting as SREBP co-activators in the promoter of several genes involved in fatty acids and cholesterol metabolism [10–14]. The region −200/−1

Abbreviations: SRE, sterol regulatory element; SREBP-2, SRE-binding protein 2; NF-Y, nuclear factor Y; LDLR, low density lipoprotein receptor; FBS, foetal bovine serum; Lov, lovastatin; 25-OH chol, 25-hydroxycholesterol; LPDS, lipoprotein deficient serum; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation

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upstream the transcription start site of *TM7SF2* gene exhibits promoter activity [4] and contains, besides a potential SRE, Sp1 and NF-Y binding motifs. Although SREBP-2-dependent transactivation of *TM7SF2* promoter is known [4], it has not been demonstrated whether the potential SRE is able to bind SREBP-2. Due to the variable sequences of SRE motifs, other not yet identified SREs could fulfil this function. In this paper, we investigate the binding of SREBP-2 to *TM7SF2* promoter. Using electrophoretic mobility shift assays (EMSA) and co-transfection of promoter–luciferase reporter constructs, we demonstrated through various mutation analyses that the previously retrieved SRE is not able to bind SREBP-2, whereas a new SRE motif exhibits this feature, leading to promoter activation. In addition, we established that a GC-box and an inverted CCAAT-box are essential for full promoter transactivation. Chromatin immunoprecipitation (ChIP) assays confirmed the ability of *TM7SF2* promoter to bind SREBP-2, NF-Y, and Sp1 in vivo.

2. Materials and methods

2.1. Materials

Minimum essential medium (MEM), Dulbecco's Modified Eagle's medium (DMEM), foetal bovine serum (FBS), and other culture reagents were from GIBCO (Invitrogen, Milan, Italy). Lipoprotein deficient serum (LPDS) was prepared as described previously [4]. Lovastatin (Lov) and 25-hydroxycholesterol (25-OH chol) were purchased from Sigma (Milan, Italy). Complete protease inhibitor cocktail tablets, T4 DNA ligase, T4 polynucleotide kinase, and Genopure Plasmid maxi-kit were from Roche Diagnostics (Milan, Italy). Hartmann Analytic GmbH (Germany) provided [γ-^{32}P]-dATP. Microspin™ G-50 columns and poly(dI–dC)/poly(dI–dC) were from Amersham Biosciences. Dual Luciferase Reporter Assay System, pGL2-basic plasmid, and pRL-SV40 plasmid were from Promega (Madison, WI). QuikChange® Site-Directed Mutagenesis Kit and pfu Turbo DNA polymerase were from Stratagene. Lipofectamine 2000 and custom oligonucleotides were purchased from Invitrogen. MBI Fermentas (Lithuania) provided all other reagents.

2.2. Plasmids

The −200/−1 promoter region upstream the transcription initiation site of human *TM7SF2* gene was amplified from p326 plasmid [4] using Pfu Turbo DNA polymerase and the following primers: forward, 5′-AT*GGTACC*AAACATGTGTGGCCTCCTCCTC-3′; reverse, 5′-*AAGCTT*GGACACGGAACGCAGACAAGG-3′. The amplified region corresponds to −326/−127 of p326, which was numbered starting from the ATG [4]. The fragment was subcloned upstream the luciferase gene in pGL2-basic expression vector, using the KpnI and HindIII restriction sites (in italics) present in forward and reverse primers, respectively. The obtained p200 construct was used as template for base substitution, insertion, or deletion mutations using the QuikChange® site-directed mutagenesis kit (Stratagene), according to the manufacturer's protocol. Oligonucleotides designed to mutate each element are indicated in Table 1. The pGC-boxes1/2/3 mut, in which all the three GC-boxes were mutated, was obtained by using first the GC-box2 primer and the pGC-box3 mut as template to obtain the pGC-box2/3 mut, and then the GC-boxes1/2 primer and the pGC-box2/3 mut as template. All the constructs were verified by DNA sequencing. The plasmid encoding aminoacids 1–481 of human SREBP-2 (pCS2-SREBP2) was a generous gift of Dr. T. F. Osborne (University of California, Irvine). The cDNA encoding SREBP-2 was amplified from linearized pCS2 plasmid using the forward primer 5′-GC*CTCGAG*ATG-GACGACAGCGGCGAGCT-3′ and the reverse primer 5′-GC*GGATCC*T-CACCGTGAGCGGTCTACCATGC-3′ by pfu Turbo DNA polymerase. The fragment was subcloned in bacterial expression vector pET-15b (Novagen), using the XhoI and BamHI restriction sites (in italics) present in forward and reverse primers, respectively. The plasmid Δ4NFYA13m29, expressing the dominant negative of NF-YA and the control plasmid Δ4NFYA13 were a kind gift of Dr. Roberto Mantovani (Milan, Italy). The CMV-Sp1 plasmid was Addgene plasmid 12097 (thanks to Dr. Robert Tjian).

2.3. Electrophoretic mobility shift assays

Human SREBP-2 was overexpressed in *E. coli* BL21(DE3) from plasmid pET-15b by induction with 0.8 mM isopropyl β-D-1-thiogalactopyranoside. After 3 h at 37 °C cells were lysed and supernatant purified from debris by centrifugation. The supernatant was used in EMSA experiments. As a control, a supernatant from cells transformed with empty pET-15b plasmid was used. Protein concentration of supernatants was determined by Bradford method [15]. Probes were designed to cover the −143/−1 region upstream the transcription initiation site (Fig. 1A), responsive to SREBP-2 [4]. LDL receptor oligonucleotide 5′-AAAATCACCCCACTGCAAACTCCTCCCCCTGC-3′, containing the SRE-1 element, was used as positive control (LDLR-SRE-1). Annealed probes were end-labelled with [γ-^{32}P]-dATP and T4 polynucleotide kinase and then purified with Microspin™ G-50 columns. For the binding reaction, *E. coli* cleared lysates (18 μg protein) were preincubated for 15 min at room temperature with 3 μg poly(dI–dC)/poly(dI–dC) in a solution containing 12.5 mM Hepes (pH 7.9), 6 mM $MgCl_2$, 50 mM KCl, 5.5 mM EDTA, 0.5 mM DTT, 0.25 mg/ml BSA, 5% glycerol, and protease inhibitor cocktail, final volume 20 μl. Equal amounts of probes (40,000 cpm, 0.1 pmol, 1 μl) were then added and incubation continued for 30 min at room temperature. For competition assays, the indicated molar excess of unlabeled LDLR-SRE-1 was added to the preincubated binding mixture and incubated for additional 30 min at room temperature prior to adding labelled probes. Protein–DNA complexes were then resolved by electrophoresis through 4.5% polyacrylamide gels for 3 h, and the dried gels were subjected to autoradiography.

2.4. Cell culture and transfections

HepG2 human hepatoma cells were grown in a 5% CO_2 incubator at 37 °C in MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. HEK293 cells were maintained in DMEM as described above. Cells grown in 24-well plates at 60–70% confluence were transfected in duplicate with 0.8 μg

Table 1
Forward primers used for mutagenesis of p200. Modified bases (indicated by numbers in brackets) are in italics bold.

SRE (−71/−68)	−89/GGCTGGTGTGCTGGGCCC***TTGT***TGGGCAGGGGGCGG/−54
SRE (−113/−109)	−128/CTGAGGCGCCCGCGT***TCACG***CCGCCCCCGAGCGCC/−94
Δ Inv CAATT	−104/CCCCGAGCGCCG - - - - - CTGGTGTGCTGGGCCCAGC/−69
inv CAATT mut (−91/−89)	−108/CCGCCCCCGAGCGCCGA***AGT***GCTGGTGTGCTGGGCCC/−72
Ins-5	−109/CCCGAGCGCCGATTGG***AATAA***CTGGTGTGCTGGGCCC/−72
GC-box1 (−168/−164)	−189/GCCTCCTCCTCTCGCTTGCTG***AATTC***GCCTTTCCGGGGGCGGGG/−146
GC-box2 (−152/−148)	−172/GCTGGGCGGGCCTTTCCGGG***AATTC***GGTTTTGAAGCTGAGGC/−131
GC-box3 (−58/−54)	−77/GGGCCCAGCATGGGCAGGG***AATTC***TTCCACTTAAAAACCCTGGG/−34
GC-boxes1/2	−189/GCCTCCTCCTCTCGCTTGCTG***AATTC***GCCTTTCCGGG***AATTC***GG/−146