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Regulation of Steroid 5-alpha reductase type 2 (Srd5a2) by Sterol Regulatory Element Binding Proteins and Statins

Young-kyo Seo[1], **Bing Zhu**[2], **Tae-Il Jeon**[1], and **Timothy F. Osborne**[1,*]
[1]Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92697-3900

Abstract

In this study, we examined sterol regulatory element binding proteins (SREBPs) regulates expression of Srd5a2, an enzyme that catalyzes the irreversible conversion of testosterone to dihydrotestosterone in the male reproductive tract and is highly expressed in androgen-sensitive tissues such as the prostate and skin. We show that Srd5a2 is induced in livers and prostate from mice fed a chow diet supplemented with lovastatin plus ezitimibe (L/E), which increases the activity of nuclear SREBP-2. The three fold increase in Srd5a2 mRNA mediated by L/E treatment was accompanied by the induction of SREBP-2 binding to the Srd5a2 promoter detected by a ChIP-chip assay in liver. We identified a SREBP-2 responsive region within the first 300 upstream bases of the mouse Srd5a2 promoter by co-transfection assays which contain a site that bound SREBP-2 in vitro by an EMSA. Srd5a2 protein was also induced in cells over-expressing SREBP-2 in culture. The induction of Srd5a2 through SREBP-2 provides a mechanistic explanation for why even though statinn therapy is effective in reducing cholesterol levels in treating hypercholesterolemia it does not compromise androgen production in clinical studies.

Keywords

SREBP-2; Srd5a2; androgen regulation; statin; transcription regulation; Chip-chip assay

Introduction

Sterol regulatory element binding proteins (SREBP) consist of a sub-family of basic-helix-loop-helix transcription factors that play key roles in the regulation of cell lipid homeostasis [1,2]. There are three major SREBP is forms in mammals that are encoded by two genes. The *Srebf-1* gene produces two overlapping mRNAs that differ only in their specific 5'-terminal exons, where unique 1a and 1c exons give rise to identical proteins except for their unique amino-terminal activation domains [3]. The *Srebf-2* gene produces a single SREBP-2 protein with a potent activation domain similar to SREBP-1a. In cultured cells at least, low cholesterol levels result in membrane release of SREBP-2, whereas low cholesterol and fatty acids trigger release of SREBP-1[4].

*To whom correspondence should be addressed: Dept. of Molecular Biology and Biochemistry, 3244 McGaugh Hall, UC Irvine, Irvine, CA 92697-3900. tfosborn@uci.edu.
[2]Present Address: Department of Pathology, University of Texas Medical Branch, Galveston, TX 77555-0144

Since cholesterol is the precursor of steroid hormones, steroid metabolic synthesis can be affected by SREBPs and conditions where cholesterol is limiting could result in compromised steroid hormone production [5]. A key step in the androgen synthetic pathway converts testosterone into the more biologically active dihydroxytesosterone [6–9]. This step is catalyzed by steroid 5α-reductases, which are membrane-associated NADPH-dependent enzyme that catalyzes the irreversible steroid specific reduction of C^{19} 3-keto-$\Delta^{4–5}$ steroid to 5α-reduced metabolites. There are two Steroid 5α-reducates isotypes, I (Srd5a1) and II (Srd5a2), in humans and they are composed of 260 and 254 amino acids, respectively, with 47% sequence identity and distinct biochemical properties [9–12].

In mice fed a chow diet supplemented with lovastatin plus ezetimibe (L/E) to limit dietary sterol absorption and decrease endogenous synthesis in the body, nuclear levels of hepatic SREBP-2 are induced [12,13]. Chip studies revealed gene-specific binding of SREBP-2 to known SREBP-responsive genes. In the current study, we searched a genomic promoter-wide Chip-chip data set for SREBP-2 binding to chromatin from livers of L/E-fed mice and this revealed that the Srd5a2 promoter was bound by SREBP-2. Further studies showed that Srd5a2 gene expression is under control of SREBP-2 in mouse liver and prostate. These results suggest that steroid hormone production is under control of SREBP-2 and this regulation could be key to maintaining androgen activity at normal levels under conditions where cellular cholesterol levels are low. There have been several studies that indicate patients undergoing statin therapy to lower serum cholesterol levels have normal androgen regulated functions [14–16] and the activation of SRD5a2 directly by SREBP-2 provides a molecular explanation for these clinical observations.

Experimental Procedures

Animal care

Male 8-week old B6/129 mice were obtained from Taconic and maintained on chow diet (2020X, Harlan Teklad Global) for one week with a 12 hr light 12 hr dark cycle for acclimatization. Then animals were separated into two groups of 6 animals each and one group was maintained on the normal chow diet and the second group was fed the same diet supplemented with a mixture of lovastatin (Mylan Pharmaceuticals Inc., 0.1%, w/w) and ezetimibe (ezetimibe from Merck/ Schering- Plough Pharmaceuticals, 0.021%, w/w). After one week of feeding, animals were sacrificed by CO_2 asphyxiation in the morning at the end of the dark cycle and tissues were immediately removed for RNA, chromatin and protein extraction as described below.

Chromatin Immunoprecipitation(ChIP) assay

ChIP assays from mouse tissues were performed as previously described [13]. Briefly, livers were pooled and placed in ice-cold PBS solution with a mixture of protease inhibitors. The tissue was minced with a razorblade and processed. Final DNA samples were analyzed by quantitative PCR for SREBP-2 binding to specific gene promoters in triplicate with a standard dilution curve of the input DNA performed in parallel. The qPCR oligonucleotide pairs for the mouse promoters were as follows: Srd5a2, forward 5'-TGAGACCCAGGAGGAATTTG and reverse 5'-CAGTTGTCCATGCTTCTCCA, HMG-CoA reductase, forward 5'-GCTCGGAG ACCAATAGGA-3' and reverse 5'-CCGCCAATAAGGAAGGAT-3', L32, forward 5'-ACATTTGCCCTGAATGTGGT and reverse 5'-ATCCTCTTGCCCTGACC TT.

ChIP-chip array

To prepare samples for the ChIP-chip array, after reversing the crosslinking and isolating the ChIP-enriched DNA, samples enriched for SREBP-2, or treated with a non-specific rabbit

IgG fraction as a control, along with input DNA were prepared for hybridization to a tiled 1.5-kb mouse promoter array (NimbleGen/Roche) using a random PCR amplification protocol [17]. The results were analyzed by the Signal Map software program from NimbleGen/Roche and Srd5a2 was picked out as having significant SREBP-2 binding.

RNA Analyses

Tissues were treated with TRIzol (Invitrogen) and total RNA was extracted according to the manufacturer's instructions. cDNA was synthesized and used as template for qPCR as described [18,19]. All qPCR reactions were performed in triplicate. Primers used for qPCR are followed: Srd5a2, forward 5'-TTTCCTGGGCGAGATTATTG, reverse 5'-CGCGCAATAAACCAGGTAAT; SREBP-2, forward 5'-ATGCTACAGTTTGTCAGCAATCAAG reverse 5'- TGCTGTTGTTGCCACTG, HMG CoA reductase, Forward 5'- ACCCTGCAGGTCAAACTCTG, Reverse 5'-TCACGAACGGTCTCCCTAAC, and L32, forward 5'-ACATTTGCCCTGAATGTGGT and reverse 5'- ATCCTCTTGCCCTGACCTT.

Plasmids

The mouse Srd5a2 promoter construct (−1,500 to +107) was cloned by PCR amplification using mouse genomic DNA as template, followed by recombination with the pDONR2.1 vector according to Gateway technology (Invitrogen). The Srd5a2 construct was then transferred by Gateway technology (Invitrogen) into the luciferase reporter vector p-LUC-GW. All constructs were verified by DNA sequencing. The plasmids, 2×flag pCDNA3.1+ SREBP-2 and pSynSRE-positive control SREBP reporter, have been previously described [3].

Transient transfection in 293T cell and Reporter assay

293T cells (2×10^5 cells/well) seeded in 24-well plates were transfected with 200 ng of the Srd5a2 promoter-luciferase reporter and 5 ng of 2×flag pCDNA3.1+ SREBP-2 plasmids using Lipofectamine 2000 reagent (Invitrogen). A pCMV–β-gal expression construct was included in every transfection as a normalization control. Twenty-four hours after transfection, cells were harvested and assayed for luciferase and β-gal activities. Results were computed based on three independent transfections.

Immunoblotting

293T cells were seeded onto 100mm-culture dishes at 2.5×10^6 cells/dish in DMEM containing 10% FBS and were transfected with 2×flag pCDNA3.1 or 2×flag pCDNA3.1+SREBP-2 and after 24 hr., cells were collected by scrapping and lysed (2% SDS, 5% β-mercaptoethanol, 125 mM Tris-HCl, pH6.8, 20% glycerol) and 30ug of total cellular proteins were analyzed by 10% SDS-polyacrylamide gel. For immunodetection of SRD5a2, the polyclonal anti-human SRD5a2 (H-100: sc-20659, Santa Cruz) was used as the primary antibody. The antibody against β-actin (A1978, Sigma) and polyclonal anit-flag antibody (M2; Sigma) were used as the primary antibodies. Using the peroxidase conjugated secondary antibody, the antigen/antibody complex was detected via chemiluminescence (ECL kit, GE) and x-ray film.

LNCaP cells, an androgen sensitive prostate cancer cell line were provided by Dr. John Krowleski, UC Irvine. Cells were seeded onto 100mm dishes at 2×10^6 cells/dish in RPMI1640 supplemented with 10% FCS, 2mM glutamine, 1mM sodium pyruvate, and 10mM HEPES buffer in an atmosphere of 5% CO_2 at 37°C. The next day, dishes were washed with 1X PBS and refed the same medium without serum and plus or minus a mixture of sterols (12 μg/ml cholesterol and 1 μg/ml 25-hydroxycholesterol) and with or

without atorvastatin (10 mM), as described previously [20]. Cells were harvested 24 hours later and 30 ug total cell protein were analyzed for immunoblotting as above.

Electrophoretic Mobility Shift Assay (EMSA)

The 293T cells were transfected with the SREBP-2 expressing construct as described above and following 24 hr., cells were harvested and nuclear extracts were prepared as described [21] above. 2 ug of total nuclear extract from mock or SREBP-2 transfected cells were preincubated (5 min at room temperature) with polydeoxyinosinic deoxycytidylic acid (0.1 ug/ul) in a DNA-binding buffer (10 mM HEPES, pH 7.6; 25 mM $MgCl_2$; 50 mM NaCl; 1mM EDTA; 5% glycerol (v/v); 1 mM dithiothreitol) after which the ^{32}P labeled oligonucleotide probe (1 ug) was added and incubation was continued for 30 min on ice. The DNA-bound proteins were analyzed on a 6% native polyacrylamide gel run in tris borate buffer (TBE). For competition, the excess (300x) unlabeled homologous or non-self double strand competitors were added at the preincubation stage. The sequence for one strand of the double strand probes used are as follow; Srd5a2, 5-GAACGAGGATCAAGGC ATCTTA GGGCCACGT, LDLR, 5'-TTTGAAAATCACCCCACTGCAAACTCCTCCCCTGCTA. Non-specific competitor (NS), 5'-GATCGTTACAGGTCAAAGCCATAGGTC.

Results

In order to identify novel SREBP-2 target genes, we performed a whole genome promoter ChIP-chip assay with chromatin isolated from livers of mice fed a diet supplemented with lovastatin and ezetimibe. The lovastatin inhibits HMG-CoA reductase, and the ezetimibe blocks endogenous cholesterol synthesis and dietary cholesterol absorption respectively. The mice respond as though they are cholesterol starved, and nuclear levels of hepatic SREBP-2 increase significantly [13]. Using DNA enriched from hepatic chromatin with an SREBP-2 antibody, we performed a chromatin immunoprecipitation and used the enriched DNA to interrogate a promoter array containing tiled oligonucleotide probes from more than 25,000 mouse promoters.

Our ChIP-chip array for SREBP-2 binding site in 1.5 kb mouse whole genome promoter array shows 1,741 unique SREBP-2 putative target genes at p value = 0.001. A Gene Ontology (GO) analysis shows a cluster of genes in lipid biosynthesis. This work is still in progress and will be reported when the analyses are complete."

However, in our analyses, we found that SREBP-2 binding to the promoter for Srd5a2 is enriched relative to a companion array hybridized with DNA prepared from chromatin incubated with a control IgG fraction (Fig. 1A). Next, we sought to confirm this observation through gene-specific ChIP analysis with oligonucleotide primers for the Srd5a2 promoter. The result in Fig. 1B demonstrates that SREBP-2 bound directly to the Srd5a2 promoter as well as to promoters for known SREBP-2 target gene including HMG CoA reductase. The binding of SREBP-2 to the Srd5a2 promoter is functional as expression of Srd5a2 mRNA was also induced in the liver of the L/E treated mice (Fig. 1C).

Fig. 1C shows that mRNA expression of Srd5a2, SREBP-2 and HMGCR in L/E treated mouse liver. Srd5a2 is expectedly induced by L/E, although CT value is lower then that of SREBP-2 or HMGCR.

Srd5a2 mRNA expression and SREBP-2 binding to the Srd5a2 promoter were also significantly elevated in samples prepared from the prostate of L/E treated mice (Fig. 2) where Srd5a2 activity is crucial for androgen production. Interestingly the binding of

SREBP-1 protein was also increased at the Srd5a2 promoter similar to what we observed for the FAS promoter in liver chromatin from L/E treated mice [13].

To determine whether this regulation is conserved for the human gene and whether SREBP-2 might increase Srd5a2 protein levels, we transfected a FLAG-eptiope tagged SREBP-2 expression vector for SREBP-2 into 293T cells and measured endogenous Srd5a2 protein using an antibody (Fig. 3A). Here, the accumulation of nuclear FLAG-tagged SREBP-2 was accompanied by an increase in Srd5a2 protein.

Next, we analyzed whether activation of endogenous SREBPs by sterol depletion in the LNCaP human prostate cancer cell line would increase endogenous Srd5a2 expression in a more physiologic cell type. LNCaP cells were cultured in medium with sterols (12 μg/ml cholesterol and 1 μg/ml 25-hydroxycholesterol) versus no sterols but with atorvastatin (10 mM) to deplete endogenous cholesterol. Fig. 3B shows that Srd5a2 protein levels were induced in the atorvastatin treated sample.

Based on similarity to a putative consensus site suggested by the alignment of a limited number of well characterized SREBP-2 binding sites [22] we identified a putative recognition site in the human Srd5a2 promoter that is also present in the promoters for the mouse and rat Srd5a2 genes (Fig. 4A). Additionally, the putative SREBP-2 binding element from the mouse Srd5a2 promoter bound specifically to recombinant SREBP-2 in an EMSA (Fig. 4B).

To determine if activation of Srd5a2 by SREBP-2 requires the newly identified SREBP binding motif, we prepared a luciferase reporter construct containing the mouse promoter with the putative SREBP response element and showed that its activity was significantly enhanced by co-transfection with a vector expressing the mature SREBP-2 protein (Fig. 5). When this element was deleted, activation by SREBP-2 co-transfection was significantly reduced (Fig. 5).

Discussion

The srebf1 and srebf2 genes encode sterol regulatory element binding proteins (SREBPs), which are a sub-class of bHLHLZ transcriptional activator proteins and are important regulators of the core pathways of cholesterol and fatty acid biosynthesis [23,24]. SREBPs are translated and targeted to the ER membrane where they reside until nutritional cues trigger their movement to the Golgi [2]. In the Golgi, two proteases cleave the precursor SREBPs releasing the mature nuclear-targeted transcription factors which rapidly accumulate in the nucleus to activate target gene expression. The srebf1 gene encodes two alternatively spliced transcripts that encode two almost identical SREBP-1 proteins that preferentially activate genes of fatty acid metabolism and the srebf2 gene encodes a single SREBP-2 that is a key cholesterol regulatory transcription factor [25].

Hepatic levels of SREBP-2 protein are elevated when mice are fed a diet supplemented with lovastatin to inhibit cholesterol production plus ezetimibe to limit cholesterol absorption [13]. Under these conditions, hepatic levels of SREBP-1 protein decline and we have been using these feeding conditions to evaluate SREBP-2 target gene activation relative to SREBP-1. We have used an antibody directed against SREBP-2 in ChIP-chip analyses to identify new SREBP-2 target genes. In a recent study, we established that the G-protein coupled T2R receptors that respond to bitter tasting and potentially toxic components of the diet are target genes for SREBP-2 in the endocrine cells of the proximal intestine [21].

In the studies reported here we have identified Srd5a2 as a new SREBP-2 target gene and show expression of its mRNA in the prostate is increased by Lovastatin/ezetimibe feeding.

We also show that SREBP activation in 293T cells and the human prostate cancer LNCaP cell line result in elevated levels of endogenous Srd5a2 protein. Srd5a2 catalyzes the conversion of testosterone to dihydroxytestosterone which is a much more potent androgen in vivo than testosterone [6,12]. Uncovering the regulation of Srd5a2 by SREBP-2 reveals an interesting metabolic response to limiting cholesterol that may be a key response in androgen producing tissues such as prostate.

SREBP-2 levels are increased when cellular cholesterol needs to be increased for cell homeostasis. In most cells cholesterol is the end of a metabolic pathway and cholesterol is used directly as an important membrane component. Thus, regulation of the pathway only needs to account for production of cholesterol directly. However, cholesterol is a precursor for the synthesis of bile acids in the liver and for steroid hormones in steroid hormone producing tissues. Thus, it would be useful to evolve regulatory processes that have adapted to the special needs of pathways in tissues where cholesterol is an intermediate and not the end-product.

At least some rodents have adapted a regulatory mechanism to respond to excess cholesterol in the liver through the stimulation of cholesterol 7-α-hydroxylase (CYP7a1) by the oxysterol activated liver X receptor (LXR)[26–28]. This helps prevent the toxic overaccumulation of cholesterol in the liver. There is also evidence that the key 12-α-hydroxylase enzyme (CYP8B1) is a target of SREBP signaling which could be significant for regulating the hydrophilic/hydrophobic character of bile produced at low flux through the hepatic synthetic pathway [29]. The regulatory process uncovered by the current studies would provide another mechanism for ensuring proper flux through to the end of the androgen synthetic pathway under limiting pathway flux from cholesterol.

Statins have proven to be clinically beneficial in treating hypercholesterolemia patients because they lower serum LDL-cholesterol levels significantly [15,30]. Because steroid hormone production relies on cholesterol as a precursor, the cholesterol lowering effects of statins were predicted to possibly limit cholesterol availability and possibly decrease steroid hormone synthesis. However, studies to date indicate that statins do not lower androgen function in any clinically significant way [30]. The activation of Srd5a2 in androgen producing tissues by SREBP-2 would ensure that DHT production could continue despite the lower flux through the pathway. Thus, our results provide evidence for a feed-forward mechanism for androgen production from limiting amounts of cholesterol and provide a molecular explanation for why clinical studies fail to correlate statin therapy with defective androgen production in patients.

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The abbreviations used are

Srd5a2	Steroid 5-α reductase type 2
SREBP	sterol regulatory element-binding protein
HMG	3-hydroxy-3-methylglutaryl
ChIP	chromatin immunoprecipitation
qPCR	quantitative PCR
L/E	lovastatin and ezetimibe

LDLR low density lipoprotein receptor

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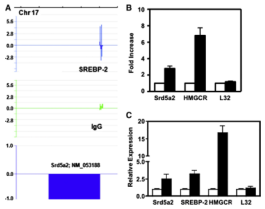

Figure 1.

Identification of mouse Srd5a2 as SREBP-2 target gene. SREBP-2 ChIP-on-chip analysis was performed using 1.5 kb mouse whole genome promoter arrays from NimbleGen/Roche. (**A**) Location of the hybridization signals on the ChIP-chip array at the Srd5a2 locus on mouse chromosome 17 (Chr 17). The top panel shows log2 ratios of hybridization signals for tiled probes for SREBP-2 antibody-enriched ChIP DNA relative to the input DNA. The middle panel shows a similar analysis when a control IgG fraction was used as antibody. The X axis of [Fig 1A] is Enrichment Score relative to Input (Log2) and the lower panel in [Fig 1A] shows the relative position of the Srd5a2 coding region on Chr 17. (**B**) Gene-specific ChIP analysis. Chromatin from chow (open bars) or L/E (filled bars) treated mouse liver was analyzed for SREBP-2 binding to the indicated promoter regions using promoter specific oligonucleotides as described in Experimental Procedures. (**C**) Total RNAs from mice fed a control chow (open) or L/E supplemented diet were analyzed for expression of Srd5a2, SREBP-2, HMGCR and L32, as described under “Experimental Procedures.” Expression of each mRNA indicated was normalized to the expression of ribosomal L32 protein RNA in each sample, and the ratio in the normal chow sample was set at 1.0. Data are mean ± SEM; $n = 3$ for 3 separate experiments.

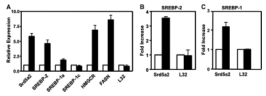

Figure 2.
mRNA expression (**A**) and binding of SREBP-2 (**B**) and SREBP-1 (**C**) by ChIP in mouse prostate. Symbols and notations were as described in the legend to Fig. 1.

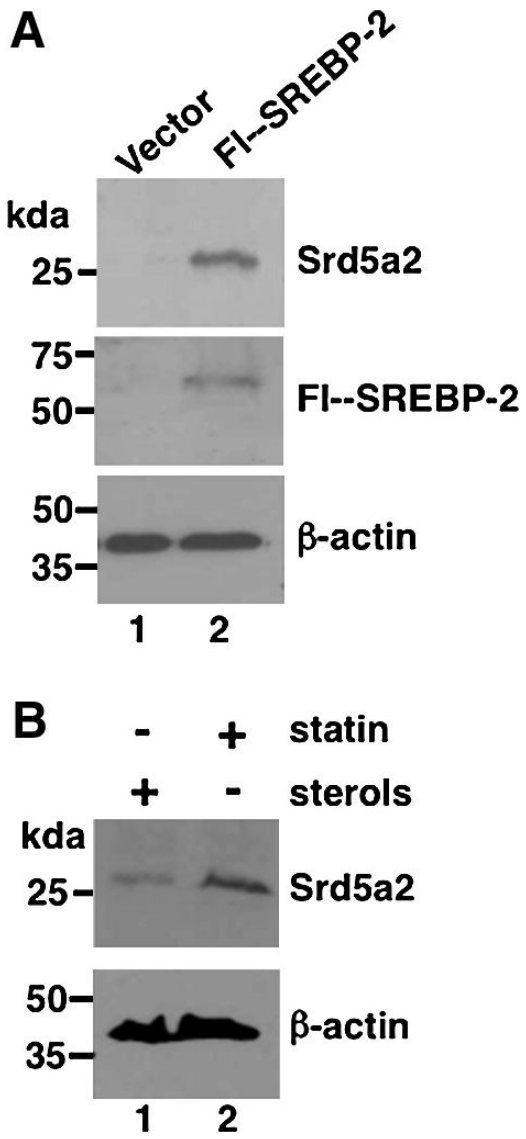

Figure 3.

Analysis of Srd5a2 protein expression human cell lines. (**A**) 293T cells were transiently transfected with vector (lane 1) or a flag-tagged SREBP-2 expression vector (lane 2). (**B**) LNCaP cells were cultured in medium with sterols (lane 1) or without sterols plus atorvastatin (lane 2). Cell lysates were prepared and equal amounts of total protein from comparative samples were analyzed by immunoblotting with antibodies directed against Srd5a2, flag-SREBP-2 or β-actin as shown. Experiments were repeated at least three times with similar results.

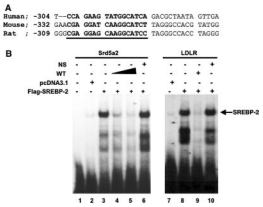

Figure 4.

SREBP-2 binds to and directly activates the Srd5a2 promoter. (**A**) Nucleotide alignment of putative SRE region in the Srd5a2 promoter from different species. Sequences in the box highlight the conservation in different species. (B) 293T cells were transfected with vector or an SREBP-2 expression vector and nuclear extracts were prepared and used in EMSA assays with a probe encompassing the putative SRE element from the mouse Srd5a2 promoter in A (lanes 1–6) or the known SREBP site from the human LDL receptor promoter (lanes 7–10). Where indicated wild type probe (WT) or non-specific (NS) oligonucleotides were used as competitors in the binding reaction.

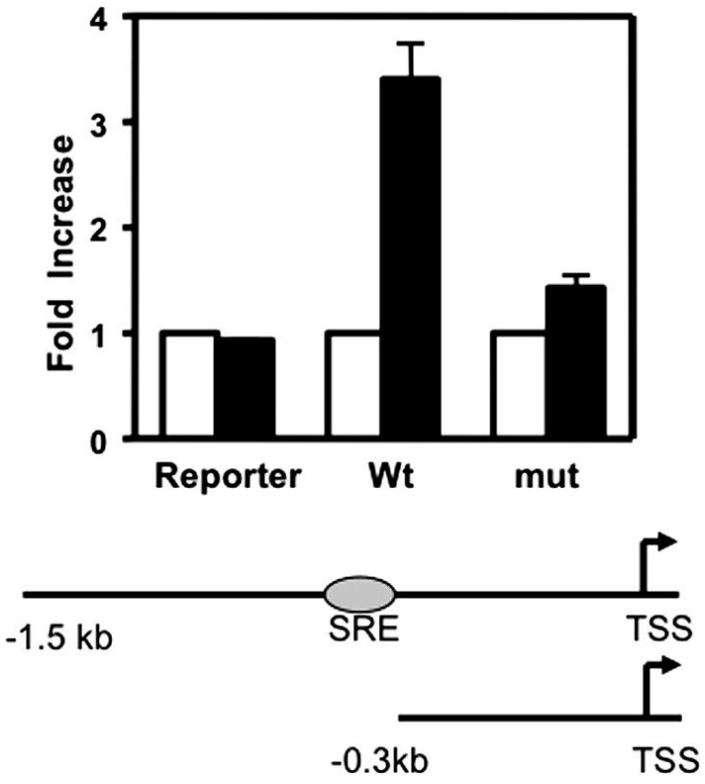

Figure 5.
293T cells were transiently co-transfected with the indicated Srd5a2 reporter construct and pcDNA3.1 empty vector (open symbols) or SREBP-2 expression construct (filled symbols) and cultured for 24 hr. Cells were harvested and extracts were used to measure luciferase and β-gal activity as described in Experimental Procedures.