

**A cAMP responsive element binding site is essential for sterol regulation of the human lanosterol 14 $\alpha$ -demethylase gene (CYP51)**

Running title: Sterol regulation of the human CYP51 gene

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## ABSTRACT

Lanosterol 14 $\alpha$ -demethylase (CYP51) is involved in the cholesterol biosynthesis pathway, producing folicular fluid meiosis activating sterol (FF-MAS). The promoter region of the human CYP51 gene contains a cluster of regulatory elements including GC box, cAMP response element (CRE) and sterol regulatory element (SRE). To understand the mechanism of sterol-dependent regulation of this gene, several constructs of the promoter with the reporter gene have been tested in JEG-3 cells containing overexpressed human SREBP-1a. The wild type construct showed maximal SREBP-dependent activation, most of which is retained when the GC-box is mutated/deleted. Activation is abolished when either CRE or SRE are removed/mutated. Furthermore, mutation of CRE abolishes SREBP-dependent activation after overexpression of SREBP-1a and CREB. This shows that CRE is essential, and that under *ex vivo* conditions CREB and SREBP cooperate in transactivating CYP51. Interestingly, protein kinase A (PKA) shows a marked stimulation of the CYP51 promoter activity when overexpressed together with SREBP-1a but not when overexpressed with CREB, suggesting phosphorylation of SREBP-1a. Using a DNA probe containing all three regulatory elements it is found that SREBP-1a, a CREB-like factor and Sp1 likely all bind the CYP51 promoter. While SREBP-1a and the CRE-bound proteins are essential for the SREBP-dependent response, Sp1 apparently functions only to maximize sterol regulation of CYP51. To date this is the first gene where co-operation between SREBP and a CREB/CREM/ATF family transcription factor is shown to be essential and sufficient for SREBP-dependent activation.

## INTRODUCTION

The CYP51 gene encodes a cytochrome P450 enzyme 14 $\alpha$ -sterol demethylase that is highly conserved in sterol synthesizing eukaryotic phyla (1). As a result of its recent discovery in *Mycobacteria* species (2), it is considered to be an ancient P450 arising before the divergence of eukaryotes. CYP51 catalyzes the first step following cyclization in sterol biosynthesis, the three-reaction 14 $\alpha$ -demethylation of lanosterol. Cholesterol biosynthesis involves at least 30 reactions. Genes involved in the presqualene portion of the pathway are well characterized, their sterol dependent transcription being well established. These include HMG-CoA reductase (3, 4) HMG-CoA synthase (5), farnesyl pyrophosphate synthase (FPP) (6) and squalene synthase (7). All require the transcription factor sterol regulatory element binding protein (SREBP) and at least one additional co-regulatory transcription factor, although the combination of factors differs from gene to gene. Besides cholesterol biosynthesis, transcription factors of the SREBP family regulate synthesis of the LDL receptor (3, 8, 9) and caveolin (10), as well as fatty acid and triglyceride synthesis (11-13).

SREBPs are a family of nuclear transcription factors that are synthesized as precursors localized in the endoplasmic reticulum. There are at least two isoforms of SREBP-1 (SREBP-1a and SREBP-1c) that are expressed from a single gene while SREBP-2 arises from a distinct gene (14-16). In the absence of sterols, the full length, membrane bound SREBPs are proteolytically processed leading to release of the bHLH-Zip amino terminal portion of the protein that directly translocates to the nucleus and activates transcription of target genes *via* binding to SREs (14, 17-19). Both SREBP-1 and SREBP-2 have the ability to bind to SREs and activate promoters of cholesterologenic and

lipogenic enzymes (20-24). SREBP-1a is the predominant form in cultured cell lines while in tissues SREBP-2 seems to be more important for regulation of cholesterologenic genes (21). Although SREBPs are the key regulatory proteins for cholesterol-dependent activation of all these genes, they are not sufficient for transcriptional activation of any, and require the simultaneous binding of other transcription factors to response elements found near the SRE (25). Some genes require a single SREBP co-regulatory protein, such as Sp1 for the human LDL-receptor gene (8) and nuclear factor (NF-Y) for both FPP synthase (6, 26) and squalene synthase (7). Several genes involved in either cholesterol or fatty acid biosynthesis require two SREBP co-regulatory proteins. Sp1 and NF-Y are needed for the fatty acid synthase (11, 12, 27) and 7-dehydrocholesterol reductase (28) activation, while NF-Y and a CREB/ATF family factor are co-regulators for the HMG-CoA reductase (3) and HMG-CoA synthase (29) genes. Recently, evidence is accumulating that different isoforms of SREBP proteins are not only involved in cholesterol-regulated events but are themselves targets for intracellular signalling pathways. For example, activation of AMP-activated protein kinase that is a major cellular regulator of lipid and glucose metabolism suppressed expression of SREBP-1a (30) while glucagon dramatically decreased the expression of ADD1/SREBP-1c through generating cAMP and activating PKA (31). Phosphorylation of SREBP-1a at Ser 117 of SREBP-1a by the Erk subfamily of MAP kinases was found to be involved in SREBP-1a mediated induction of the LDL receptor gene by insulin and PDGF (32).

The CYP51 gene encodes lanosterol 14 $\alpha$ -demethylase that belongs to the post-squalene portion of cholesterol biosynthesis and metabolizes lanosterol, which is the first cyclic intermediate of the pathway. Beyond expression and regulation studies of CYP51

(33-36), regulation of only one other gene of the late cholesterol biosynthetic pathway has been studied (7-dehydrocholesterol reductase in (28)). Our previous studies have shown that SREBPs are not essential for a high-level expression of the lanosterol 14 $\alpha$ -demethylase gene in male germ cells, where CREM $\tau$  is essential, but are involved in transcription of CYP51 in somatic cells (35). However, the molecular mechanism of the SREBP-dependent activation has not been established. The CYP51 promoter contains both a GC box and a CRE in close proximity to SRE, indicating that either the Sp or CREB/CREM/ATF family of transcription factors, or perhaps a combination of both, is involved in the SREBP-dependent activation.

In the current study we have used JEG-3, a human choriocarcinoma placental cell line, as a model for somatic cells to understand sterol-mediated regulation of human CYP51. Results show that SRE is crucial for regulation but is itself not sufficient in transactivating the CYP51 promoter. Data suggest that sterol-response of the lanosterol 14 $\alpha$ -demethylase gene depends not only on the availability of SREBPs but also on proteins of the CREB/CREM/ATF family.

## RESULTS

### *SREBP-dependent activation of human CYP51 requires SRE and CRE elements while the GC-box is not essential*

The proximal promoter region of the human CYP51 gene is evolutionarily highly conserved (37). It lacks TATA or CCAAT boxes and is GC-rich (38), characteristic of housekeeping genes. It contains at least three nuclear transcription factor binding sites that are conserved in sequence and are found at identical positions in human, mouse, rat and pig promoters (37). In human CYP51, a SRE (ATCACCTCAG) is located at -232/-223 (Fig. 1). This SRE is not identical to SREs studied in other cholesterologenic genes, but it is 100% identical to SREs present in rat and mouse CYP51 promoters (37). A GC box (GGGGGCGC) also identical in all mammalian CYP51 promoters is located 40 bp upstream from SRE and a highly conserved CYP51 cAMP responsive element CRE (TGACGCGA) is found at -252/-244, immediately upstream of the SRE.

Results obtained after cotransfection of JEG-3 cells with the wild type and deletion/mutation reporter constructs (Fig. 2A) and the plasmid pCMV-hSREBP-1a that expresses the mature, transcriptionally active form of SREBP-1a (amino acids 1-460) are presented in Fig. 2. The reporter construct -282/+314 showed a similar level of activation as the wild type promoter (Fig. 2B, lanes 1, 2 ). Removal of the GC-box (-272/+314) reduces activity by 20% (lane 3). Deletion of the next 20bp (lane 4) drops the activation to 50% of wild type, although no recognizable transcription binding sites are present within the -272/-252 region. However, this region is immediately upstream the 8 bp CRE site that is essential for SREBP response. Construct -237/+314 that still contains an intact

SRE element but lacks the CRE site, is unable to mediate the SREBP-dependent transactivation of the CYP51 promoter (lane 5).

Similar data were obtained after cotransfection of pCMV-hSREBP-1a and the -334/+314 constructs containing mutations in these regulatory elements of interest (Fig. 2C). Mutation of SRE shows as expected a very low level of activation (lane 3). Mutation of the GC box results in approximately 46% activity loss (lane 4). Mutation in the CRE sequence reduces the activity to a similar extent as does mutation of SRE (compare lanes 3 and 5). Experiments where individual elements have been deleted from the promoter-reporter constructs are in complete agreement with the mutation data (not shown). In the presence of overexpressed SREBP-1a, deletion of SRE diminished the reporter gene activity to near basal level. Deletion of the 8 bp GC box reduced the CAT activity by only 30% while deletion of both SRE and GC box led to basal regulation that was observed also after the deletion of the entire proximal promoter region. Therefore, SRE in the human CYP51 promoter is crucial for sterol-mediated regulation. Modest reduction in CAT activity observed after deletion of the GC-box suggests that Sp family transcription factors do not play a major role in SREBP-dependent transcription of CYP51. Mutation and deletion data clearly show that an intact CRE element is essential for the SREBP-dependent activation of CYP51. The presence of an intact GC-box is beneficial and optimizes transcriptional activation, but it is not essential for the SREBP-mediated transactivation of CYP51.

*SREBP-1a, a CRE-binding protein and Sp1 bind to their corresponding elements.*

To investigate the DNA-protein interactions we used *in vitro* gel shift binding assays (Fig. 3). The CYP51 SRE probe interacts efficiently with purified SREBP-1a to form a complex similar to that of the human LDL receptor SRE (Fig. 3A, lanes 2 and 4). Complexes are supershifted in the presence of anti-SREBP-1 (lanes 3 and 5). However, the mutation examined in Fig. 2C, completely abolished formation of the DNA/protein complex (lane 6).

The CYP51-CRE probe forms a complex with CRE-binding proteins from nuclear extracts in JEG-3 cells (Fig. 3B, lane 1). We were unable to supershift the complex but the addition of two different anti-CREB antibodies (lanes 2, 3) and an anti-ATF1 antibody (lane 4) ablated the binding while anti-ATF2 antibody shows no effect (lane 5). Presence of a complex formed between CRE and the bZip binding domain of CREB (lane 6) also confirmed that a nuclear protein of the CREB/CREM/ATF family binds to this site. Binding was reduced in the presence of cold CRE competitor (lane 7) and the complex did not form on the mutated CRE element (lane 8). We have shown previously that this CYP51-CRE sequence (CYP51-CRE2 in (35)) binds CREM $\tau$  which is the predominant cAMP-dependent transcriptional activator in haploid male germ cells. The DNA/CREM $\tau$  complex was disrupted in the presence of both anti-CREB and anti-CREM antibodies (D. Rozman, unpublished). CREM $\tau$  is rarely present in somatic cells and its presence in JEG-3 cells is not documented.

The CYP51 GC box forms a complex with Sp1 similar to that of the consensus GC box probe and the complex is supershifted with anti-Sp1 antibody (Fig. 3C, lanes 4, 6 and 8). Cold competition with either the consensus GC box or the CYP51 GC box competed



formation of the complex (lanes 9,10). The mutated GC-box (lane 11) was unable to compete the formation of the DNA/Sp complex. Furthermore, the DNA/Sp complex was also not observed when the mutated CYP51-GC-box was taken as a probe and either nuclear extracts or the purified Sp1 protein were applied (data not shown). Besides Sp1, Sp3 can bind to the CYP51-GC box however, the intensity of Sp1 binding is much greater, indicating that Sp1 is the major Sp family transcription factor in JEG-3 cells.

*SREBP-dependent activation of the human CYP51 promoter in the presence of overexpressed Sp1 and CREB.* Results described in Fig. 2 show the essential role of CRE for the SREBP-mediated transcription of CYP51 and that the GC box is not essential but contributes to the maximal activation of the promoter. To further evaluate the functional role of Sp1 in SREBP-dependent activation, SREBP-1a and Sp1 were overexpressed in a *Drosophilla* cell line SL-2, together with CYP51 or LDL-receptor luciferase reporter constructs. SL2 cells do not express functional homologues of several mammalian transcription factors, including Sp1. These cells have been used previously to understand regulation of the human LDL receptor gene where SREBP and Sp1 are involved in synergistic activation (8). SREBP-1a alone weakly activates the LDL-receptor promoter (Fig. 4A, lane 3) and the addition of Sp1 (lane 4) results in a 10-fold increase of the SREBP-dependent activation. SREBP-1a alone also weakly activates the CYP51 promoter (lane 7) but the presence of Sp1 results in a much smaller increase of the SREBP-dependent activation (compare lanes 4 and 8).

The functional role of the CRE-bound protein on SREBP-dependent transcription of CYP51 was studied in JEG-3 cells. Wild type CYP51 promoter or the CRE element

mutated promoter were cotransfected together with 10 or 100 ng of the SREBP-1a expression plasmid with or without the addition of CREB. Activation of the wild type CYP51 promoter increases with increased amount of SREBP-1a (Fig. 4B, black bars in lanes 2,4) and some SREBP-1a dose-response is still observed with the CRE-mutated construct (dashed bars in lanes 2, 4). However, the CRE-mutated construct failed to show SREBP-dependent dose-response when CREB is overexpressed together with SREBP-1a (dashed bars in lanes 3,5). A SREBP-1a dose-dependent (black bars, lanes 3 and 5) and SREBP-1a/CREB co-operative action (black bars, lanes 2 and 3; 4 and 5) is observed with the wild type promoter when both transcription factors are overexpressed.

#### *The role of PKA in SREBP-1a dependent transcription of CYP51*

Both CREB as well as SREBP-1a were shown to be activated by phosphorylation at specific serine residues (32, 39). Since CREB and SREBP-1a are involved in transactivation of CYP51 *ex vivo*, we wanted to evaluate whether the PKA signalling pathway influences CYP51 transcriptional activation. Overexpression of CREB stimulates the wild type CYP51-CAT reporter (black bars) (Fig. 4D, lane 2). Surprisingly, overexpression of PKA did not influence the CREB-dependent activation of CYP51 (Fig. 4D, compare lanes 2 and 3), although with the somatostatin-CAT reporter that served as a positive control, overexpression of PKA stimulated the CREB-dependent activation (Fig. 4C, compare lanes 2 and 3). Overexpression of SREBP-1a also stimulated the wild type CYP51-CAT reporter (Fig. 4D, lane 4) and the addition of PKA resulted in a 3-fold increase of the SREBP-1a dependent activation (Fig. 4D, lanes 5 and 4). As shown already in Fig. 4B, lane 4, the CRE-mutated promoter exhibits a markedly

diminished activity compared to the wild type promoter after overexpression of SREBP-1a. Overexpression of PKA does not stimulate the residual SREBP-1a –dependent activation of the CRE-mutated CYP51-CAT reporter (Fig. 4D, compare dashed bars in lanes 4 and 5).

*SREBP-dependent activation of CYP51 is mediated by a DNA/multi-protein complex composed of SREBP-1a, a CRE-binding protein and Sp1*

To investigate whether transcription factors can bind at the same time to the GC-box, CRE and SRE elements in the 58 bp-region of the human CYP51 promoter, a longer probe covering the three binding regions has been studied in mobility shift experiments. The DNA/protein interaction between the 99 bp CYP51 promoter probe and nuclear proteins from JEG-3 cells resulted in two major complexes (lane 1 in Figs. 5 A, B). The cold SRE probe removed both complexes and generates a new, intermediate complex (Fig. 5A, lane 3). A similar effect is observed with the GC-box competitor (Fig. 5A, lane 4). Presence of both SREBP and Sp1 was confirmed by adding specific antibodies. The upper complex was supershifted in the presence of anti-Sp1 (Fig. 5B, lane 2) while anti-SREBP-1a disrupted both DNA/protein complexes. The cold CRE-probe failed to show an effect in competition studies (Fig. 5A, lane 2). However, supershift analysis using the anti-CREB antibody suggests binding of a CREB-like protein to the 99 bp CYP51 promoter region (Fig. 5 B, lane 4).

## DISCUSSION

From the standpoint of evolution, lanosterol 14  $\alpha$ -demethylase is the most conserved gene in the cytochrome P450 (CYP) superfamily. Not only do the coding regions of mammalian CYP51 genes share over 90% identity, the proximal promoter region is also conserved, containing the same regulatory elements at identical positions in the mouse, rat, pig and human promoters (37). This suggests that molecular mechanisms of CYP51 regulation are conserved across mammalian species and might be indispensable for mammalian development, since all nucleated mammalian cells synthesize cholesterol. The CYP51 proximal promoter contains three conserved elements: GC-box, CRE and SRE within 58 bp (Fig. 1). Additionally, a conserved GCAAT sequence is located 12 bp downstream of SRE but does not seem to be important in the SREBP-dependent regulation since a construct containing SRE and the GCAAT box failed to show a SREBP-dependent activation (Fig. 2).

We have shown previously that the CRE element (CYP51-CRE2 in (35)) binds CREM $\tau$ , a transcription activator of the CREB/CREM/ATF family. CREM $\tau$  is the major cAMP-dependent activator in haploid male germ cells where it controls expression of multiple genes that are needed for normal sperm development (40). CREM $\tau$  is not present in most somatic cells, but other CREM isoforms are found in many cell types (41). Interestingly, studies in CREM  $-/-$  mice (42) revealed that CREM $\tau$  is sufficient to drive high-level expression of CYP51 in haploid male germ cells *in vivo* (35). In wild type mice fed a normal chow diet, the CYP51 mRNA level in testis exceeds that in the liver by at least one order of magnitude, due to the presence of highly expressed testis-specific

CYP51 transcripts (35). In contrast, testis of CREM  $-/-$  mice (42) show a low level of somatic CYP51 mRNAs, that is comparable to CYP51 mRNA in the liver. Since DNA-bound SREBP proteins are below the limit of detection in rodent germ cells (35), it is proposed that the cAMP-dependent pathway and CREM $\tau$  can activate CYP51 promoter independently of SREBPs, in a tissue-specific manner. Herein it is shown that this same CYP51-CRE site is essential also for sterol-regulated expression of the lanosterol 14 $\alpha$ -demethylase gene in somatic cells, yet in somatic cells SREBP is required as well. SREBP-dependent activation is lost when this CRE site is deleted or mutated. We had difficulties establishing precisely which nuclear protein(s) of JEG-3 cells binds to the CYP51-CRE site. Experiments with antibodies indicate that the CYP51-CRE bound protein has a bZIP domain and is likely a member of the CREB/CREM/ATF family of transcription factors. The protein is closely related to CREB and ATF-1 since two different anti-CREB antibodies and the anti-ATF-1 antibody ablate the intensity of the DNA-protein complex. Indications of a close relation of the CRE-bound protein from JEG-3 cells and CREB arise also from promoter-reporter studies. When overexpressed, CREB can functionally interact with the overexpressed SREBP-1a and act as a co-regulatory protein in the SREBP-dependent transcription of CYP51. The involvement of transcription factors of the CREB/ATF family in the SREBP-dependent activation has also been reported for the HMG-CoA synthase gene (29), where the 5'-portion of the CRE site partially overlaps with one of the two SRE sites. Mutations within CRE abolished sterol-regulated transcription of HMG-CoA synthase, but overexpression of SREBP-1a in combination with either CREB or ATF-2 did not lead to transactivation of the synthase promoter in a *Drosophila* SL-2 cell model. Only when NF-Y was expressed

together with SREBP-1a and a CRE-binding factor, could transactivation of the HMG-CoA synthase promoter be observed, suggesting that simultaneous action of SREBP, a CRE-binding factor and NF-Y is essential for activation of the HMG-CoA synthase promoter (29). Similarly, in the case of HMG-CoA reductase, chromatin immunoprecipitation showed the efficient binding of both CREB and NF-Y under SREBP-induced conditions, while binding of both co-regulatory proteins was diminished in cholesterol loaded CHO cells (3). In the case of CYP51 we were able to show a co-operative effect of SREBP-1a and CREB in transactivating the CYP51 gene in JEG-3 cells, activation being abolished when the CRE-site was mutated (Fig. 4B). This functional assay seems to rule out the requirement of other potential co-regulatory proteins in SREBP-dependent transactivation of the CYP51 gene. Thus CYP51 is the first gene where co-operation with a CREB/CREM/ATF family transcription factor is *essential and sufficient* for the SREBP-dependent response. Interestingly, PKA stimulates the SREBP-1a induced promoter activity but does not stimulate the CREB-induced promoter activity. This suggests that non-phosphorylated CREB and phosphorylated SREBP might be involved in CYP51 transactivation complex, which would rule out CBP as a potential coactivator. Apparently the well known PKA phosphorylation site in CREB is blocked by interaction with bound SREBP-1a.

It is not clear to us why Sp1 is involved in the CYP51 transactivation complex since SREBP and a CRE-binding factor alone are able to drive up to 80% of the sterol-dependent activation. In the case of the LDL receptor promoter where Sp1 has a defined role in SREBP-mediated transactivation, it was proposed that SREBP recruits Sp1 to the promoter (3). In the case of the fatty acid synthase I (FAS I) promoter, where the Sp1 site also seems to be largely dispensable, in a similar manner as found for CYP51, it was

proposed that Sp1 recruits SREBP to the SRE (11). In another study Magana and coworkers established that while Sp1 is largely dispensable for sterol regulation in immortalized cell lines, it is required for the carbohydrate activation of the FAS promoter in primary hepatocytes (12). This difference is explained by different isoforms of SREBP transcription factors in cultured cells *versus* in tissues, and by the fact that different SREBP-isoforms utilize distinct co-regulatory proteins in order to activate expression of sterol-responsive genes (12). Our data show Sp1 as a contributing factor in order to achieve a maximal promoter activity of CYP51. Since Sp1 seems to bind simultaneously with SREBP-1 and the CRE-binding factor to the promoter of CYP51, it could have a role in stabilization of the DNA/multiprotein transactivation complex.

In conclusion, we propose that the essential role of a CRE-bound factor and the non-essential role of Sp1 in CYP51 transactivation occurs in tissues as well in established cell lines. This assumption is supported by the fact that a CRE-binding factor CREM $\tau$  alone is sufficient to drive high-level expression of the CYP51 gene in mouse germ cells, where Sp1 is abundant (35). Further studies are needed to determine which CYP51-CRE-bound transcription factors are required for SREBP-dependent activation in different cell lines and tissues and how they interact biochemically with SREBP, as well as to understand the physiological role of Sp1 in this process. Most recent data indicate that other genes of the cholesterol biosynthetic pathway are also regulated differently in somatic cells and in male germ cells (43). What is particularly interesting about CYP51, however, is that the same CRE element is essential in both cases, even though the biochemical details of activation of transcription are clearly different.

## MATERIALS AND METHODS

*Construction of Reporter Gene Plasmids* – Preparation of the wild type (WT) human

CYP51 promoter CAT construct (-334 to +314 bp) was described previously (35). To prepare a shorter promoter construct (-121/+314), a *NheI* and *SmaI* digested fragment was removed from the WT construct, blunt-ended by Klenow and self ligated.

The site-specific deletion constructs, delSRE, delGC box, and delSRE/GC box were prepared using Quick Change<sup>TM</sup> Site-Directed Mutagenesis Kit (Stratagene) with the following oligonucleotides 1) SRE deletion, sense 5'-GACGC GATGT AGGCC GAGGC GCTCG CGGTG CAATC ACAGA GC-3', antisense 5'-GTGAT TGCAC CGCGA GCGCC TCGGC CTACA TCGCG TCAGC GGGGC C-3'; 2) GC box deletion, sense 5'GTACC CTGCG TCCGG ACATG CTCAC GCCCA AGGCC CCGC-3', antisense 5'GGGCC TTGGG CGTGA GCATG TCCGG ACGCA GGGTA CTGGG GCACC-3'. The plasmid used as a template for mutagenesis was generated by digestion of the WT promoter CAT vector by *KpnI* and *SmaI*, followed by cloning the -334 to -121 insert into the Bluescript (Stratagene). Inserts were recovered from Bluescript using the same restriction enzymes and reinserted into the same sites of the WT promoter vector. Site-specific deletions were confirmed by sequencing.

The substitution mutation constructs, mutSRE, mutGC box, mutCRE, and mutGC/CRE were prepared as above using oligonucleotides that contain the following mutations: SRE - ATCACCTACAG to TTTTTTTTTT, GC box - GGGGGCGG to GGGTTTTG, and CRE - TGACGCCA to AATGGCCA. An additional construct containing a CRE mutation (CYP51-CRE2-mut1) was prepared by changing



TGACCGCGA to TGATTCCGA. Effects of the two CRE mutations were indistinguishable. All modifications were confirmed by sequencing.

The 5' nested deletion constructs, -282/+314, -272/+314, -252/+314, and -237/+314 were generated by PCR using *Pfu* turbo polymerase (Stratagene). Using individual sense primers having a *KpnI* site at the 5' end and the same antisense primer, we amplified promoter fragments from the WT-CAT promoter construct. Each fragment was subcloned into Bluescript at the *SmaI* site and sequenced. Clones having the correct orientation were used in construction of 5'-deletion CAT-reporter constructs.

*Cell culture and DNA Transfection.* JEG-3 cells were cultured in Dulbecco's modified eagle's medium (GIBCO-BRL) containing 10% (v/v) bovine calf serum with or without the addition of 1% L-glutamine and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin sulfate) or fungizone (GIBCO-BRL). In some experiments 10% delipidated serum was applied (35). Cells were grown to 90% confluency in T75 tissue culture flasks and were plated at  $1 \times 10^6$  cells/60 mm dish 24 h prior to transfection. At 50 - 70% confluency, cells were transiently transfected by a standard calcium phosphate co-precipitation method (44). The total amount of plasmid DNA was adjusted to 15 µg with a pCAT-basic vector. Following transfection, cells were washed twice with PBS followed by addition of fresh medium and allowed to grow for 24 h at 37°C. Cells were then harvested and total cell extracts isolated by two freeze-thaw cycles. Protein concentration in each extract was measured using a protein assay kit (Pierce or Biorad) and 20 µg protein was used to measure the choramphenicol acetyl transferase (CAT) activity (44). pCAT-control plasmid (Promega) was used as a control of CAT activity.

In SREBP-1a/CREB and PKA/CREB/SREBP-1a cotransfection studies in JEG-3 cells the total amount of plasmid was adjusted to 21  $\mu\text{g}$ : 10  $\mu\text{g}$  of the human WT CYP51-CAT construct or CYP51-CRE2mut plasmid, 5  $\mu\text{g}$  of RSV $\beta$ -gal (for normalization), 10 ng or 100 ng of pCMV SREBP, 3  $\mu\text{g}$  pRSV-CREB and 1  $\mu\text{g}$  pSV PKA. pCAT basic was used as the DNA carrier up to 21  $\mu\text{g}$ . Conditions for transfections with the somatostatin CAT reporter were the same. Cells were harvested 48 hours after transfection and analyzed as described previously (35). Every transfection was performed at least three times with two parallel samples in each experiment. The average value and SEM were calculated with the Excel program (Microsoft Corp.).

*Drosophilla* SL2 cells were cultured at 27°C in Shields and Sang medium (Sigma) containing 10% (v/v) fetal bovine serum and supplemented with 100 units/ml penicillin, 100  $\mu\text{g}/\text{ml}$  each streptomycin sulfate and fungizone (GIBCO-BRL). Cells were plated at  $1 \times 10^6$  cells/well in 6-well plates and 16 h later transfected by effectene transfection reagent according to the manufacturer's instructions (QIAGEN). Each reporter (200 ng) was transfected alone or with 50 ng of pPAC-Sp1 or pPAC-SREBP expression vectors, or a combination of both. Total DNA for each transfection was adjusted to 0.4  $\mu\text{g}$  with pPAC empty vector. 48 h after transfection cells were harvested, and extracts prepared by freezing and thawing. The -314/+343 CYP51 promoter region was recloned from pCAT-basic to pGEM-luc and used in transfections. Firefly luciferase activity was measured as described by the manufacturer (Promega).

*Nuclear Extract Preparation and Gel Shift Assay* – JEG-3 cells were split into 10 cm dishes and allowed to grow to 80% confluency. Cells were harvested in PBS and

nuclear extracts prepared (35). Pairs of oligonucleotides that were used as probes in gel shift assay or as competitors are listed in Table 1. Oligonucleotides were purified on a 15% - 20% acrylamide gel under denaturing conditions and pairs mixed in equimolar concentrations. Each double stranded probe (0.1  $\mu$ g) was end-labeled using [ $\gamma$ - $^{32}$ P]ATP and T4 polynucleotide kinase. They were then allowed to anneal at 70°C for 10 min and gradually cooled to room temperature. Radiolabeled probes were purified on Sephadex columns or by electrophoresis followed by ethanol precipitation. The gel-shift binding buffer contained 20 mM HEPES (pH 7.9), 80 mM KCl, 5 mM MgCl<sub>2</sub>, 0.2 mM DTT, 0.1 mM EDTA, 2% ficoll, 5% glycerol, 0.1% Nonidet P-40, 10  $\mu$ g aprotinin, 2 $\mu$ g of yeast tRNA, 0.5  $\mu$ g of poly dI-dC, and 5  $\mu$ g of bovine serum albumin (BSA) in a final volume of 20  $\mu$ l. Different antibodies have been used in supershift analysis. Anti-CREB and anti-SREBP-1a were prepared as described previously (35). Additional anti-CREB, anti-ATF-1 anti-ATF-2 and anti-Sp1 antibodies were purchased from New England Biolabs and from Santa Cruz. 2  $\mu$ l of each antibody was added into the binding reaction and incubated on ice for 10 min. Labeled probe (10,000 CPM) was used for each incubation (5 min on ice) and the DNA-protein complex was resolved on 5% native acrylamide gels by electrophoresis in 0.5X TBE buffer at room temperature. In the experiments with antibodies gels were run at 4°C. The gel was dried by vacuum and exposed to X-ray film at -80°C with an intensifying screen.

A 99 bp long gel shift probe of the human CYP51 promoter region (-297/-198) was amplified by the cloned *Pfu* polymerase (Stratagene, USA) from the CYP51 CAT construct using sense (5'-CCCTG CGTCCG GACAG GGGGCG GTGC-3') and antisense (5'-GCGCTC TGTGA TTGCA CCGCG AGCG-3') primers. PCR conditions

were 94°C, 2 min, 94°C, 1 min, 60°C, 1 min, 72°C, 1 min, with 35 cycles. The amplified fragment was purified using a QiAquick PCR purification kit prior to labeling. In some experiments 1 µg of the CREB DNA binding domain (Santa Cruz) was used. BSA was added as a carrier protein to 10 µg.

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ABBREVIATIONS: SREBP, sterol regulatory element binding protein; SRE, sterol regulatory element; CRE, cAMP-responsive element; CREB, cAMP-responsive element binding protein; HMG-CoA, 3-hydroxy-1-methyl-glutaryl coenzyme A; LDL, low density lipoprotein; FAS, fatty acid synthase; FPP, farnesy pyrophosphate synthase; NF-Y, nuclear factor Y; CBP, CREB binding protein. PKA, protein kinase A.

## FIGURE LEGENDS

Fig. 1. Structure of the functional promoter region of human CYP51. The SRE (-232/-223), GC box (-280/-273), and CRE (-251/-244) sequences are boxed and the arrow indicates the major transcription start site (45).

Fig. 2. Role of different regulatory elements on the SREBP-1a-mediated transactivation of CYP51. A: schematic presentation of the studied promoter-reporter constructs that were transfected in JEG-3 cells together with pCMV-hSREBP1a (amino acids 1-460) or an empty pCMV vector. B: the effect of 5'-nested deletions. Note that the -237/+314 construct (lane 5) contains SRE but lacks CRE. C: The effect of substitution mutations introduced into regulatory elements. Note that SRE (lane 3) and CRE (lane 5) mutations show similar effects on the SREBP-dependent transactivation. Data are an average of three (C) or two (B) independent transfections.

Fig. 3. Detection of transcription factors that bind to CYP51 regulatory elements by *in vitro* mobility shift assay. Sequences of the DNA probes are described in Materials and Methods and in Table 1. A: SREBP1a binding. 0.2  $\mu$ g of SREBP1a protein was incubated with the wild type human LDL SRE probe (lanes 1-3) or wild type human CYP51 SRE probe (lanes 4-5), and with the mutant CYP51 SRE (lane 6). Lane 1, LDL receptor SRE probe with no protein. B: CRE binding. The CYP51-CRE probe (lanes 1-7) was incubated with JEG-3 nuclear proteins (lane 1). Antibodies from New England Biolabs, anti-CREB (lane 2) and Santa Cruz, anti-CREB (lane 3), anti-ATF1 (lane 4) and anti-ATF2 (lane 5) have been added. CYP51-CRE was incubated with the bZip domain of the

CREB protein (lanes 6, 7). A 1000-fold excess of cold CYP51-CRE is added in lane 7. In lane 8 the mutated CYP51-CREmut1 probe was incubated with 1 $\mu$ g of the CREB bZip domain. C: Binding of Sp family of transcription factors. JEG-3 nuclear extract (5  $\mu$ g) was incubated with consensus GC box (lanes 3-4), and with CYP51 GC box (lanes 5-11) probes. Lane 1, consensus GC box probe with 0.5  $\mu$ l of purified Sp1 (Promega); lane 2, consensus GC box probe with no extract; lanes 3 and 5, nuclear extract; lanes 4 and 8, with antibodies against both Sp1 and Sp3; lane 6, with Sp1 antibody; lane 7, with Sp3 antibody; lanes 9 and 10, cold competition (500X) with consensus GC box and CYP51-GC box, respectively; lane 11, cold competition (500X) with CYP51-GC box mutant probe. Each antibody was diluted 2.5 fold and 1  $\mu$ l added to each incubation. Unbound probes are indicated as Free.

Fig. 4. Role of co-regulatory proteins in SREBP-1-dependent activation of CYP51. A: The role of Sp1. *Drosophilla* SL2 cells were transiently transfected using luciferase constructs for both wild type LDL receptor (lanes 1 to 5) and human CYP51 (lanes 5 to 8) promoters. Each reporter was transfected alone or with expression vectors for SREBP or Sp1, or a combination of both, as indicated at the bottom the figure. Data are presented as fold induction where the value of luciferase light units is normalized to total cell protein and the reporter alone is set at 1. B: The role of CREB. Human JEG-3 cells were transiently transfected using CAT constructs containing either the wild type (black bars) or CRE-mutated (CYP51-CREmut1, dashed bars) CYP51 -334/ +314 constructs. 10 ng (+) or 100 ng (++) of the SREBP-1a expression plasmid were transfected alone (lanes 2 and 4) or in combination with 3  $\mu$ g of the CREB expression plasmid (lanes 3 and 5). C:

Control experiments for PKA activation. JEG-3 cells were transfected with the somatostatin CAT reporter, cotransfections were made with 3  $\mu$ g CREB with or without 1  $\mu$ g of PKA. D: The role of PKA in CYP51 activation. Human JEG-3 cells were transiently transfected with CAT constructs containing either wild type (black bars) or CRE-mutated (CYP51-mutCRE, dashed bars) CYP51  $-334/+314$  constructs. Cotransfections were made with 3  $\mu$ g CREB or 100 ng SREBP and 1  $\mu$ g PKA. CAT activities in Figures 4 B, C and D were normalized to  $\beta$ -gal and to the amount of protein as described previously (35). Each transfection was performed at least three times with two parallel samples in each experiment.

Fig. 5. Protein complexes bound to a 99 bp CYP51 promoter in sterol-depleted medium. Supershift or competition analyses were performed by adding antibodies or competitors to nuclear extracts of JEG3 cells grown in media containing delipidated serum for 48 h. Sequences of competitors are shown in Table 1. A: competition analysis: no competitor (lane 1), 1000-fold molar excess of unlabeled CRE (lane 2), SRE (lane 3) and GC-box (lane 4), B: Supershift assay; no antibodies (lane 1), anti-Sp1 antibody (lane 2), anti SREBP-1a antibody (lane 3), anti-CREB antibody (lane 4) prepared as described previously (35). Nuclear extract was pre-incubated with specific antibodies as indicated at the top of the figure. Supershifted complexes are marked with white arrows.

Table 1. Pairs of oligonucleotides used to prepare probes and competitors for mobility shift studies.

<b>PROBE</b>	<b>Sense primer 5' – 3'</b>	<b>Antisense primer 5' – 3'</b>
CYP51-SRE	AGGCCGAGATCACCTCAGGCGCTCG	CGAGCGCCTGAGGTGATCTCGGCCT
CYP51-SRE-a	GGCCGAGATCACCTCAGGCGCT	GCGAGCGCCTGAGGTGATCTCGCG
SREmut	AGGCCGAGTTTTTTTTTTGCGCTCG	CGAGCGCAAAAAAAAAAACTCGGCCT
LDL-SRE	CTAGCAAAATCACCCCACTGCAAC	TCGATCGAGTTGCAGTGGGGTGATTTTG
CYP51-GCbox	GTCCGGACAGGGGGCGGTGCTCACG	CGTGAGCACCGCCCCCTGTCCGGAC
CYP51-Gcbox-a	CCTGCGTCCGGACAGGGGGCGGTGCTCACGC	GGGCGTGAGCACCGCCCCCTGTCCGG
GCbox-mut	GTCCGGACAGGGTTTTGTGCTCACG	CGTGAGCACAAAACCCTGTCCGGAC
Cons. GCbox	GATCGATCGGGGCGGGGCGATC	CATCGCCCCGCCCCGATCGATC
Cons. CRE	AAATTGACGTCATGGTAAG	CTTACCATGACGTCAATTT
CYP51-CRE	GCCCCGCTGACGCGATGTAGGCCGA	GATCTCGGCCTACATCGCGTCAGCGG
CYP51-CREmut1	GCCCAAGGCCCCGCTGATTGATGTAGGC	CGGCCTACATCGAATCAGCGGGGCCTTGG
CYP51-CREmut2	GCCCAAGGCCCCGCAATGGCCATGTAGGC	CGGCCTACATCGCCATTGCGGGGCCTTGG





-334 GGAGAAGGGTGAGGTGCCACA  
 GTTCGAGGTGCCCCAGTACCCTGCGTCCGGACA **GC-box** GGGGGCGGTGCTCACGCCCAAGG  
 CCCCCTGACGCGATGTAGGCCGAGATCACCTCAGGCGCTCGCGGTGCAATCACAG  
 -257 **CRE** **SRE**  
 -201 AGCGCGCCGTCGCCGCTCGAGCTCGCCCTGAGATCCCTCCGCTCAGCGCCTTCAA  
 -146 CGCCGTGTCCCGGCACCCCGCACCCGGGCACACAAAAACGGGTCGCCCGCGATTC  
 -90 TCAGGGATTGATCGCCTCTTCAGGTAAGTTATCTTCGGCCCCGTACCACTGTGCC  
 -35 ACAGGCGCAGCCCGCTTCCTCAGGTGCCCTATCCCGCGCAAGAAGACCACGGCTT  
 →+1

**Fig. 1**

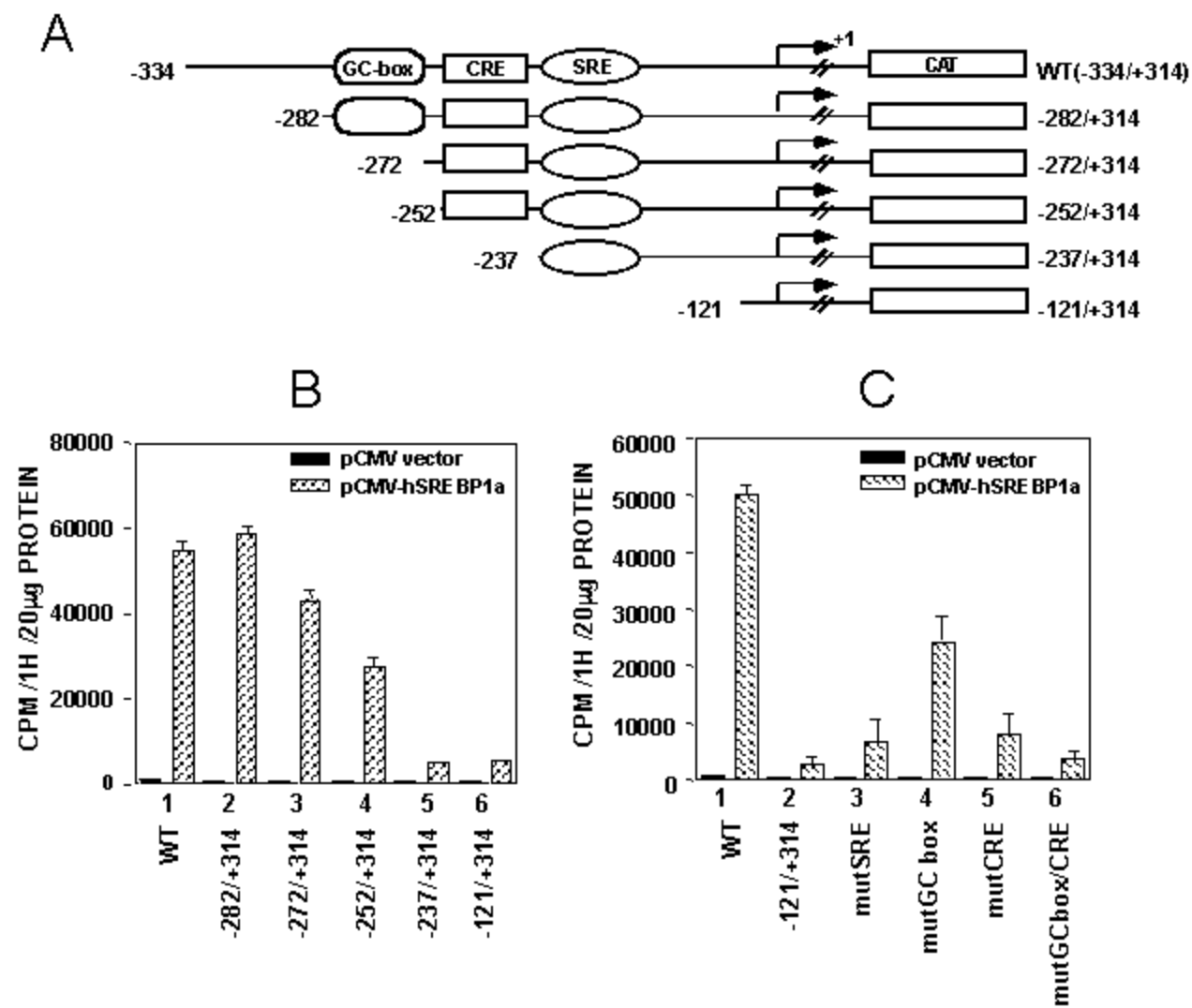


Fig. 2

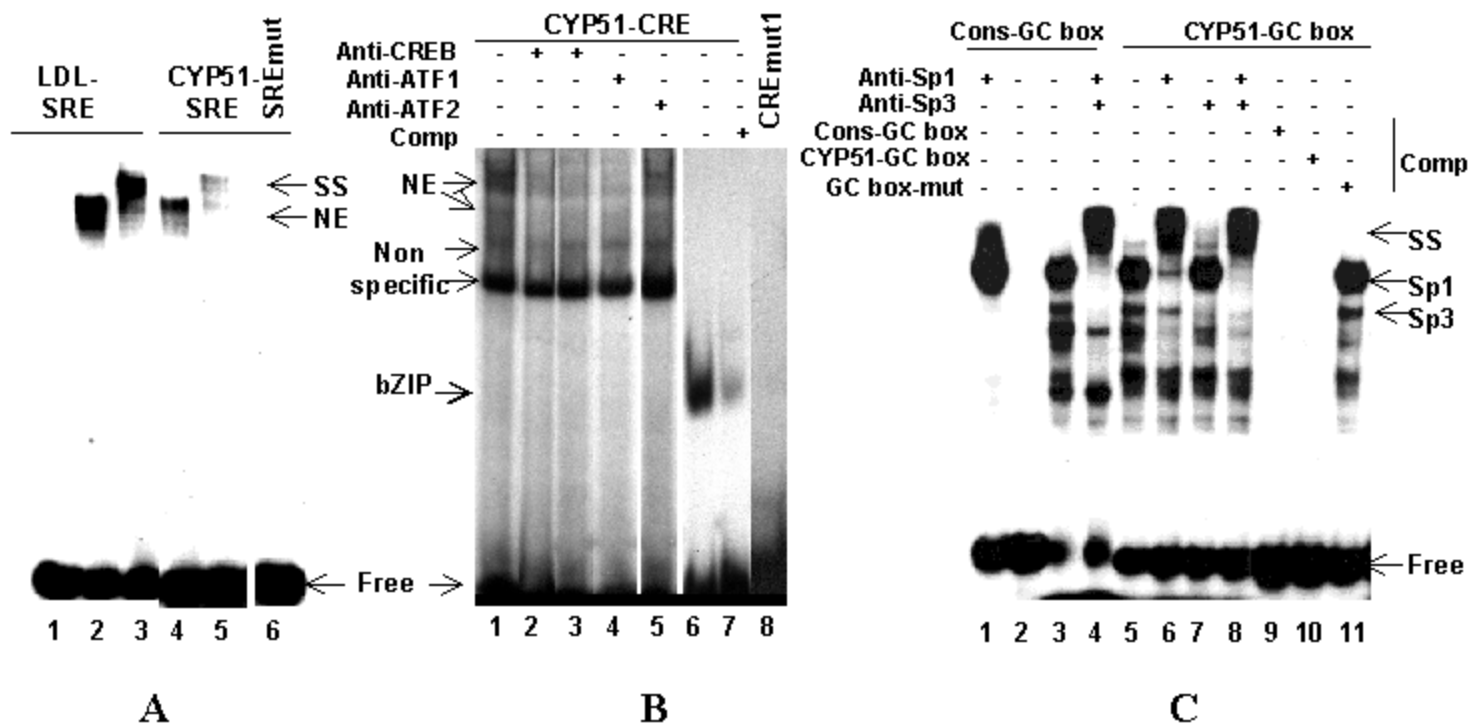
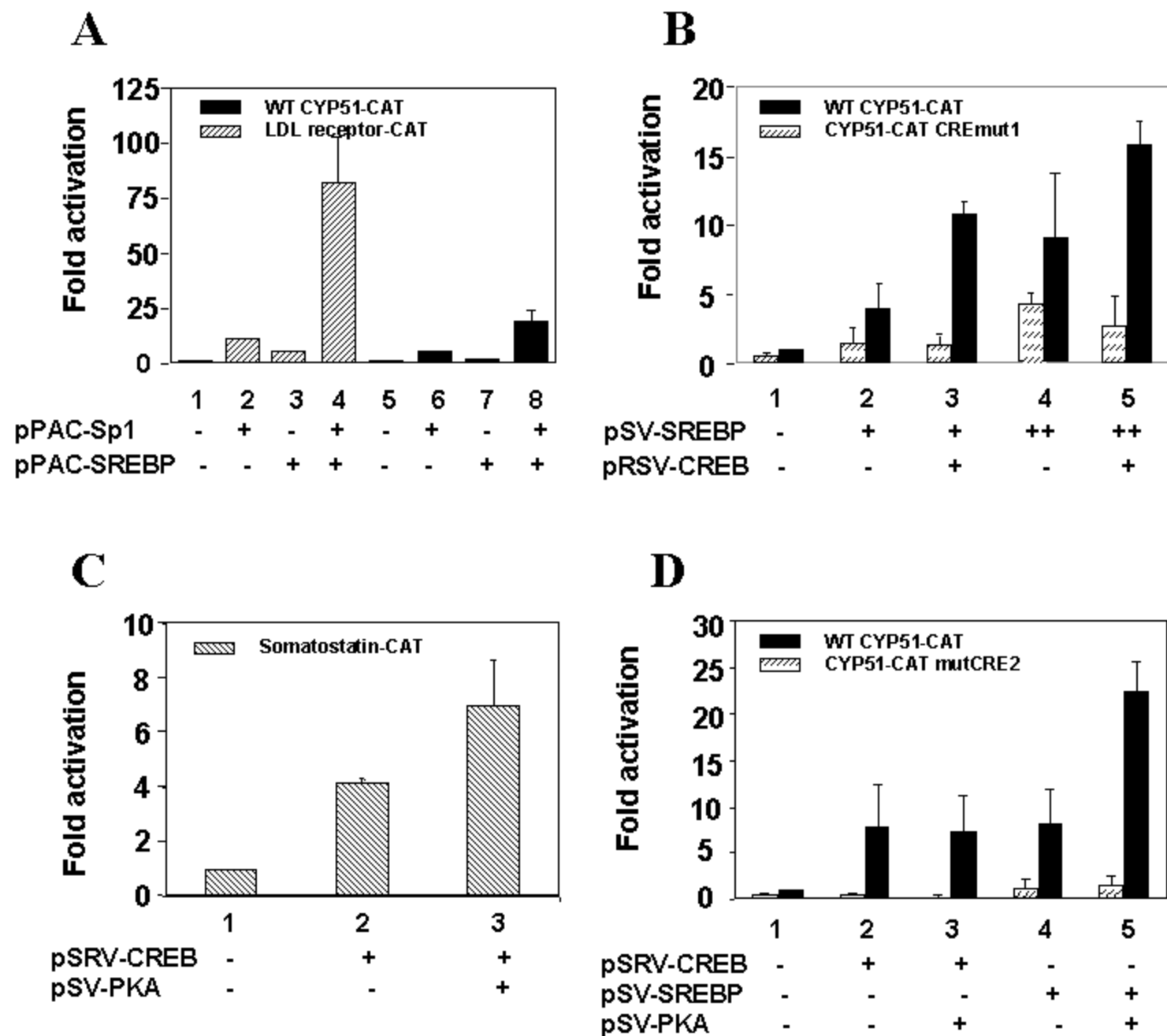


Fig. 3

**Fig. 4**



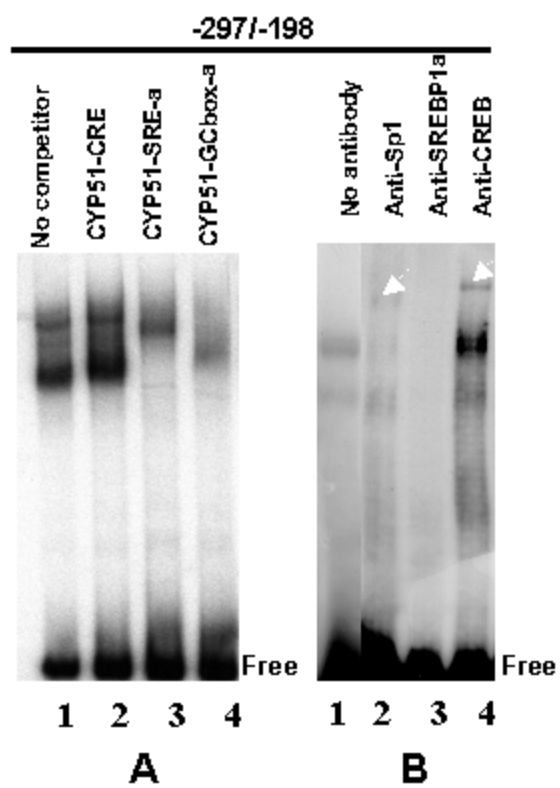


Fig. 5