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NF-Y and Sp1 are involved in transcriptional regulation of rat SND p102 gene

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Abstract

SND p102 is a rat liver endoplasmic reticulum cholesterol ester hydrolase recently described as a member of a conserved family of transcriptional coactivators that promotes phospholipid secretion into lipoproteins when overexpressed in hepatocytes. In this work, we report first evidence for a mechanism of transcriptional regulation for the SND p102 (Snd1) gene. Promoter activity of 5′ deletion fragments determined in human HepG2 and rat McA-RH7777 hepatoma cells by luciferase reporter gene assays showed a minimal promoter involving two inverted CCAAT boxes. EMSA demonstrated specific binding of Sp1 to GC boxes in the proximal, highly active promoter region besides that of NF-Y to CCAAT boxes reported earlier. Site-directed disruption of such CCAAT and GC boxes led to reduction in transcriptional activity, confirming the functional implication of NF-Y and Sp1 in SND p102 gene transcription.
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Keywords: SND p102; Gene promoter; NF-Y; Sp1; Lipid regulation

SND p102 (GenBank Accession No. AAU05374) is a 102 kDa member of an evolutionarily conserved family of proteins that contains four repeated staphylococcal nuclease domains (SND) and a single Tudor domain. In the liver, SND p102 is localised in the endoplasmic reticulum (ER) as shown by western blotting of subcellular fractions of rat hepatocytes and mouse and human liver, confocal microscopy of human HepG2 cells and high-resolution immunoelectron microscopy of rat liver samples [1]. Numerous studies describe p100, the human protein homologue of SND p102, as a transcriptional coactivator and localise it to the nucleus [2,3]. More recent works, however, encounter homologues of p100 in the ER and cytosolic lipid droplets from cow and mouse lactating mammary gland, adipocytes and liver [4,5]. These data suggest additional tissue-specific functions for these family members rather than being transcriptional coactivators and point to a role in lipid homeostasis. Indeed, SND p102 was demonstrated to be responsible for more than 90% of the hydrolysis of cholesteryl esters in rat hepatocyte microsomes [6]. Very recently, by using recombinant adenovirus-mediated SND p102 differential expression in rat primary hepatocytes, we have shown that this protein is involved in controlling the amount of phospholipid secreted into very low density lipoproteins [7].

To further understand the physiological role of SND p102, it is relevant to characterise the regulation of the SND p102 (Snd1) gene (GenBank Accession No. AY697864), located in rat chromosome 4 [8]. Since nothing is known about its transcriptional regulation, a 1688 bp promoter region (GenBank Accession No. AY957585) was recently isolated and characterised, showing the lack of typical TATA box and locating transcription start site 216 pb upstream the ATG translation start codon [9]. We cloned deletion fragments of the promoter and their transcriptional activity was determined in human HepG2 and rat McA-RH7777 hepatoma cells by a dual luciferase reporter assay. We demonstrate that the proximal promoter is mainly responsible for transcriptional activity in both cell lines and, by gel shift assays, that the two ubiquitous proteins nuclear factor-Y (NF-Y) and specificity

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protein 1 (Sp1) bind to specific sites activating the SND p102 gene promoter. NF-Y is a heterotrimeric transcriptional activator which complexes with CCAAT boxes [10,11]. Sp1 is a member of the Sp/KLF family of transcription factors that recognises and specifically binds to GC boxes via three Cys_2His_2 zinc-finger motifs [12]. Both transcription factors have relevance in the transcriptional regulation of a growing number of genes related to different cellular processes including lipid homeostasis.

Materials and methods

Chemical and reagents. TaKaRa LA Taq™ polymerase was obtained from TaKaRa Bio Inc. (Shiga, Japan) and Pfu DNA Turbo polymerase from Stratagene (La Jolla, CA). Oligonucleotides were synthesised by Isogen Life Science (Ijsselstein, The Netherlands). Human HepG2 and rat McA-RH7777 hepatoma cell lines, Eagle's minimum essential medium (EMEM), Dulbecco's modified Eagle's medium (DMEM), and horse and fetal bovine sera were obtained from ATCC (Manassas, VA). L-Glutamine, penicillin and streptomycin were from Sigma–Aldrich (St. Louis, MO).

Bioinformatic tools. Putative binding sites for transcription factors in the promoter sequence were identified using TESS [13], MatInspector [14] and Jaspar [15]. CpG islands were identified using CpG Island Searcher [16] and CpGPlot [17]. Sequence alignment with *Rattus norvegicus* and *Homo sapiens* genomes was performed using NCBI BLASTN 2.2.15 and Ensembl Release 42.

Reporter plasmids and site-directed mutagenesis. The isolated promoter sequence [9] was divided into six 5′ deletion fragments by amplification with specific sense oligonucleotides and a common antisense oligonucleotide, introducing restriction sites for *Mlu*I and *Bgl*II (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), respectively, (Table 1). The fragments comprising promoter regions (−1091, +208), (−638, +208), (−422, +208), (−275, +208), (−112, +208) and (−8, +208) were introduced into the *Firefly* luciferase reporter vector pGL3-Basic (Promega, Madison, WI) using the DNA Rapid Ligation System (Promega). The generated constructs pGL3-SND/1091, pGL3-SND/638, pGL3-SND/422, pGL3-SND/275, pGL3-SND/112 and pGL3-SND/8 were purified using the DNA Plasmid Midi kit (Qiagen, Hilden, Germany) and verified by DNA sequencing. A series of SND p102 promoter mutants were constructed using oligonucleotides with point mutations in CCAAT or GC boxes of the promoter, underlined in Table 2. Non-mutated vectors were digested with the endonuclease *Dpn*I (New England Biolabs, Ipswich, MD).

Transient transfection and luciferase reporter assays. HepG2 and McA-RH7777 cells were seeded in 35-mm wells at a density of 4×10^5 cells/well 24 h prior to transfection. HepG2 cells were maintained in EMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% fetal bovine serum. McA-RH7777 cells were maintained in DMEM supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 20% horse serum and 5% fetal bovine serum. Transfections were done using FuGene 6 transfection reagent (Roche Applied Sciences, Mannheim, Germany). To each culture well was added 900 ng of the appropiate pGL3-SND reporter vector and 700 ng of *Renilla* luciferase pRL-TK vector (Promega) as internal control for transfection efficiency. After 48 h (HepG2) or 24 h (McA-RH7777), cells were washed, harvested in Passive Lysis Buffer (Promega) and luciferase activity measured using the Dual-Luciferase Reporter Assay System (Promega). *Firefly* luciferase activity from promoter constructs was normalised to *Renilla* luciferase activity. Assays were performed in triplicate.

Electrophoretic mobility-shift assay (EMSA). Nuclear extracts from HepG2 and McA-RH7777 cells were obtained using the Nuclear Extraction kit (Panomics, Fremont, CA) [9]. DNA double-stranded oligonucleotides corresponding to putative Sp1 binding regions of the SND p102 gene promoter listed in Table 2 were labelled with digoxigenin using the DIG Gel shift kit second generation (Roche). For mobility shift assays, 2–4 μg of HepG2 or 6 μg of McA-RH7777 nuclear extract protein was incubated at room temperature for 15 min with 1 pmol of the labelled probe, according to the manufacturer's instructions. For specific or non-specific competition assays, 20- to 100-fold molar excess of unlabelled probe was used. For supershift assays, anti-Sp1 antibody (Calbiochem, EMD Biosciences, San Diego, CA) was added and incubated 15 min more at room temperature. Electrophoresis, blotting, crosslinking and chemiluminescent detection were performed as described elsewhere [9].

Statistical analysis. Results are presented as means ± SD. Statistical significance was determined using the Student's *t*-test. A *p*-value of <0.05 was considered significant.

Results and discussion

Characterisation of the SND p102 gene promoter

A region of 1688 pb corresponding to the 5′ flanking region of the cDNA sequence of rat SND p102 gene (GenBank Accession No. AY697864) was recently isolated [9] and submitted as the promoter region of the gene (GenBank Accession No. AY957585). The isolated sequence, with the exception of a number of nucleotides in the region (−789, −770), matched with entries NW_047689.2 and NW_001084827.1 from NCBI and AC128239.4 from Ensembl for *Rattus norvegicus* chromosome 4.

A bioinformatic survey predicted multiple *cis*-elements in the SND p102 gene promoter [9]. As Fig. 1A shows, it contains several CCAAT boxes and CG-rich sequences in the proximal upstream region of transcription start site. Most TATA-less promoters contain reverse CCAAT boxes in the (−80, −40) region [11]; therefore, those previously identified as NF-Y-binding sites at positions −74 and −41 might likely play a role in recruiting the transcriptional machinery. Further sequence analysis showed a CG-rich region at (−594, −14) with a tandem of seven

Table 1
Oligonucleotides used for amplification of 5′ deletion fragments of rat SND p102 promoter

SND(−1108, −1076).s	5′-GGAGCTGAGATTT*ACGCGT*ACTGGGCAAACCAG-3′[(1)]
SND(−657, −627).s	5′-CTCAGTGAGCAGG*ACGCGT*GTTATCTAAGAG-3′[(1)]
SND(−440, −408).s	5′-AGATTCCTTCAGG*ACGCGT*TCCTTAGTTCCAGG-3′[(1)]
SND(−292, −269).s	5′-GGCCCCGCCTTC*ACGCGT*CCCATG-3′[(1)]
SND(−131, −101).s	5′-CTGGAGCTCCGCAC*ACGCGT*TGGGACCAAGG-3′[(1)]
SND(−28, −4).s	5′-AGGTGGCGCTCTGGC*ACGCGT*GCGC-3′[(1)]
SND(+188, +221).as	5′-GCCATGTGT*AGATCT*GGAAAGACAGAGAGTGGGG-3′[(2)]

Numbers correspond to the recognised promoter sequence. Restriction sites for *Mlu*I[(1)] and *Bgl*II[(2)] are shown in italics. s, sense; as, antisense.

Table 2
Oligonucleotides used in EMSA for Sp1 and site-directed mutagenesis of Sp1 and NF-Y binding sites

Sp1(−337, −306).wt	5′-CTTGAG*GGGCGGG*GCAG*GGGCGGA*ACCAGGCA-3′
Sp1(−308, −278).wt	5′-GCAGG*AGGGCGAGG*CCGG*CCCCGCCT*TGAGG-3′
Sp1(−276, −247).wt	5′-CTCCCATGGC*TCCCCGCGCCC*CACGGCCAG-3′
Sp1(−194, −165).wt	5′-ACTTCCCCGGAA*GTCCCGCCT*CAAAATTTG-3′
Sp1(−114, −85).wt	5′-TTTTGGGACCAAG*GGGCGGA*GCTTCATAAA-3′
Oct2A.wt	5′-GTACGGAGTATCCAGCTCCGTAGCATGCAAATCCTCTGG-3′
Sp1(−331).mut	5′-CCGCTTGAGGATCGGAGCAGGGGCGGA-3′
Sp1(−320).mut	5′-GCGGGGCAGGATCGGTACCAGGCAGGA-3′
Sp1(−303).mut	5′-CAGGCAGGAGATCGAATCCGGCCCCGC-3′
Sp1(−289).mut	5′-CGAGGCCGGCTCCGATTTCAGGGCTC-3′
Sp1(−182).mut	5′-CCCCGGAAGTTCCGATTCAAAATTTGCCC-3′
Sp1(−101).mut	5′-GGGACCAAGGATCGGTGCTTCATAAACAG-3′
NFY(−154).mut	5′-GCCCGTCACCGAAAGGCAGCTCTTTCAGC-3′
NFY(−74).mut	5′-AAACAGAGTGCCTCCTGTCTTGAGTTCTA-3′
NFY(−41).mut	5′-GTTCTGGACGCTACCTGTCCAGAGGGAGG-3′

GC boxes are shown in italics in wild-type (wt) oligonucleotides. Replaced bases of mutated (mut) oligonucleotides are underlined. Numbers indicate the first position of the corresponding GC (Sp1) or CCAAT (NF-Y) box.

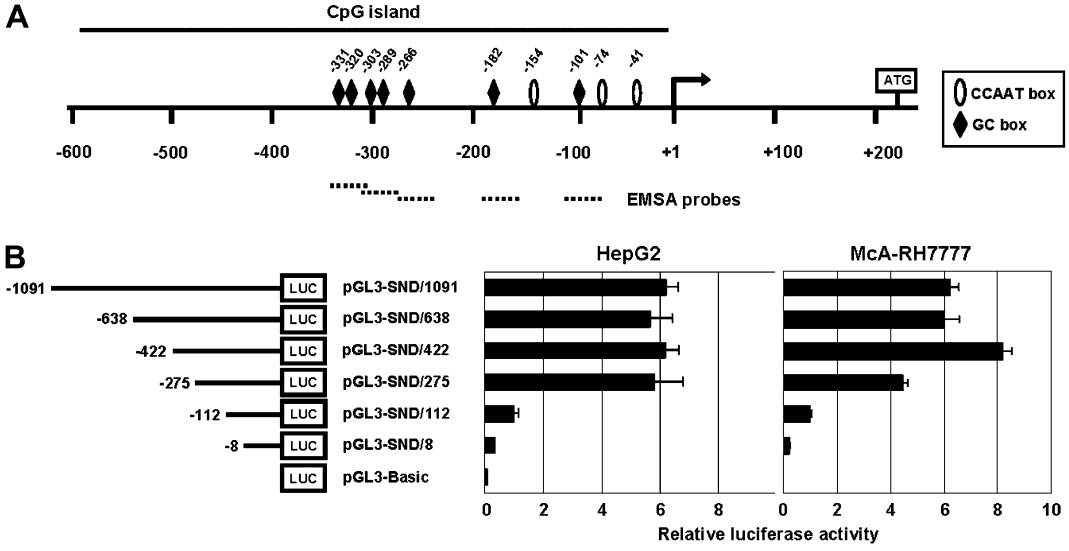

Fig. 1. Diagram and deletion analysis of rat SND p102 gene promoter. (A) Schematic diagram of region (−600, +230) of the promoter. The arrow indicates the transcription start site (+1), located 216 pb upstream the ATG codon. The identified CpG island includes one forward (−154), two inverted (−74 and −41) CCAAT boxes and seven GC boxes (−331, −320, −303, −289, −266, −182 and −101). Dotted lines correspond to probes used in EMSA. (B) Deletion analysis of the SND p102 promoter in HepG2 and McA-RH7777 cells. Cells were transfected with the constructs (left of figure) and assayed for luciferase activity. Results are given as means ± SD for at least three independent experiments, and expressed as fold increase compared with the value for pGL3-SND/112.

Sp1 sites, fitting with the description of a CpG island as a hypomethylated CG dinucleotide-rich element with multiple Sp1 binding sites [18], ranging in size from 500 to 2000 pb [19]. CpG islands are associated with 40–50% of the genes encoding proteins in mammals [20].

When compared with *Homo sapiens* genome databases, the last 311 pb at the 3′ end of the isolated sequence matched (84%) with entries NT_007933.14, NW_923640.1 and NT_079596.2 from NCBI and AC073934.1.1.131165 from Ensembl for human chromosome 7. Since significant expression of SND p102 and its homologue p100 protein was detected in rat McA-RH7777 and human HepG2 cells, respectively, (unpublished observation), the sequence similarities between the rat and human promoters suggest the presence within region (−311, +1) of conserved binding elements critical for promoter activity.

Functional analysis of the SND p102 gene promoter

Transcriptional activity was determined in HepG2 and McA-RH7777 cells transiently transfected with a series of constructs obtained as 5′ deletion fragments of the SND p102 promoter cloned upstream a luciferase reporter gene (Fig. 1B). Resulting activity data revealed the presence of a minimal promoter at region (−112, +208), containing the two reverse −74 and −41 CCAAT boxes and the −101 GC box. Full promoter activity was detected in the constructs extending to −275 in HepG2 and to −422

in McA-RH7777 cells. Analysis of the region spanning from −275 to −112 revealed the presence of the direct −154 CCAAT box and two GC boxes at −266 and −182. Four additional GC boxes were located in the fragment from −422 to −275, providing a multiple Sp1 binding site which is likely to be essential for promoter activity in rat cells. This agrees with the observation that binding of NF-Y alone is usually not able to exert an effect on transcription unless it interacts with other relevant nuclear factors, such as Sp1 [21]. In human hepatoma cells, luciferase activity remained similar in the constructs extending to positions −422, −638 and −1091 of the promoter. However, transcriptional activity decreased by 25% in rat hepatoma cells transfected with the constructs containing fragments −638 and −1091, pointing to the involvement of other transcription factors. Binding sites for hepatic nuclear factors, upstream stimulatory factors, CCAAT/enhancer binding proteins, signal transducers and activators of transcription, sterol regulatory element-binding proteins (SREBPs) or glucocorticoid receptors were predicted (data not shown), suggesting a complex regulation for the gene. Overall, these data indicate that the SND p102 promoter is active at comparable strengths in both cell lines and that the elements required for transcription in HepG2 and McA-RH7777 cells are located within −275 and −422 regions of the promoter, respectively. This is in line with the general observation that elements involved in transcription are located close to the origin of transcription for most of the genes.

NF-Y can activate transcription from the SND p102 gene promoter

A statistical analysis of over 500 sequences revealed that the CCAAT box is one of the most ubiquitous elements in all sort of promoters, with or without TATA box [22]. In an earlier work, we identified the specific binding of NF-Y to forward −154 and reverse −74 and −41 CCAAT boxes of the SND p102 promoter [9]. The functional relationship between such elements and promoter activity was confirmed in the present study by performing single and combined point mutations of the highly conserved positions C^1 and A^4 of the CCAAT consensus sequence [11] in vectors pGL3-SND/112 (Fig. 2A), pGL3-SND/275 (Fig. 2B) and pGL3-SND/422 (Fig. 2C). Mutation of the −74 and −41 CCAAT boxes separately decreased transcriptional activity in all the analysed deletion fragments, the effect being more accentuated in rat than in human hepatoma cells. In contrast, mutation of the −154 CCAAT box decreased activity only in the longest construct pGL3-SND/422. As combined mutations of both reverse CCAAT boxes resulted in maximal reductions (50–80%) of luciferase activity, regardless of whether −154 CCAAT box was or was not mutated, a minimum contribution of the latter to the SND p102 gene transcription might be suggested. Thus, these results indicate that the three CCAAT boxes analysed are functional, and that NF-Y binding to reverse CCAAT boxes accounts for most of basal promoter activity of the SND p102 gene. NF-Y binding was implicated in transcriptional regulation of a number of higher eukaryote genes [10,11]; hence, SND p102 should be added to the list.

Binding of Sp1 to the SND p102 gene promoter

To examine the Sp1 binding to the seven GC boxes of the SND p102 promoter, we performed EMSA using digoxigenin-labelled oligonucleotides complementary to regions (−337, −306), (−308, −278), (−276, −247), (−194, −165) and (−114, −85), each of them with one or two close GC boxes (Fig. 1A). Incubation of the probe for region (−337, −306), containing the −331 and −320 GC boxes, with nuclear extracts from HepG2 (Fig. 3A) or McA-RH7777 (Fig. 3B) cells produced one specific DNA-protein complex (lane 1 of A and B), which was eliminated by an excess of unlabelled specific probe (A, lanes 2 and 3; B, lanes 3 and 4). Neither the non-specific probe for transcription factor Oct2A (A, lanes 4 and 5; B, lanes 5 and 6) nor the probes mutated for either of GC boxes (B, lanes 7 and 8) avoided this complex formation. The specificity of Sp1 binding to this region was confirmed using anti-Sp1 antibody. Although a supershift was not observed, the antibody avoided the formation of the DNA–protein complex (A, lane 6; B, lane 2). A second, non specific complex was observed with HepG2 nuclear extracts, which was eliminated by either of the competitors, but not by the anti-Sp1 antibody.

Similar results to those above were obtained for promoter regions containing the −303, −289, −182 and −101 GC boxes (data not shown). However, no Sp1 binding to region (−276, −247) was observed (data not shown), suggesting that −266 GC box is a non-functional or relatively weak binding site for Sp1.

Sp1 can activate transcription from the SND p102 gene promoter

The role of the identified Sp1-binding GC boxes in transcriptional regulation was addressed by site-directed mutagenesis of elements located at −331, −320, −303, −289, −182 and −101. Highly conserved positions G^3, G^4 and G^8 [13,15] of the GC box consensus sequence GGGGCGGGG were mutated in plasmids pGL3-SND/112 (Fig. 4A), pGL3-SND/275 (Fig. 4B) and pGL3-SND/422 (Fig. 4C). Alteration of the −101 element had no effect on the minimal promoter activity in human or rat cells, and modification of the −182 GC box in region −275 produced a slight decrease of activity in HepG2 but not in McA-RH7777 cells. However, mutations of any of the GC boxes in the promoter region −422 resulted in significant decreases of transcriptional activity ranging from 10% (−331 box) to 50% (−303 box) in both cell lines. Combined mutations did not produce further change in activity from that caused by single mutations. Therefore, our

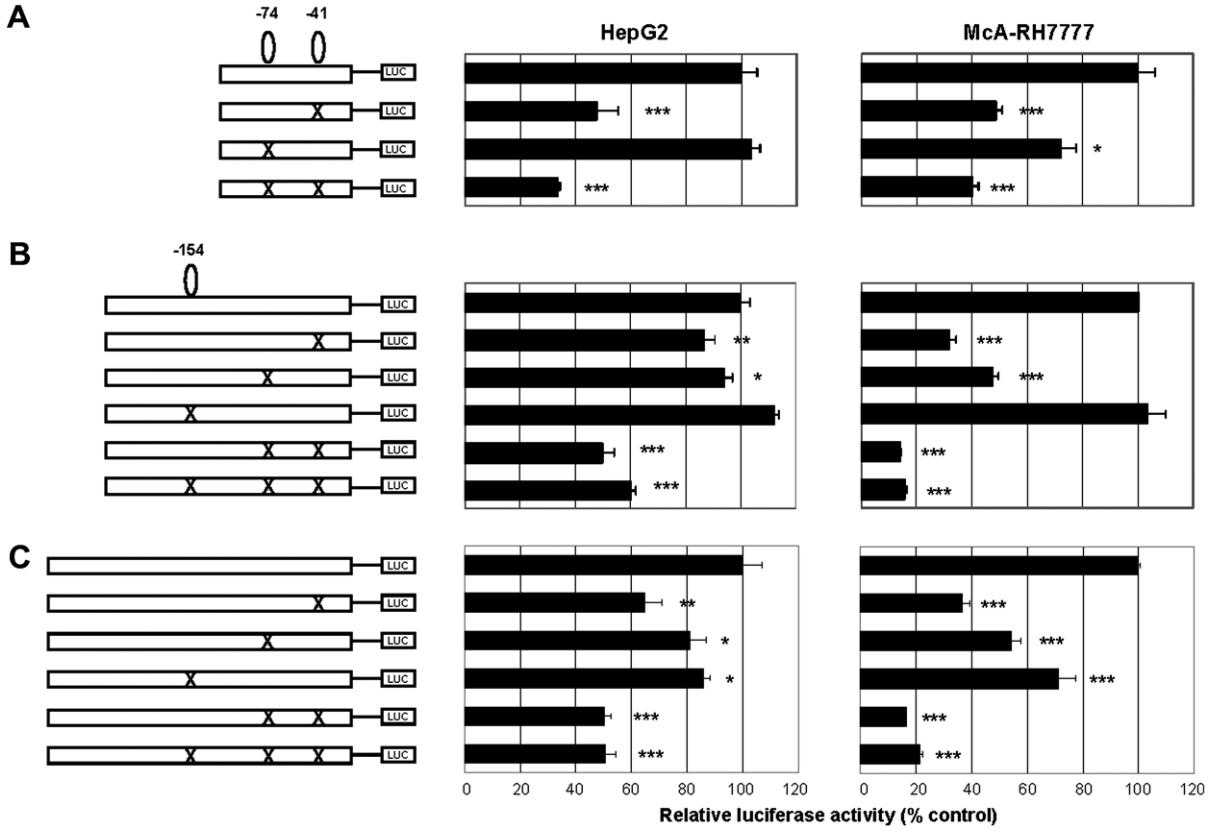

Fig. 2. Implication of NF-Y in transcriptional regulation of SND p102 gene. Single and combined mutations of CCAAT boxes located at −154, −74 and −41 were performed in plasmids pGL3-SND/112 (A), pGL3-SND/275 (B) and pGL3-SND/422 (C), and then transfected into HepG2 and McA-RH7777 cells. Luciferase activity of cells transfected with non-mutated control vectors was assigned 100% of transcriptional activity. Results are given as means ± SD for three independent experiments. $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.001$.

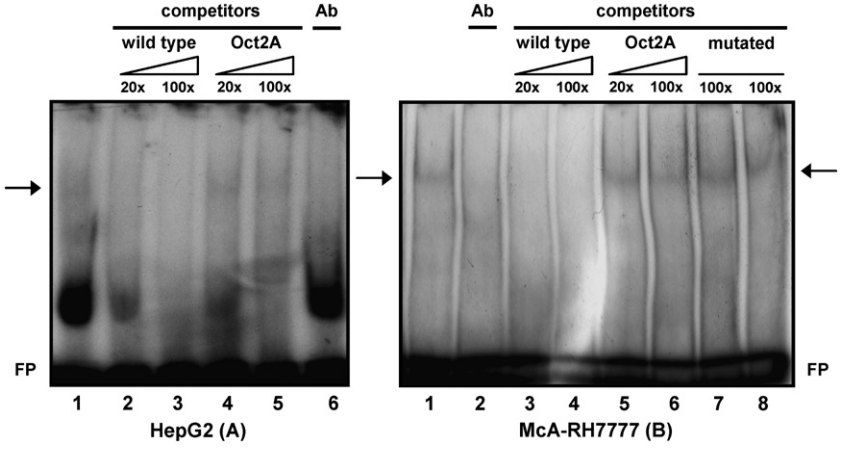

Fig. 3. Binding of Sp1 to region (−337, −306) of SND p102 gene promoter. Electrophoretic mobility shift and supershift assays were performed using nuclear extracts from HepG2 (A) and McA-RH7777 (B) cells. Incubation of nuclear extracts with digoxigenin-labelled oligonucleotide corresponding to promoter region (−337, −306) resulted in a shift, indicated by an arrow (A, lane 1; B, lane 1). Competition assays using 20- and 100-fold molar excess of specific (wild-type) competitor resulted in the elimination of the protein–DNA complex (A, lanes 2 and 3; B, lanes 3 and 4). Non-specific probe for transcription factor Oct2A (A, lanes 4 and 5; B, lanes 5 and 6) or mutated probes for −331 GC box (B, lane 7) and −320 GC box (B, lane 8) did not inhibit the formation of the shifted complex. Although anti-Sp1 antibody (Ab) did not produce a supershift, it eliminated the specific DNA-protein complex (A, lane 6; B, lane 2). FP, free probe.

findings indicate that the SND p102 promoter contains at least five binding sites for transcription factor Sp1 that are implicated in transcriptional regulation of the gene.

The results presented here complement our previously reported studies on the SND p102 gene promoter [9] and point to a role for NF-Y and Sp1 in transcription of the

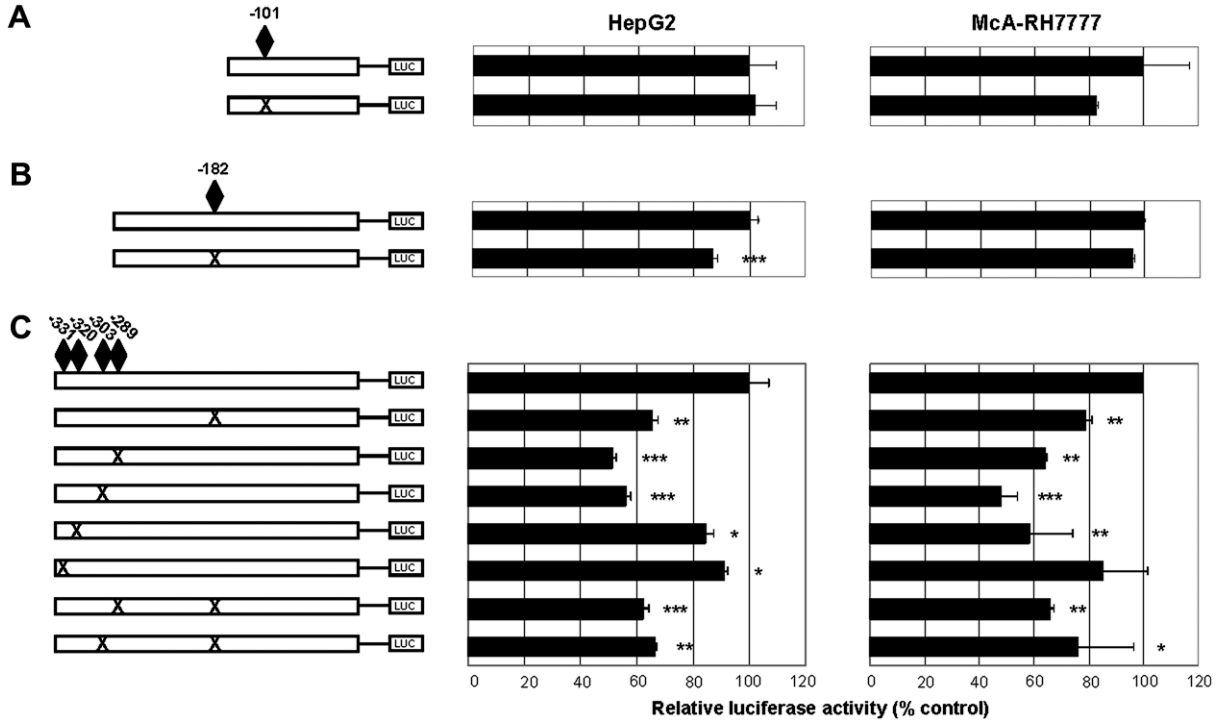

Fig. 4. Implication of Sp1 in transcriptional regulation of SND p102 gene. Single and some combined mutations of the GC boxes located at −331, −320, −303, −289, −182 and −101 were performed in plasmids pGL3-SND/112 (A), pGL3-SND/275 (B) and pGL3-SND/422 (C), and then used for transfection of HepG2 and McA-RH7777 cells. Luciferase activity of cells transfected with non-mutated control vectors was assigned 100% of transcriptional activity. Results are given as means ± SD for three independent experiments. $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.001$.

gene, providing the first evidence for its transcriptional regulation mechanism. Both transcription factors are highly conserved in human, mouse and rat [23], playing key roles for basal and regulated transcription of diverse genes. The structure of SND p102 promoter exhibits some characteristics in common with that of promoters of genes involved in cholesterol and fatty acid homeostasis, such as the SREBP targets 3-hydroxy-3-methylglutaryl-CoA reductase [24], squalene synthase [25], cytosolic cholesterol ester hydrolase [26] and fatty acid synthase [27]. They all have no canonical TATA box, and contain functional GC and inverted CCAAT boxes. In conclusion, minimal promoter activity of the SND p102 gene resides within the 112 pb upstream transcription start site and is strongly dependent on two reverse CCAAT boxes. Maximal promoter activity requires one additional CCAAT box and one GC box at (−275, −112) region in HepG2 cells, and there is also a significant contribution of the (−422, −275) region containing four GC boxes in McA-RH7777 cells. Ongoing characterisation studies of the SND p102 promoter will provide clues to identify which of the additional predicted transcription factors operate in the mechanism of its regulation.

Acknowledgments

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