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DNA binding specificity and transactivation properties of SREBP-2 bound to multiple sites on the human apoA-II promoter

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ABSTRACT

DNase I footprinting of the apoA-II promoter using sterol regulatory element binding protein-2 [(SREBP-2 (1–458)] expressed in bacteria identified four protected regions, designated AIIAB (–64 to –48), AIICD (–178 to –154), AIIDE (–352 to –332) and AIIK (–760 to –743), which bind SREBP-2 and contain either palindromic or direct repeat motifs. Potassium permanganate and dimethyl sulfate interference experiments using the AIIAB region as probe showed that the nucleotides of a decameric palindromic repeat RTCAMVTGMY and two 5′ T residues participate in DNA–protein interactions. SREBP-2 transactivated the intact (–911/+29) apoA-II promoter 1.7-fold and truncated apoA-II promoter segments which contain one, two or three SREBP-2 sites 11- to 17-fold in HepG2 cells. Transactivation of a promoter construct containing the binding site AIIAB and the apoA-II enhancer, which includes the binding site AIIK, was abolished by mutations in element AIIAB. An SREBP-2 mutant defective in DNA binding caused a dose-dependent repression of the apoA-II promoter activity. Repression was also caused by an SREBP-2 mutant which lacks the N-terminal activation domain (residues 1–93) but binds normally to its cognate sites. In contrast, a double SREBP-2 mutant which lacks both the DNA binding and the activation domains has no effect on the apoA-II promoter activity. Overall, the findings suggest that SREBP-2 can transactivate the apoA-II promoter by binding to multiple sites. Furthermore, the repression caused by the DNA binding deficient mutants results from squelching of positive activator(s) which appear to recognize the activation domain of SREBP-2.

INTRODUCTION

Sterol regulatory element binding protein-2 (SREBP-2) is a member of the membrane bound basic helix–loop–helix transcription factors that control cellular cholesterol homeostasis (1,2). Other major members of the family are SREBP-1a and SREBP-1c isoforms. SREBP-2 has 47% overall homology and 71% homology with bHLHZip motif of SREBP-1a (1). All members of the family have a highly acidic N-terminal activation domain which is recognized by the transcriptional coactivator CREB-binding protein (CBP) (3). Upon cholesterol depletion, SREBP-2 family members are cleaved by two proteases at the lumen and the cytoplasmic site of the ER, respectively, to generate the N-terminal fragment (4–8). The SREBP-2 cleavage activating protein (SCAP) cleaves SREBP-2 between Leu 522 and Ser 523 (4). The processed N-terminal fragment of SREBP-2 containing the bHLHZip motif and the activation domain translocates to the nucleus and induces transcription of several genes involved in cholesterol biosynthesis and transport (9–12). The importance of SREBP-2 in cholesterol homeostasis has been further demonstrated in CHO cells which contain a 460 amino acid long truncated SREBP-2 form that activates constitutively the LDL receptor and HMG-CoA synthase genes and confers resistance to 25-hydroxy cholesterol (13).

In the current study we demonstrate that SREBP-2 can transactivate the human apoA-II promoter in HepG2 cells by binding to four distinct sites. The activity of this promoter is controlled by 14 regulatory elements designated AIIA to AIIN. Important roles for the activity of this promoter in HepG2 cells play the proximal element AIIAB and the distal enhancer region which contains elements AIII to AIIN (14). The regulatory elements AIIAB, AIIK and AIIL are recognized by the transcription factor USF (15), and element AIIJ by nuclear hormone receptors (16–18). These factors play an important role in apoA-II gene transcription (14,16–19). Two of the sites that are important for the SREBP-2 mediated transactivation contain palindromic RTCAMVTGMY (R = A or G, M = A or C, Y = C or T) repeats and overlap with the binding sites of CIIIB1/USF (15,19). In addition, utilization of SREBP-2 mutants defective in DNA binding and/or transcriptional activation provided new insights on the mode of binding and the mechanism of activation of the apoA-II promoter by SREBP-2.

MATERIALS AND METHODS

Materials

Reagents were purchased from the following sources: the Klenow fragment of the DNA polymerase I restriction enzymes, T_4 ligase,

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Table 1. Oligonucleotides used in PCR amplification and mutagenesis

5-rev-26	TCACACAGGAAACAGCTATGACCATG	pUCSHCAT vector (sense)
3-CAT-26	CTCCATTTTAGCTTCCTTAGCTCCTG	CAT gene (antisense)
AII3APaI	TTTTGGGCCCTGAACATACCCTACCCCCAGTAAAA	-640/-616(antisense) contains an Apa site
AIIAB Apa	TAAAGGGCCCAGTCCTGTCAACCTGACAGGGGGTGGGTAAACAGA	-67/-34 (sense)
AIIABM7	TAAAGGGCCCAGTCCTTCACAACTCAAGGGGGTGGGTAAACAGA	-67/-34 (sense)
AIIK3	AGGACCCGTGTTCTTGTCTCACCTTATCAGTTCTAG	-770/-735 (antisense)
AIIK4	GAGACAAGAACACGGGTCCTTCAACCTTTACCCTG	-754/-720 (sense)
HLHmut1 F	GGG CTG ACA ACC CTT CCT ATC ATT GAG AAA CGA TAT CGC TCC	nt 1108/1149 (sense)
HLHmut2 R	GAT AGG AAG GGT TGT CAG CCC TTC TCC TTC TTT GGG GGG CT	nt 1363/1383(antisense)
HLHmut3 F	GCG GCA CGA TAT GCC TCC TCC ATC AAT GAC AAA ATC	nt 1132/1167 (sense)
HLHmut4 R	GGC ATA TCG TGC CGC AAT GAT AGG AAG GGT TGT CCG	nt 1111/1146 (antisense)
SREBP2-3F	GTAT AAGCTT ATG GAC TAC AAG GAC GAC GAT GAC AAG GAC GAC AGC GGC GAG CTG	nt 121/138 (sense)
SREBP2-3EF	GTAT GAATTC ATG GAC TAC AAG GAC GAC GAT GAC AAG GAC GAC AGC GGC GAG CTG	nt 121/138 (sense)
SREBP-2-4R	CATA GAA TTC CAA TAG AGG GCT TCC TGG CTC	nt 1471/1491 (antisense)
SREBP2-13F	GTAT GAATTC ATGGAC TACAAG GACGAC GATGACAAG CAGGTC ACA TTA CCT TCC TTC	nt 397/417 (sense)
SREBP2-246	AAA AGG ATC CCA TAT GCA GAT CAT CAA GAC AGA TTC C	nt 853/873 (sense)
SREBP2-1F	AAA CTC GAG CAT ATG GAC GAC AGC GGG GAGCT	nt 118/127 (sense)
SREBP2-1R	TGG GAA ACT CGA GCA ATA GAG GGC TTC CTG GCTC	nt 1491/1471 (antisense)
USF1-5	CAT TAC TCG AGC ATA TGA AGG GGC AGC AGA AAA CAG CTG AA	nt 121/147 (sense)
USF-1	ATA CAC TCG AGG AAT TCT TAG TTG CTG TCA TTC TTG ATG AC	nt 1090/1113 (antisense)

±, Oligonucleotide position relative to the transcription initiation site.
nt, Nucleotide number of the published SREBP-2 cDNA sequence (1).

T_4 polynucleotide kinase and Vent polymerase from New England Biolabs (Beverly, MA); [γ-^{32}P]ATP (5000 Ci/mmol), [α-^{32}P]dCTP and [α-^{32}P]dGTP (3000 Ci/mmol) were from New England Nuclear (Boston, MA). DNaseI was from Worthington (Freehold, NJ). Anti-Flag monoclonal antibody from (Kodak, IBI; New Haven, CT). The secondary antibody from Santa Cruz (Santa Cruz, CA). *O*-nitrophenyl-1-D-galacto-β-pyranoside (ONPG) from Sigma (St Louis, MO). The CAT ELISA kit was from Boehringer Mannheim (Indianapolis, IN). Sequenase 2.0 and the ECL system from Amersham (Arlington Heights, IL). The Sequenase sequencing kit was purchased from United States Biochemicals (Cleveland, OH). Bactotryptone and bacto yeast extracts from Difco (Detroit, MI). Double-stranded poly(dI–dC) from Pharmacia LKB Biotechnology, Inc (Piscataway, NJ). Acrylamide, sodium dodecyl sulfate (SDS), urea and Tris from International Biotechnologies, Inc (Rochester, NY). Bacterial XL-1 Blue cells from Stratagene (La Jolla, CA). Full-length cDNA of SREBP-2 from ATCC (Rockville, MD). Oligonucleotides were synthesized and purified as described previously (20). Reagents for automated DNA synthesis from Applied Biosystems, Inc (Foster City, CA).

The expression vector containing human USF-1 cDNA under the control of the CMV promoter was a generous gift of Dr Axel Kahn (21).

Plasmid constructions

ApoA-II promoter mutants. To remove the middle elements from the apoA-II promoter the –911 to –616 and –67 to +29 regions were amplified separately by PCR using the –911/+29 apoA-II pUCSHCAT plasmid as a template (14). The region of the apoA-II enhancer (–911 to –616) was amplified with the 5-rev-26 and AII3ApaI primers (Table 1). The proximal region of the apoA-II promoter was amplified with the primers AIIAB Apa and 3-CAT-26 (Table 1). The primers AII3ApaI and AIIAB Apa contain an *Apa*I site. The PCR products were digested with *Xba*I and *Apa*I (–911 to –616) and with *Apa*I and *Xho*I (–67 to +29). The parental pUCSHCAT vector (20) was also digested with *Xba*I and *Xho*I. The two PCR fragments and the pUCSHCAT vector were ligated in a triple ligation to produce apoA-II promoter with deletion of the region –616 to –67 (AIIAB wild-type AIIK wild-type). The mutation of the AIIK element was produced by amplification of the apoA-II enhancer with two sets of overlapping primers (5-rev-26 and AIIK3) and (AIIK4 and AII3*Apa*I). An aliquot containing 1% of each of the PCR products was mixed and amplified with the external 5-rev-26 and AII3*Apa*I primers. The mutant enhancer was digested with *Xba*I and *Apa*I. The AIIAB element was mutated by amplification of the wild-type apoA-II promoter with the primers AIIABM7 and 3-CAT-26. The PCR

product was digested with *Apa*I and *Xho*I. The mutant apoA-II enhancer was ligated to the wild-type proximal (–67 to +29) region and the *Xba*I and *Xho*I digested pUCSHCAT vector to produce the AIIAB wild-type AIIK mut construct. Alternatively the mutant apoA-II enhancer was ligated to the mutated –67 to +29 region and the *Xba*I and *Xho*I digested pUCSHCAT vector to produce the AIIAB mut AIIK mut construct. Finally, the wild-type apoA-II enhancer was ligated to the mutated –67 to +29 region and the *Xba*I and *Xho*I digested pUCSHCAT vector to produce the AIIAB mut AIIK wild-type construct. The presence of the mutations in the elements AIIAB and/or AIIK in the final constructs were verified by DNA sequencing.

Wild type and mutant SREBP-2 expression plasmids. A plasmid pEXLOX(+) SREBP-2 containing the SREBP-2 cDNA was purchased from ATCC. The flagged version of SREBP-2 was constructed by PCR amplification using the SREBP-2 plasmid as template and SREBP2-3F and SREBP2-4R as primers (Table 1). Primer SREBP2-3F contained a *Hin*dIII restriction site followed by oligonucleotides coding for Met-1 followed by the flag sequence DTKDDDDK and residues two to seven of SREBP-2. Primer SREBP2-4R contained an *Eco*RI restriction site followed by nucleotides corresponding to the antisense sequence of amino acids 458–452 of SREBP-2 (Table 1). To construct the flagged versions of SREBP-2 (1–458) containing mutation in the DNA binding domain, two regions of SREBP-2 cDNA (nt 118–1128 and nt 1149–1108) were amplified separately with two sets of primers (SREBP2-3F and HLHmut2R) and (SREBP2-4R and HLHmut1F). The primers HLHmut1F and HLHmut-2R contained nucleotide changes which substituted Gly331 for Arg, Leu332 for Arg, Leu335 for His and Pro336 for Asn. An aliquot containing 1% of the two amplification products was mixed together with and amplified with the outside primers (SREBP2-3EF and SREBP2-4R) (Table 1) to produce the final product. The mutated sequence thus obtained was amplified and mutagenized using similarly two separate reactions. In these reactions, SREBP2-3EF containing an *Eco*RI site and SREBP2-4R were utilized as external primer and (HLHmut3F and HLHmut4R) as mutagenic primers. These primers contain nucleotide changes with substituted Ala339 for Glu, Ala340 for Lys and Ala343 for Arg. An aliquot containing 1% of the two amplification products was mixed and amplified with the external primers (SREBP2-3EF and SREBP2-4R) to produce the final product. The amplified fragment was digested with *Eco*RI and cloned in the correct orientation into the *Eco*RI site of plasmid pcDNAI. The SREBP-2 deletion mutant lacking the activation domain was constructed using the same strategy utilized to construct the wild-type flagged SREBP-2 but it employed a different 5′ external primer (SREBP2-13F) (Table 1). This primer contains an *Eco*RI site followed by nucleotides encoding the flag sequence and residues 94–100 of SREBP-2. The wild type SREBP-2 cDNA was used as a template to generate the mutant lacking the activation domain [SREBP-2Δ(1–93)]. The mutant SREBP-2 cDNA containing substitution in the DNA binding domain was used as template to obtain the double mutant in the DNA binding and the activation domain [SREBP-2DBPmutΔ(1–93)].

Expression of the SREBP-2 and USF-1 proteins in BL21(DE3) cells

The region 118–1492 nt of the SREBP-2 cDNA, that encodes a protein of 458 amino acids was amplified using nucleotides SREBP2-1F and SREBP2-1R as primers (Table 1), which contain *Nde*I and *Xho*I restriction sites, respectively. The amplified fragment was digested with *Nde*I and *Xho*I and cloned in the corresponding sites of the bacterial expression vector pAED4 (pET3a derivative) to generate the pAED4 SREBP-2 (1–458) plasmid. These plasmids were used to transform *Escherichia coli* BL21(DE3). To generate the SREBP-2 (1–401) expression derivative the pAED4 SREBP-2 (1–458) plasmid was digested with *Hin*dIII and re-ligated. To generate SREBP-2 (246–458) the original pEXLOX+SREBP-2 plasmid was amplified with primers SREBP2-246 and SREBP2-1R (Table 1) by PCR. The amplified fragment was then digested by *Nde*I and *Xho*I and cloned in the corresponding sites of the pAED4 vector to generate pAED4 SREBP-2 (246–458). To generate the USF-1 bacterial expression plasmid, the USF-1 cDNA was amplified with oligonucleotides USF1–5 and USF1–3 (Table 1). The 5′ sense oligonucleotide USF1–5 contains *Xho*I and *Nde*I restriction sites followed by oligonucleotides coding for the first 10 amino acids of USF-1. The 3′ antisense oligonucleotide USF1–4 (Table 1) contains the restriction sites *Xho*I and *Eco*RI followed by the antisense sequence of the stop codon and the codons specifying the last eight amino acids of USF-1. The amplified DNA sequence was digested with *Nde*I and *Xho*I and cloned into the corresponding sites of the pAED4 bacterial expression vector. An overnight culture of bacteria was diluted 80-fold in 4 ml of LB medium containing 100 μg/ml ampicillin and incubated for ~1.5 h. Isopropyl-β-D-thio-galactopyranoside (IPTG) was then added to final concentration 1 mM and the culture was maintained for an additional 3 h. The bacteria were spun for 5 min at 5000 r.p.m. (2000 *g*) and the pellet was dissolved in 0.5 ml denaturation buffer (20 mM HEPES pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 8% glycerol, 1 mM DTT, 6 M GnHCl, pH 7.6) supplemented with protease inhibitors [1 mM aprotinin, 1 mM benzamidine, 1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The bacterial lysates were rotated on a platform for 30 min at 4°C, centrifuged for 5 min in a microfuge and the supernatant was dialyzed twice for 1 h against an excess of the denaturation buffer without GnHCl and containing 1 mM PMSF. To form SREBP-2, SREBP-1 heterodimer cell lysates prepared from different cell clones expressing these factors were mixed prior to denaturation. After dialysis, the extracts were centrifuged for 5 min at 4°C in a microfuge (12 000 *g*) to remove precipitated material, and the supernatant containing SREBP-2 was stored at –80°C.

DNaseI footprinting assays

The –911/+29 apoA-II pUCSHCAT constructs (16) were amplified with ^{32}P-labeled 5-rev-26 and unlabeled 3-CAT-26 primer (Table 1). This PCR product was used to footprint the distal (AIIK) SREBP-2 binding site. To footprint the proximal (AIIAB and AIICD) sites the –230/+29 apoA-II pUCSHCAT (16) was digested with *Xho*I, end labeled with T_4 polynucleotide kinase in the polylinker region proximal to residue –911 and then digested with *Sal*I. To footprint the AIIDE site, the –440/+29 apoA-II pUCSHCAT (14) was digested with *Xho*I, labeled with T4 polynucleotide kinase, and digested with *Sal*I. All the labeled promoter fragments were separated by 5% PAGE and purified by electroelution. Footprinting was performed with 4 μl extracts of bacteria expressing SREBP-2 prepared as described above. The positions of the binding sites of SREBP-2 in the apoA-II

promoters were determined by comparing the SREBP-2 footprints with the G+A ladder produced by chemical cleavage of the same DNA fragment.

Gel electrophoretic mobility shift assays

This analysis was performed as described (20). Excess of unlabeled competitor oligonucleotide was added into 20 µl reaction mixture which contained 4 µl of extracts of SREBP-2 in 1× binding buffer [3 µg poly(dI–dC) in 10 mM HEPES pH 7.9, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA and 5% glycerol] and preincubated for 15 min at 4°C. Labeled, double-stranded oligonucleotide (0.5 ng; specific activity 5×10^8 c.p.m./µg) was then added to the preincubation mixture and the incubation continued for 30 min at 4°C. The final probe concentration in all reactions was 2.5 µM (50 000 c.p.m.). Free DNA and DNA complexes were separated on a 5% native polyacrylamide gel in 1× TAE buffer (6.7 mM Tris–HCl pH 7.9, 3.3 mM sodium acetate and 1 mM EDTA). The gel was dried and exposed to X-ray film.

Transient transfection and CAT assays

Human hepatoma HepG2 cells and monkey kidney COS-1 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. HepG2 cells were plated in 30 mm dishes at density 5×10^5/plate in DMEM supplemented with 10% fetal bovine serum. They were transfected by the calcium phosphate coprecipitation method (22). Forty-eight hours post-transfection, the cells were washed three times with cold PBS and lysed with a lysis buffer (Boehringer Mannheim) for 30 min at room temperature. The lysates were collected and used for CAT and β-gal assays. All experiments were done in duplicate and repeated at least three times. The relative amounts of the CAT enzyme in the cells was determined by sandwich ELISA assays using the CAT ELISA kit. The cell lysate was added to 96-well plates coated with anti-CAT antibody for 1 h and incubated at 37°C (23). After five washes with the wash solution, anti-CAT antibody labeled with digoxigenin was added to the wells and the mixture was incubated for an additional 1 h. The excess antibody was then removed by five washes and the secondary anti-digoxigenin Fab fragment conjugated with horseradish peroxidase (HRP) was added for 1 h. After the incubation, the samples were washed five times and the 2,2′-azino-di-3-ethylbenzthiazolinesulfonate-(6)-diammonium salt (ABTS) substrate was added to the wells. The absorbance of the solution was then measured at 410 nm (23). The β-galactosidase activity of the extracts was used to normalize for the efficiency of transfection (24). Three to six independent transfections in duplicate were performed for each of the promoter constructs.

SDS–PAGE and western blotting

Protein extracts were analyzed on standard (10% resolving, 4% stacking) mini SDS–polyacrylamide gels (Bio-Rad) in Tris–glycine buffer (0.025 mM Tris, 0.2 M glycine, 0.1% SDS). Following electrophoresis, the proteins were either stained with Coomassie brilliant blue for direct visualization or transferred to nitrocellulose filters for 1 h at 100 V in transfer buffer (0.025 mM Tris, 0.2 M glycine, 20% EtOH). The bound proteins, carrying the Flag epitope at the N-terminuss (DTKDDDDK), were detected using monoclonal anti-Flag M2 antibody (Kodak, IBI). Briefly, the membranes were blocked with 5% non-fat dried milk in 50 mM Tris–HCl pH 7.4, 150 mM NaCl for 30 min at room temperature. The primary antibody was then added to final concentration 10 µg/ml for 30 min. After the incubation, the membranes were washed briefly twice with the same solution without milk. The secondary antibody conjugated with HRP (Santa Cruz) was then added in dilution 1:2000 in blocking solution and the membranes were incubated for 30 min. The membranes were washed three times in the same solution without milk for 5 min each time and the proteins were detected with the enhanced chemiluminescence (ECL) system (Amersham).

Dimethyl sulfate (DMS) and potassium permanganate ($KMnO_4$) interference assays

For the methylation interference assay, single-stranded oligonucleotides corresponding to the coding and non-coding strands of element AIIAB (Table 2) (5 pmol) were end-labeled with T_4 polynucleotide kinase and annealed to their complementary unlabeled strands. Double-stranded DNA (10^7 c.p.m.) was treated with DMS for 3 min at room temperature in the presence of 2 µg of salmon sperm DNA (20). For permanganate interference assay, single-stranded DNA (10^7 c.p.m.) was treated with 2.5 mM $KMnO_4$ for 10 min at room temperature in the presence of 4 µg of salmon sperm DNA followed by annealing to its complementary strand (25). The treated probes were incubated with bacterial extracts expressing SREBP-2 (1–458) and the complexes were resolved by a preparative mobility shift gel analysis. Following electrophoresis the protein–DNA complexes and the free probe were excised from the gel, electroeluted and treated with 1 M piperidine for 30 min at 90°C. The samples were then dried, the dry pellets were counted and dissolved in 98% formamide dye. Equal counts from all the samples were fractionated by electrophoresis on sequencing 6% urea–polyacrylamide gels and the bands were visualized by autoradiography.

RESULTS

Identification of SREBP-2 binding sites of the human apoA-II promoter

Initial binding of SREBP-2 on the element AIIAB of the apoA-II promoter was determined by screening of a human liver cDNA library (Clontech; Palo Alto, CA) for transcription factors that recognize various regulatory elements of the human apolipoprotein promoters. This analysis resulted in the isolation of one clone that corresponded to the nucleotide sequence 669–1761 of SREBP-2 based on the GenBank sequence information (1).

To assess the presence of other binding sites of SREBP-2 on the human apoA-II promoter, we expressed a 1.5 kb segment cDNA encoding for the first 458 amino acids of SREBP-2 in *E.coli* BL21(DE3) strain. DNaseI footprinting analysis using extracts of bacteria expressing SREBP-2 (1–458) identified four protected regions. Two of these regions, AIIAB (–64 to –48) and AIIK (–760 to –743), were previously identified as binding sites for rat liver nuclear proteins (14). The other two sites designated AIICD (–178 to –154), AIIDE (–352 to –332) are unique binding sites for SREBP-2 and are located between the previously defined footprints AIIC and AIID, and AIID and AIIE, respectively (Fig. 1A–D).

The binding specificity of SREBP-2 to these sites was characterized by gel electrophoretic mobility shift assays using SREBP-2 (1–458) expressed in bacteria. For this purpose, double-stranded oligonucleotides corresponding to the protected

Table 2. Oligonucleotides used in DNA binding competition DMS and $KMnO_4$ interference experiments

Oligonucleotide	Oligonucleotide Sequence	Ref.
AIIAB[a]	$^{-67}$AGTCCTGTCACCTGACAGGGGGTGGGTAAACAGACA^{-32}	20
AIIABmut[b]	$^{-67}$AGTCCTTCACAACTCAAGGGGGTGGGTAAACAGA^{-34}	20
CIIIB[b]	$^{-92}$GGTCAGCAGGTGACCTTTGCCCAGCG^{-67}	20
AIICD[b]	$^{-180}$ACCCCCTGCCTCTCACCCCATCACCATGAG^{-151}	20
AIIDE[b]	$^{-358}$GAGCCTTCCATGGTATGATGGGTTGAT^{-332}	20
AIIK[b]	$^{-766}$AACTGATAAGGTGATCAAATGACCAGGTGCCTT^{-734}	20
AIIKmut[b]	$^{-766}$AACTGATAAGGTGAGACAAGAACACGGGTCCTT^{-734}	20
SRE-1[b]	$^{-68}$AAAATCACCCCACTGCAA^{-51}	33
CIII-G	$^{-669}$TCG ACC TTG GCTT CTC CAC CAA CCCC^{-648}	20

The nucleotides mutated in elements AIIABmut and AIIKmut are underlined.
[a]Sense strand of oligos used in DMS and $KMnO_4$ assays.
[b]Sense strand of oligos used in DNA binding and competition assays.

regions defined by DNaseI footprinting were synthesized and used as probes in gel electrophoretic mobility shift assays (Table 2). Extracts from bacteria transformed with the empty expression vector were used as a negative control. This analysis showed that SREBP-2 (1–458) can bind to all four footprinted regions of the apoA-II promoter, AIIAB, AIICD, AIIDE, AIIK as well as to elements CIIIB of the apoCIII gene and SRE-1 of the LDL receptor (Table 2). Binding is competed out by excess of unlabelled oligonucleotide but not by an unrelated oligonucleotide, indicating that it is specific. No binding was observed in lanes containing bacterial extract from the cells transformed with the empty vector pAED4 (Fig. 2A).

Additional DNA binding experiments were performed that were designed to test whether a SREBP-2 can heterodimerize with another bHLH transcription factor USF (26–28) which binds to identical sites on element AIIAB and AIIK (15,19). These experiments showed that SREBP-2 or SREBP-1 forms a single DNA–protein complex and USF-1, one major complex and several faster migrating complexes which probably represent degradation products of USF-1. Mixing of USF-1 and SREBP-2 did not produce any new band migrating between USF-1 and SREBP-2 (Fig. 2B) indicating that these two factors do not heterodimerize. Control experiments using SREBP-2 (1–458) and an N-terminal truncated form SREBP-2 (246–458) showed that each SREBP-2 form produced a single DNA–protein complex. However, when the two truncated SREBP forms were used together in DNA binding experiments, they generated the two initial DNA–protein complexes and a new complex with intermediate electrophoretic mobility of those corresponding to SREBP-2 (1–458) and SREBP-2 (246–458). This new complex represents a heterodimer of the long and short SREBP form (Fig. 2B). To observe formation of heterodimers it was necessary to dissolve both bacterial extracts in 6 M GnHCl and slowly dialyze out the GnHCl to allow dimerization. On the other hand, mixing SREBP-1 and SREBP–2 that were denatured with 6 M GnHCl and renatured separately by dialysis prevented formation of the heterodimers. The findings suggest that the dissociation constant of heterodimers formed in solution is small and, once formed, the dimers are stable.

The relative affinities of the four SREBP-2 binding sites on the apoA-II promoter were assessed by competition assays using the SRE-1 element from the LDL promoter as a probe (29) (Table 2). Competition experiments were performed using as competitors unlabeled oligonucleotides containing SREBP-2 binding sites from the apoA-II as well as apoCIII promoters, and the original SRE-1 of the LDL receptor promoters and as probe, the ^{32}P-labeled SRE-1. Element CIIIB of the human apoCIII promoter was utilized in these experiments since it is highly homologous to element AIIAB of the human apoA-II promoter (Table 2). The concentrations of the competitor oligonucleotides used were 10- to 200-fold molar excess over the probe. Figure 2C shows that the ability of the different oligonucleotides to compete for binding of SREBP-2 to SRE-1 follows the order SRE-1 > AIIAB > AIICD ≈ AIIDE ≈ AIIK > CIIIB.

Comparison of the SREBP-2 binding sites found in the apoA-II promoter to previously reported SREBP binding sites

Table 3A and B shows the alignment of SREBP-2 binding sites found in the apoA-II promoter identified by DNaseI footprinting to previously reported consensus SREBP binding sites as well as to the original SRE-1 found on the promoter of the LDL receptor and other genes (10,29–35).

As indicated in this table the SREBP-2 binding sites on the apoA-II promoter consist of two types of repeats. Elements AIICD and AIIDE contain direct repeats that are 80% homologous to the ATCACCCCAC decanucleotide repeat found in the LDL receptor promoter (29) that is recognized by SREBP-2 and SREBP-1 (Table 3A). On the other hand, elements AIIAB and AIIK contain a palindromic decameric repeat (Table 3A). A similar repeat is found on the regulatory element CIIIB of the apoCIII promoter that, as shown below, weakly binds SREBP-2. Included in this

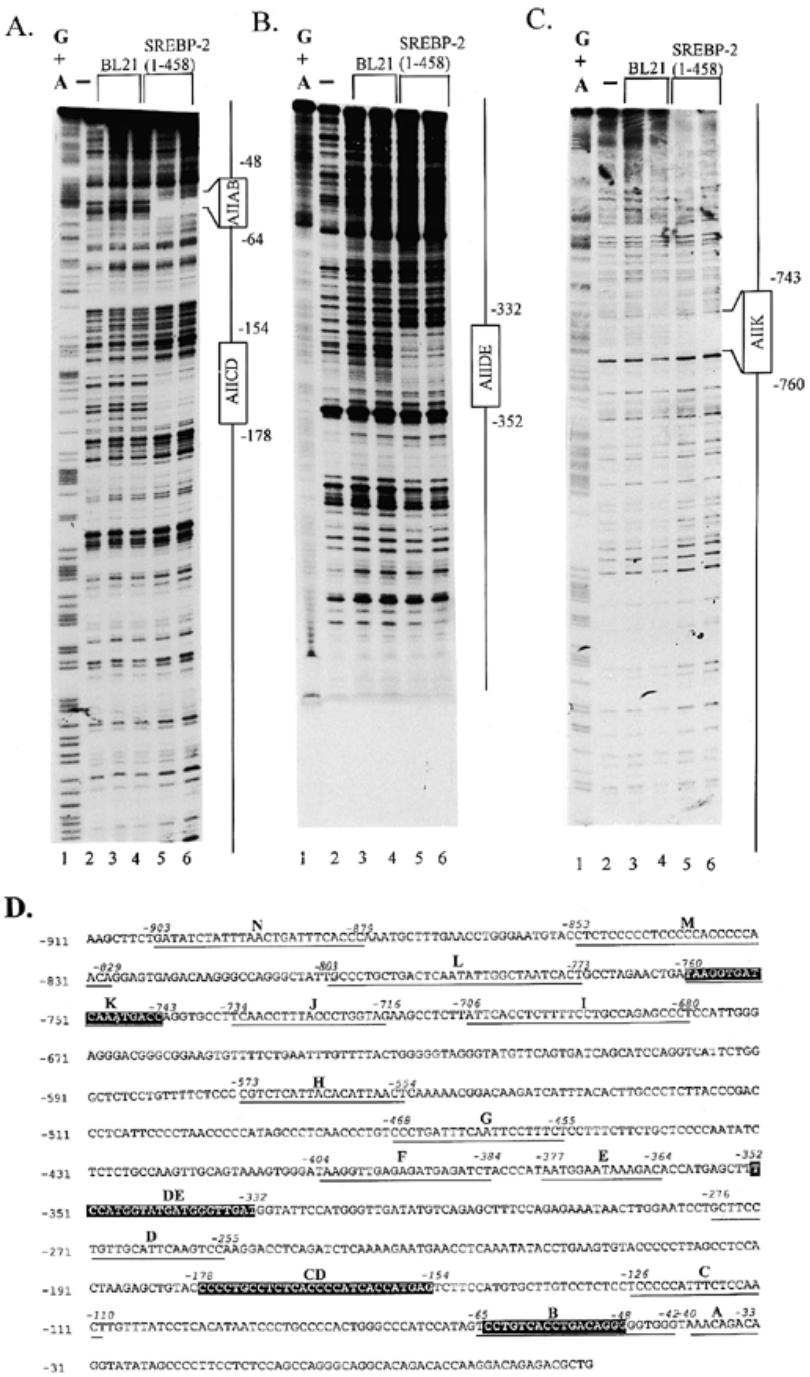


Figure 1. Definition of the binding sites of SREBP-2 on the entire human apoA-II promoter by DNase I footprinting. The DNA fragments utilized and the site of ^{32}P-labeling were as follows: (**A** and **B**) –230/+29 apoA-II promoter labeled at the pUC polylinker region in the vicinity of residues –230 and –440, respectively; (**C**) –911/+29 apoA-II promoter labeled at the pUC polylinker region in the vicinity of residue –911. (A–C) Left to right: lane 1, G+A reactions; lane 2, no extracts; lanes 3 and 4, extracts of bacteria transformed with the empty pAED4 vector; lanes 5 and 6, 4 µl extracts of bacterial cells expressing SREBP-2 (1–458). The protected regions and their relative position in the apoA-II promoter are indicated by boxes. The preparation of the bacterial extracts and the footprinting analysis was performed as described in Materials and Methods. (**D**) Summary of apoA-II promoter region protected from DNase I digestion in the presence of SREBP-2. Underlined are previously identified footprints in the apoA-II promoter (20).

palindromic decameric repeat is the E-box type CANNTG motif that is recognized by other members of the bHLHZip family of transcription factors. The consensus sequence of the motif palindromic decameric repeat based on these and previous findings is RTCAMVTGMY (Table 3A).

The oligonucleotides of the palindromic repeat which participate in DNA–protein interactions were identified with $KMnO_4$ and DMS interference experiments. This analysis showed that all the nucleotides of the palindromic decameric repeat as well as two 5′ T residues participate in DNA–protein interactions. These

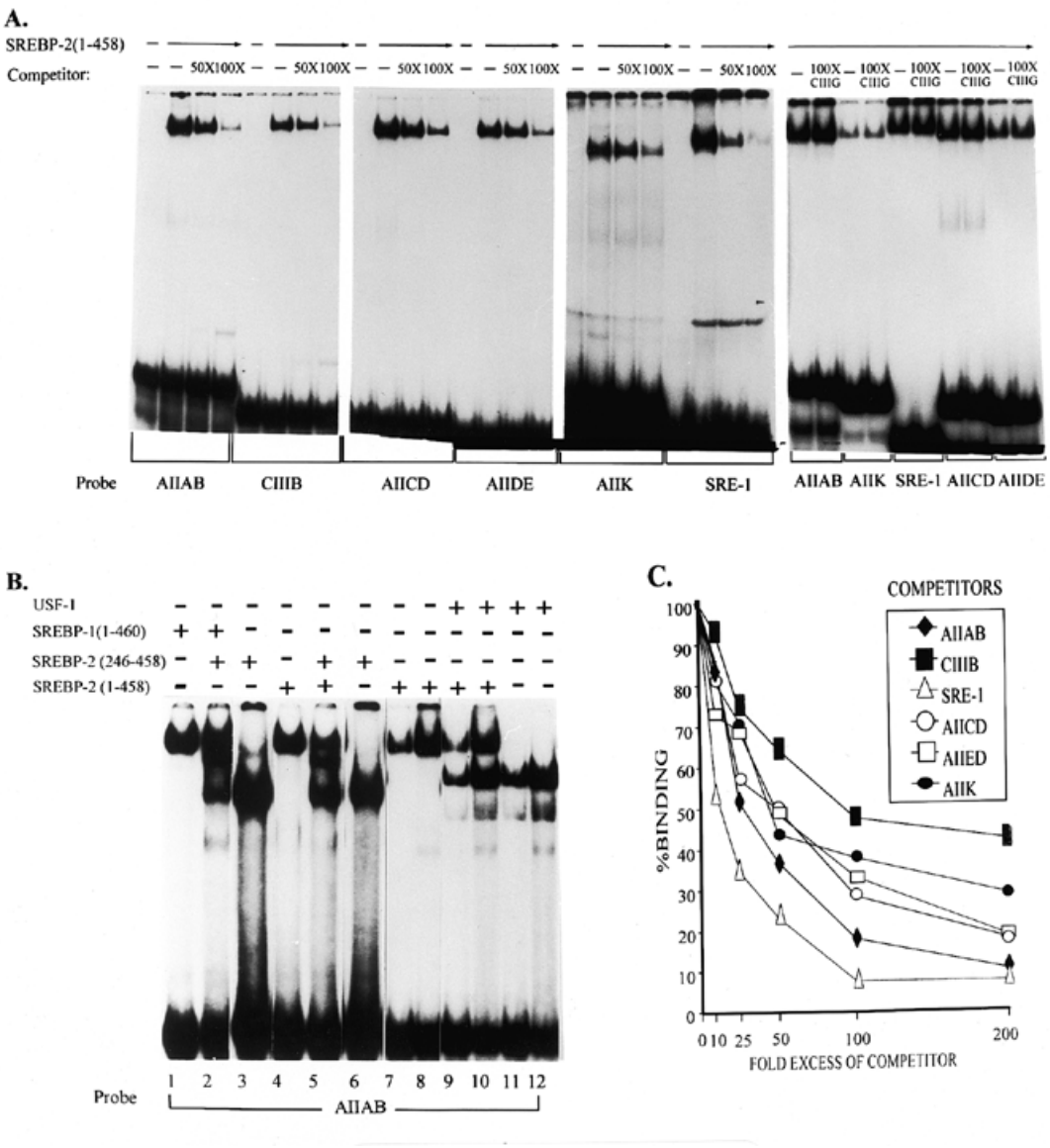
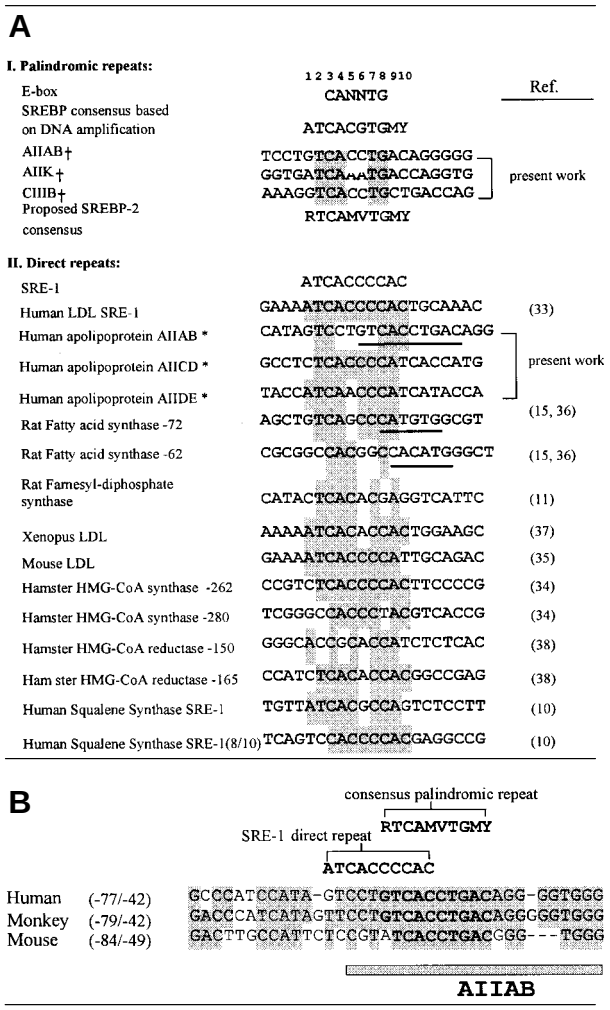


Figure 2. (**A**) Gel electrophoretic mobility shift and competition assays using several SREBP-2 binding sites as probes or competitors. DNA binding and competition assays were performed with SREBP-2 (1–458) expressed in bacteria as explained in Materials and Methods. The competitor oligonucleotides were used at 50 or 100-fold molar excess relative to the ^{32}P-labeled probe. The probes and the competitor used are indicated by abbreviations at the top of the figure and are described in Table 2. (–) indicates control bacterial extract [BL21(DE3)]. Each lane contains 50 fmol probe (50 000 c.p.m.) and 1 µl of bacterial extract prepared as described in Materials and Methods. (**B**) Gel electrophoretic mobility shift assays using different truncated forms of SREBP-1 and SREBP-2 and USF-1 expressed in bacteria and AIIAB as probe as shown on the top of the figure. Lanes 1–6 and 7, 9 and 11 contain 50 fmol probe (50 000 c.p.m.) and 1 µl each of the indicated bacterial extracts. Lanes 8, 10 and 12 contains 4 µg of the indicated extracts that were prepared as described in Materials and Methods. Note that SREBP-1 and SREBP-2 form heterodimers whereas SREBP-2 does not form heterodimers with USF-1. (**C**) DNA binding of SREBP-2 (1–458) to the SRE-1 probe and competition of binding by unlabeled oligonucleotides corresponding to the regulatory elements SRE-1 of the LDL receptor gene, AIIAB, AIICD, AIIDE and AIIK of the apoA-II gene, and CIIIB of the apoCIII gene. DNA binding and competition assays were performed with SREBP-2 (1–458) expressed in bacteria as explained in Materials and Methods. The competitor oligonucleotides were used at 10- to 200-fold molar excess relative to the ^{32}P-labeled SRE-1 probe. The competitors used are indicated by abbreviation in the insert and are described in Table 2. *y*-axis shows the percent of binding compared with the binding without competitor arbitrarily set to 100%. *x*-axis shows the fold excess of competitors used.

oligonucleotides are numbered –4 to +10 (Fig. 3A–C). More specifically, nucleotides –1 and –4 of the coding strand and 4, 6, 9 and 10 of the non-coding strand participate in strong DNA–protein interactions. In addition, nucleotides 1, 2, 7 and 8 of the coding strand, and 3 and 5 of the non-coding strand participate in weak DNA–protein interactions (Fig. 3A–C). Based on the $KMnO_4$ and DMS interference experiments it appears that the SREBP-2 binding site on element AIIAB extends beyond the palindromic RTCAMVTGMY repeat. As shown in Table 3B, the sequence of the AIIAB probe has homology to the direct repeat SREBP binding motif and may allow SREBP-2 to bind to either of the direct or the palindromic repeat motifs.

Table 3. (**A**) Comparison of the binding sites found in the apoA-II and other promoters with previously reported SREB binding sites and (**B**) alignment of the AIIAB element of mammalian species



Underlining in the AIIAB and rat fatty acid synthase direct repeat motifs indicates homology to palindromic decameric and E-box type repeats, respectively.

†The homologies of AIIAB, AIIK and CIIIB to the SREBP consensus palindromic repeat motif are all 80%.

*The similarities of AIIAB, AIICD and AIIDE to SRE-1 are 50, 80 and 80%, respectively.

Binding of SREBP-2 (1–458) to multiple sites results in transactivation of the human apoA-II promoter

To evaluate the effect of SREBP-2 (1–458) on the activity of the human apoA-II promoter, plasmids expressing the N-terminal portion of SREBP-2 cDNA encoding the amino acids 1–458, along with different apoA-II promoter constructs were used in the cotransfection experiments of HepG2 cells. It was shown previously that such N-terminal segments of SREBP-2 (1–460) translocate directly into the nucleus and are transcriptionally active (13,36). This analysis showed that SREBP-2 (1–458) transactivates the human apoA-II promoter 1.7-fold (Fig. 4A). The transactivation reaches a plateau at ~400 ng of SREBP-2 expression plasmid, suggesting either that saturation of the apoA-II promoter occurs, or that another factor may become limiting or that transfection is saturated at 400 ng plasmid. This 1.7-fold transactivation is significant given the very strong activity of the apoA-II promoter in HepG2 cells (16) (Fig. 4A). Transactivation is reduced slightly by further increase in SREBP-2.

Similar cotransfection experiments show that SREBP-2 could transactivate four truncated apoA-II promoter segments extending to nucleotides –80, –230, –440 or –614 were also tested for transactivation by SREBP-2 (Fig. 4B and C). These promoter segments contain either one, two or three SREBP-2 sites that are underlined in Figure 4C. The activity of these truncated promoters is small compared with the full-length –911/+29 promoter but it increased from 11- to 17-fold in the presence of SREBP-2. The greatest increase, 17-fold, is observed for the –230/+29 promoter segment which contains one direct and one palindromic SREBP-2 binding site. This level of activity is comparable with the activity of weaker apolipoprotein promoters such as apoA-I (37). These truncated apoA-II promoter constructs provide a useful set of reporter plasmids to explore further the structure and functions of SREBP family members and their functional interactions with coactivators or corepressors. The combined data of Figures 1–4 demonstrate that binding of SREBP-2 to multiple sites results in the transactivation of the human apoA-II promoter. The transactivation of the full-length –911/+29 apoA-II promoter is the result of complex interactions among SREBP-2 and other factors bound to the proximal promoter and distal enhancer sites.

Previous mutagenesis analysis indicated that elements AIIAB and AIIK, which are SREBP-2 binding sites, are very important for promoter activity in HepG2 cells. Point mutations or deletions of these elements reduced the promoter activity to 10–20% of the control in HepG2 cells (14). To test the importance of these elements in the SREBP-2 mediated transactivation, four new apoA-II promoter constructs were made carrying internal deletion that removed the elements AIIC to AIIH and linked the enhancer region (AIII to AIIN) to the element AIIAB containing either the wild-type sequence or mutations in elements AIIAB, AIIK or both. The same mutations, designed to abolish the binding of SREBP-2, were introduced into elements AIIAB and AIIK and the mutated elements were utilized as probes or competitors in DNA binding assays (Table 2). In agreement with previous findings (16), transient cotransfection assays using these constructs showed that the internal deletion of the region (–616 to –67) does not decrease the apoA-II promoter activity in HepG2 cells, but rather increased significantly (~1.7-fold) the activity of the mutant promoter $P < 0.025$ (Fig. 5A and B). The increase in the promoter activity is consistent with previous findings which indicated that the deleted region may contain weak negative regulatory elements (38,39). The introduction of mutations in the elements AIIAB and AIIK decreased by ~40–50% the activity of the mutant apoA-II promoter constructs. The promoter activity of the constructs carrying mutations in elements AIIAB or AIIK was comparable with that of the wild-type promoter. The extent of transactivation of these constructs was not affected by mutations in element AIIK but it was abolished by mutations in element AIIAB indicating the importance of this element in the SREBP-2 mediated transactivation (Fig. 5A and B). Mutations in both elements AIIAB and AIIK reduced the promoter strength to approximately 20% of its original value and also abolished the SERBP-2 mediated transactivation (Fig. 5A). To assess the effect

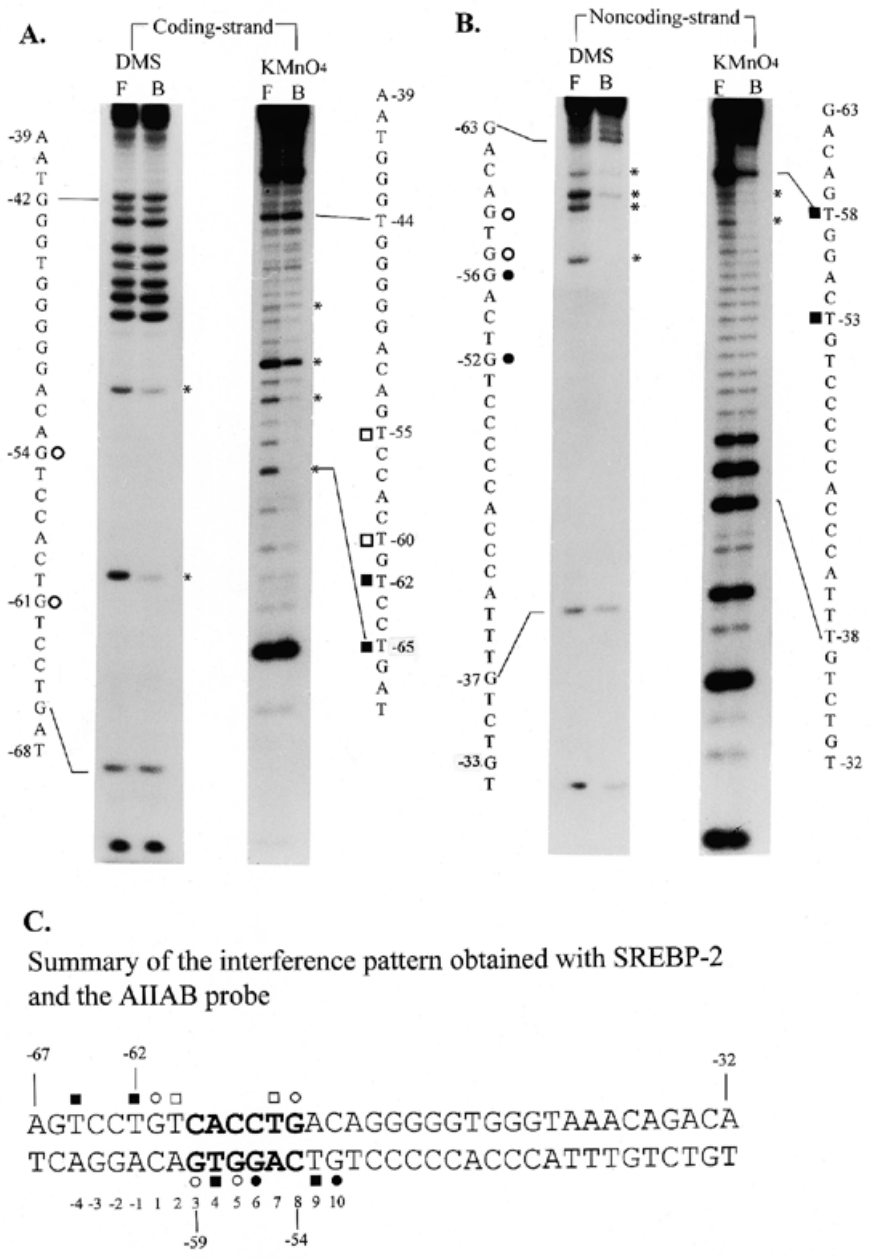


Figure 3. DMS and $KMnO_4$ modification pattern of the DNA–protein complex formed with SREBP-2 expressed in bacteria, using element AIIAB as a probe (Table 2). The DMS and $KMnO_4$ modification pattern of SREBP-2 with the coding and non-coding strand of element AIIAB is shown in (**A**) and (**B**), respectively. The nucleotide sequence of the coding and non-coding strand of element AIIAB is indicated on each side of (A) and (B). Nucleotides which participate in DNA–protein interactions are indicated by circles or squares, and correspond to the DMS and $KMnO_4$ data, respectively. (**C**) Summary of the interference pattern deduced from the findings of (A) and (B). Strong interactions are illustrated by closed circles (●) or squares (■), and weak interactions by open circles (○) and squares (□). Numbers 1–10 are assigned in the previously identified palindromic decameric motif and –1 to –4 in the T residues located 5′ to this motif.

of mutations on the regulatory elements AIIAB and AIIK on the binding of SREBP-2, we performed direct binding and competition experiments using the wild-type or the mutated oligonucleotides as probes as indicated at the bottom of the figure, or competitors as indicated at the top of the figure. The direct binding assays showed that the mutations abolished the binding of SREBP-2 to the mutant oligonucleotides (Fig. 6). The competition experiments showed that the mutated oligonucleotides (AIIABmut and AIIKmut) (Table 2) do not compete for the binding of SREBP-2 to the AIIAB and AIIK oligonucleotides.

SREBP mutants defective in DNA binding or transcriptional activation domains repress the apoA-II promoter activity

To elucidate the mechanism of transactivation of the apoA-II promoter by SREPB-2 we introduced mutations in the SREBP-2 cDNA. Three SREBP-2 cDNA mutants were generated: one defective in the DNA binding domain carrying substitution of Gly331 for Arg, Leu332 for Arg, Leu335 for His, Pro336 for Asn, Ala339 for Gly, Ala340 for Lys, Ala343 for Arg (SREBP-2 DBP

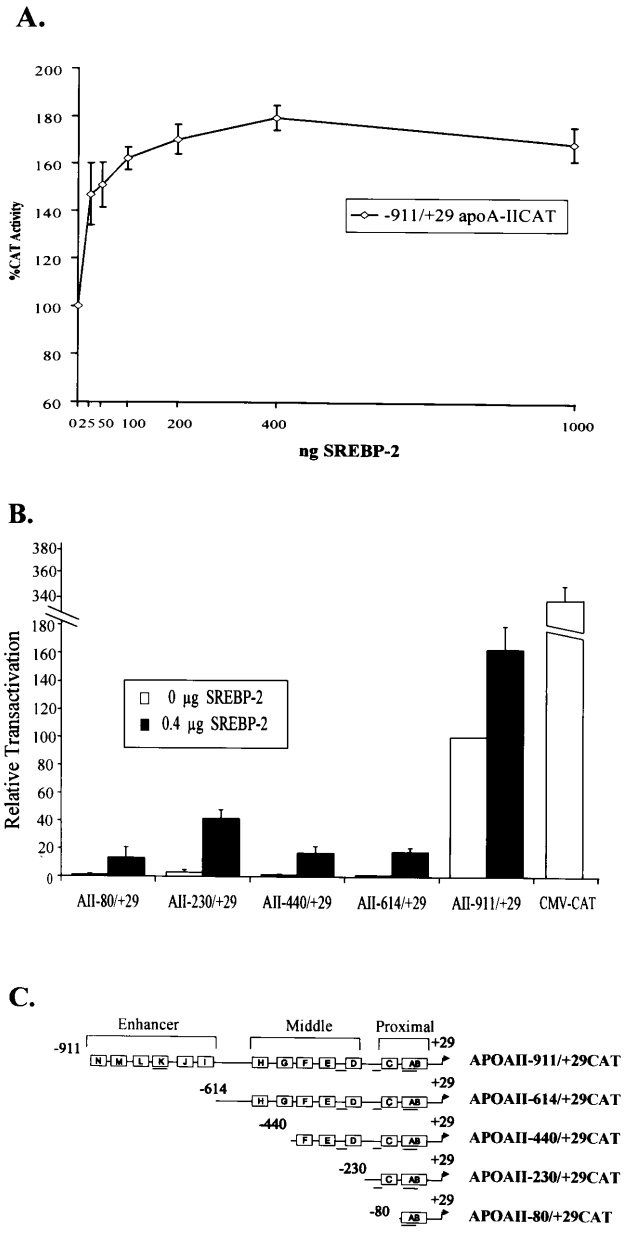


Figure 4. (**A**) Transactivation of the wild-type (–911/+29) apoA-II promoter in HepG2 cells by increasing concentration of SREBP-2 (1–458) expression plasmid. The CAT values at 25 and 50 ng SREBP-2 are statistically lower than those at 100 and 200 ng ($P < 0.05$ to 0.01). Also, the CAT value at 100 ng SREBP-2 is statistically lower ($P < 0.01$) than that at 200 ng SREBP-2. (**B**) Transactivation of the apoA-II promoter segments extending to nucleotides –80, –230, –440 or –614 in HepG2 cells by SREBP-2 expressing plasmids. In (A) and (B), HepG2 cells were cotransfected transiently with 3 µg of the wild-type –911/+29 promoter or one of the truncated apoA-II promoter constructs (AII–80/+29, AII–230/+29, AII–440/+29 and AII–614/+29), 0.4 µg of the pcDNAI.Amp SREBP-2 (1–458) plasmid and 1 µg of the CMV β-gal plasmid. Forty hours following transfections, cells were harvested and the amount of the CAT enzyme was determined as described in Materials and Methods. The mean values (± SD) from six independent transfections performed in duplicates are presented in the form of a bar graph. (**C**) Schematic representation of the promoter constructs used in cotransfection experiments with SREBP-2 promoter plasmids shown in (A). The location of the SREBP binding sites in each construct is underlined.

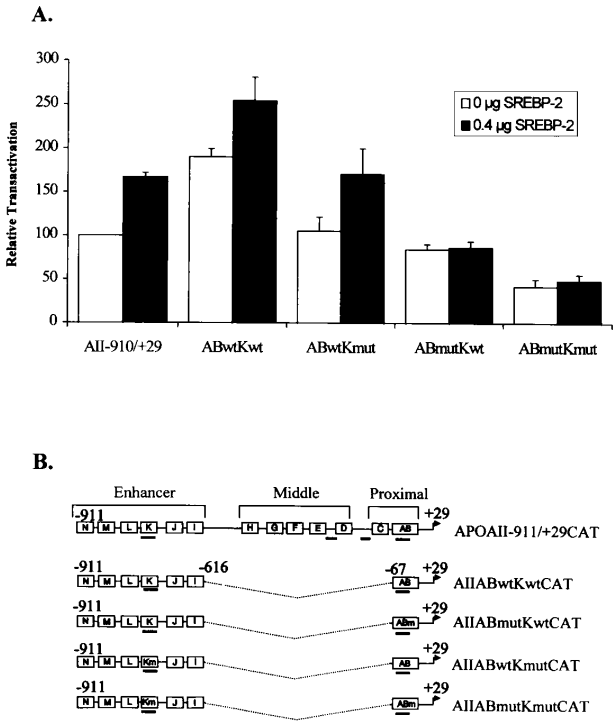


Figure 5. Transactivation of the apoA-II promoter carrying mutations in elements AIIAB and AIIK by SREBP-2 (1–458). HepG2 cells were cotransfected transiently with 3 µg of the apoA-II promoter constructs of (B), 0.4 µg of the pcDNAI.Amp SREBP-2 (1–458) plasmid and 1 µg of the CMV β-gal plasmid. Forty hours following transfections, cells were harvested and the amount of the CAT enzyme was determined as described in Materials and Methods. (**A**) Mean values (± SD) from six independent transfections performed in duplicate, in the form of a bar graph. The abbreviated names of the mutants used are shown on the *x*-axis. (**B**) Schematic representation of the apoA-II promoter constructs containing deletion of the region AIIH to AIIC and having the wild-type or mutated sequences in elements AIIAB and AIIK. The location of the SREBP-2 binding sites in each construct is underlined.

mut); a second carrying a deletion of the N-terminal residues 1–93 that are involved in transcriptional activation [SREBP-2 Δ(1–93)]; and a third carrying both the mutations in the DNA binding domain and the deletion of residues 1–93 [SREBP-2 DBP mut Δ(1–93)]. To monitor the expression of the mutant forms, the flagged version of different SREBP-2 mutants were cloned in mammalian vectors. Immunoblot analysis of COS-1 cells showed that the wild-type and the mutant SREBP-2 forms were expressed efficiently in COS-1 (Fig. 7A and B).

Transient cotransfection assays showed that SREBP-2 mutant carrying several amino acid substitutions in the DNA binding domain caused a dose-dependent decrease in the –911/+29 apoA-II promoter activity in HepG2 cells, reaching 20% of the initial promoter activity at 1000 ng SREBP-2 DBPmut as compared with 1.7-fold transactivation observed with the wild-type SREBP-2 (1–458) (Figs 5A and 7C).

Cotransfection of the SREBP-2 Δ(1–93) mutant similarly caused a dose-dependent repression of the –911/+29 apoA-II promoter activity in HepG2 cells reaching 50% of the initial promoter activity at 1000 ng SREBP-2 Δ(1–93) mutant (Fig. 7C). In contrast, cotransfection using the double mutant with deletion of the N-terminal activation domain and the substitutions in the

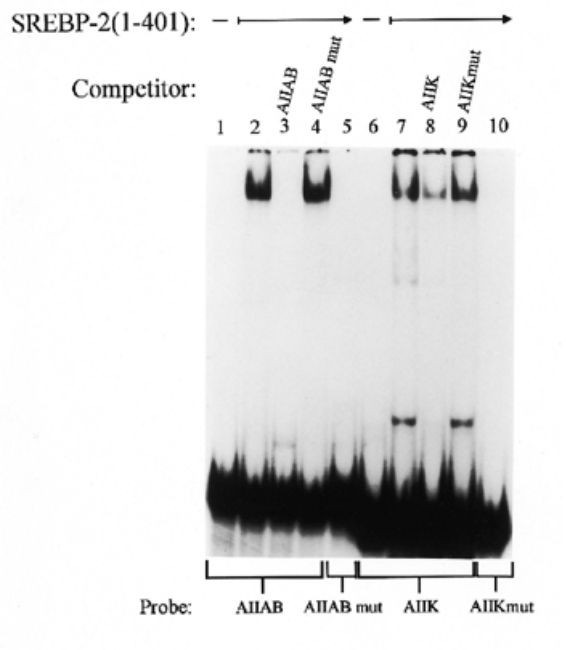


Figure 6. Gel electrophoretic mobility shift and competition assays using wild-type and mutated oligonucleotides AIIAB and AIIK as probes. DNA binding and competition assays were performed with SREBP-2 (1–401) expressed in bacteria as explained in Materials and Methods. Each lane contains 50 fmol probe (50 000 c.p.m.) and 1 µl of bacterial extract prepared as described in Materials and Methods. The competitor oligonucleotides were used at 100-fold molar excess relative to the ^{32}P-labeled probe. They are indicated by abbreviations at the top of the figure and are described in Table 2. The figure establishes that mutations in elements AIIAB and AIIK abolished the binding of SREBP-2 to these sites.

DNA binding domain [SREBP-2 DBP mut Δ(1–93)] did not affect the apoA-II promoter activity (Fig. 7C).

The transactivation of the wild-type apoA-II promoter by SREBP-2 (1–458) correlated with the ability of the SREBP-2 mutant proteins expressed in bacteria to bind to the elements AIIAB, AIIK, AIICD, AIIDE and SRE-1 in gel electrophoretic mobility shift assays. Similar amounts of the SREBP-2 mutant proteins were used in these assays (as assessed by immunoblotting of the bacterial extracts) (Fig. 7A and B). This analysis showed that substitution of several residues in the DNA binding of SREBP-2 (SREBP-2 DBPmut) abolished the binding of the mutant protein to the regulatory elements AIIAB, AIIK, AIICD and SRE-1 (Fig. 8A and B). Nevertheless it could efficiently repress the apoA-II promoter activity (Fig. 7C).

SREBP-2 mutant carrying a deletion at the N-terminal residues 1–93 SREBP-2 Δ(1–93) could bind efficiently to its cognate sites on element AIIAB, AIIK, AIICD, AIIDE and SRE-1 (Fig. 8A and B) and could likewise repress the apoA-II promoter activity (Fig. 7C). In contrast, the double mutant [SREBP-2 DBP mut Δ(1–93)] did not bind to its cognate sites (Fig. 8A) and had no effect on the apoA-II promoter activity (Fig. 7C). The findings of Figures 7C and 8A and B suggest that repression by the DNA binding deficient mutants requires the activation domain of SREBP-2.

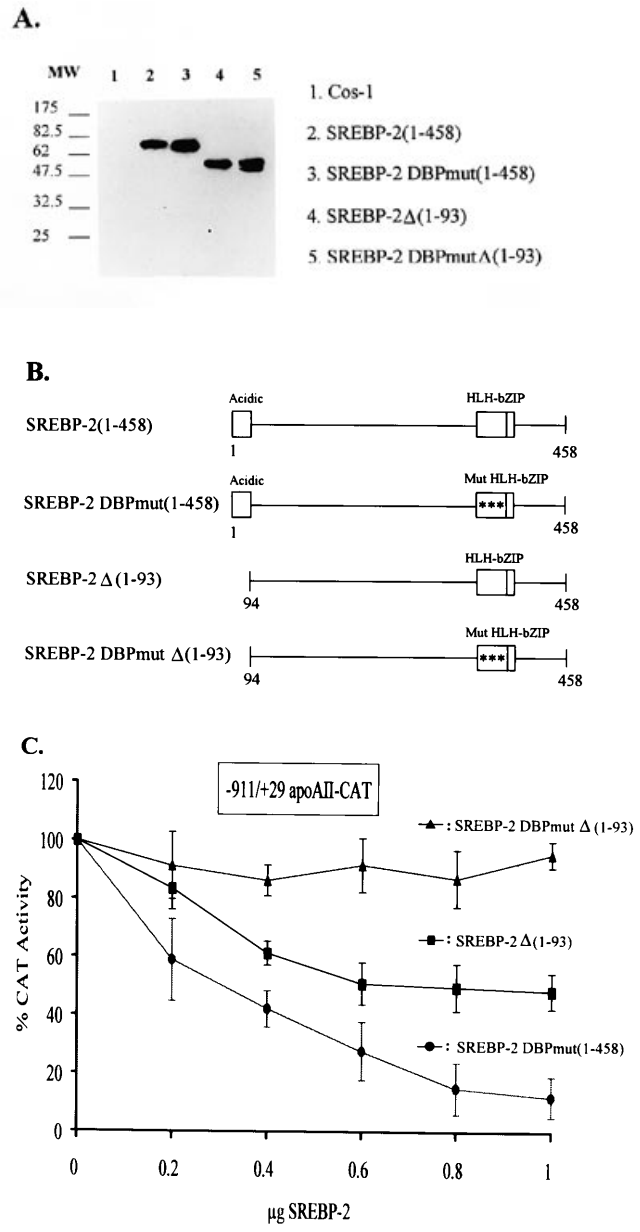


Figure 7. (**A**) Expression of wild-type and mutant SREBP-2 forms in COS-1 cells. The figure shows immunoblotting of extracts of COS-1 cells transiently transfected with expression plasmids carrying the flagged version of either the wild-type or mutant SREBP-2 (1–458) cDNA sequences. The blot was treated with mouse monoclonal anti-Flag antibodies and anti-mouse secondary antibody conjugated to HRP. (**B**) Schematic representation of the SREBP-2 (1–458) forms expressed in COS-1 cells shown in (A). The site of the mutation is indicated by an asterisk (*). (**C**) Effect of increasing concentration of SREBP-2 mutants defective in either the DNA binding or the activation domain or both on the wild-type apoA-II promoter activity in HepG2 cells. The cotransfection titration experiments were performed with 3 µg of the –911/+29 apoA-II promoter construct, 1 µg of the CMV β-gal plasmid and increasing concentration 0–1 µg of the mutant SREBP-2 plasmids. Forty hours following transfections, cells were harvested and the amount of the CAT enzyme was determined as described in Materials and Methods. The mean values (± SD) from three independent transfections performed in duplicates are presented. The SREBP-2 forms used are indicated by abbreviations in the Figure and are described as in (B).

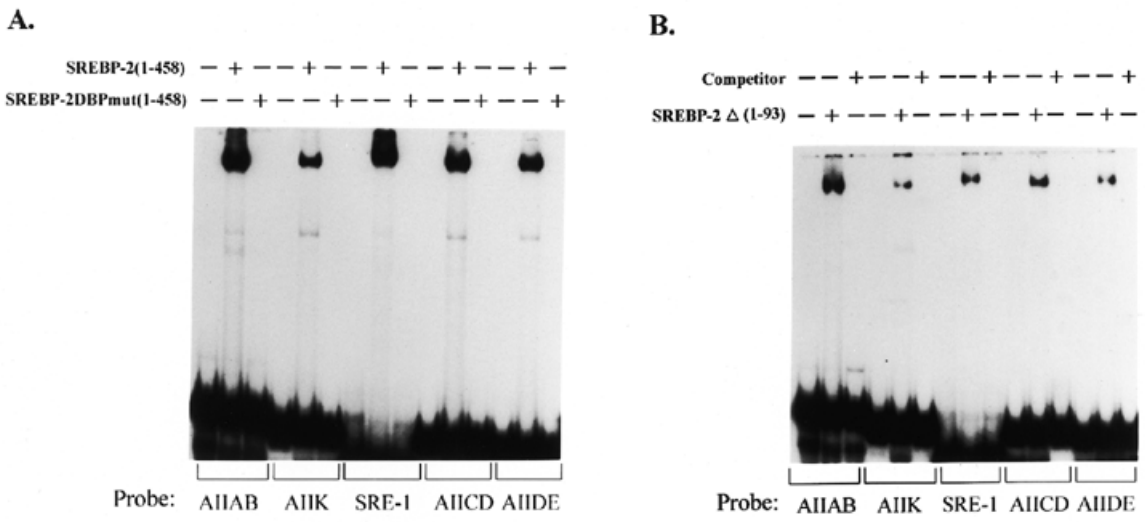


Figure 8. Gel electrophoretic mobility shift assay using the AIIAB, AIIK, AIICD, AIIDE and SRE-1 elements as probes and wild-type or mutant SREBP-2 forms expressed in bacteria. The SREBP-2 forms used are indicated by abbreviations at the top of the figure and described in Figure 7B. Each lane contains 50 fmol probe (50 000 c.p.m.) and 1 µl of bacterial extract prepared as described in Materials and Methods. The figure establishes that the mutation in the DNA binding domain of SREBP-2 abolishes the binding of the mutant protein to all the cognate sites on the apoA-II promoter as well as on SRE-1.

DISCUSSION

SREBP-2 binds to multiple sites on the human apoA-II promoter

Since the original cloning (12,40), it has been shown that SREBP-2 and SREBP-1 regulates several promoters of genes involved in cholesterol metabolism (9–12). The present study establishes that the human apoA-II promoter contains four binding sites for SREBP-2. It is possible that the availability of multiple binding sites may enable SREBP-2 to contribute to an increase in the overall transactivation of this strong hepatic promoter under conditions of cholesterol deprivation.

SREBP-2 binds to two direct repeats present in footprints AIICD and AIIDE. In addition, it binds to two palindromic repeats present in elements AIIAB and AIIK. These two distinct classes of binding sites were identified previously by gel electrophoretic mobility shift assays and selective amplification of the SREBP binding sites (29,41). The consensus palindromic repeat motif which is derived by comparison of all the reported SREBP binding sites appears to be RTCAMTVTGMY (Table 3A).

To identify the residues within the palindromic repeat motif which participate in DNA–protein interaction, we performed $KMnO_4$ and DMS interference analysis using the AIIAB motif as probe. It was found that all the nucleotides of this palindromic decameric repeat and two 5′ T residues participate in DNA–protein interactions. A unique feature of the regulatory element AIIAB is that it contains overlapping motifs homologous to both the palindromic and the direct repeats that are recognized by SREBP-2. Thus, it is possible that SREBP-2 may bind to either of these sites and this could explain the participation of T residues at positions –1 and –4 in DNA–protein interactions. It is interesting that the decameric palindromic repeat and the four 5′ nucleotides are highly conserved among mammalian species (Table 3B). A similar but not identical interaction has been described in the fatty acid synthase promoter. In this promoter SREBP-2 and SREBP-1 can bind to two tandem binding sites flanking the E-box motif. Both sites are required for optimum activation of the promoter by sterols (32).

Previous studies established that the ability of SREBP to recognize either palindromic or direct repeat motifs depends on a Tyr residue present in the basic domain of SREBP. Other bHLHZip proteins have Arg in this position. When the Tyr of SREBP was substituted by Arg at this position. SREBP could no longer recognize the direct repeat motif (41). The potential functional difference between these two classes of SREBP binding sites is not known. We have examined the possibility that SREBP-2 could heterodimerize on the E-box palindromic repeats AIIAB and AIIK with another bHLHZip proteins; the USF-1, which can bind to the same site (15,19). This analysis showed that USF-1 could not form heterodimers with SREBP-2. Nevertheless, the possibility that other bHLHZip transcription factors may heterodimerize with SREBP-2 cannot be excluded.

Binding of SREBP-2 to multiple sites results in upregulation of the apoA-II promoter activity in HepG2 cells

The full-length apoA-II promoter is transactivated ~1.7-fold by SREBP-2. This level of transactivation is significant given the strong activity of this promoter in HepG2 cells (16). On the other hand, the activity of the weak truncated promoters that lack the enhancer region AIII to AIIN depends largely on SREBP-2 and its transactivation increases up to 17-fold for the –230/+29 apoA-II promoter.

Out of the four SREBP-2 binding sites identified on the apoA-II promoter, the AIIAB and AIIK are of special interest since they have been shown to bind another nuclear factor designated CIIIB1 which is a positive regulator of the apoA-II promoter (19). It was shown recently (15) that several properties of CIIIB1 correspond to the properties of the previously described transcription factor USF (26–28). CIIIB1 purified from hepatic nuclear extracts binds on element CIIIB of the apoCIII promoter which

contains an E-box type motif CACCTG on the antisense strand between nucleotides –71 to –76. Nucleotide substitutions within this sequence abolished the binding of the purified factor (19). Purified CIIIB1 protein also protects the region –65 to –48 of the apoA-II promoter which contains an identical E-box type CACCTG motif on the sense strand between nucleotides –59 and –54 (19) as well as to the regulatory elements AIIK and AIIL of the apoA-II promoter. The binding of SREBP-2 to elements AIIAB and AIIK overlaps completely with the binding site of the factor CIIIB1/USF (19). The contribution of elements AIIAB and AIIK in the SREBP mediated transactivation of the apoA-II promoter was tested in a synthetic promoter which contains the element AIIAB and the entire apoA-II enhancer (elements AIIH to AIIK). Transactivation of this promoter by SREBP-2 was altered only by mutations in element AIIAB, thus demonstrating the importance of this element for the observed transactivation. In contrast, previous studies showed that the activities of the intact apoA-II promoter were affected mostly by mutation which altered the binding of CIIIB1 to element AIIK, and to a lesser extent by mutations in element AIIAB (19). The finding suggests that although CIIIB1/USF and SREBP-2 occupy identical sites on elements AIIAB and AIIK of the apoA-II promoter the mechanism of transactivation of the apoA-II gene by these factors may be different. In the former case binding of the CIIIB1/USF to element AIIK is the most important whereas in the latter case binding of SREBP-2 to element AIIAB appears to be the most important. Collectively, our findings suggest that the binding of SREBP-2 to its cognate sites in the apoA-II promoter is associated with the transactivation of the apoA-II promoter. In contrast, promoters that contain weak SREBP-2 binding sites such as the apoC-III promoter or promoters that lack SREBP-2 sites are not transactivated by SREBP-2 (V.I.Zannis, H.-Y.Kan and P.Pissios, data not shown). Previous studies have shown that other transcription factors including SP1 (42,43) and NFY (44–46) synergize with SREBP in the transactivation of target promoters. We have shown previously that oligonucleotides containing the binding site of NFY compete for the binding of activities which recognize the regulatory elements C, D, F and L of apoA-II (38,47). Computer analysis also indicated that the apoA-II promoter contains three NFY binding motifs ATTGG between nucleotides –786 and –782, –677 and –673 and –439 and –435 respectively. In addition, an SP1 binding site was identified between nucleotides –665 and –660. Thus, it is possible that the SREBP-2 mediated transactivation of the apoA-II promoter may result from synergism with NFY or other factors. Such interactions were not pursued in the current study. RNase protection experiments on HepG2 cell cultures grown in lipoprotein deficient serum or in the presence or absence of cholesterol and 25-OH cholesterol showed small relative increase in the apoA-II mRNA/glycerol-3 phosphate dehydrogenase mRNA in cholesterol depleted cells as compared with the cholesterol overloaded cells. However, no difference was observed in the apoA-II mRNA/β-actin mRNA ratio (data not shown). Thus, the role of SREBP-2 in the regulation of the apoA-II gene *in vivo* requires further studies.

Mutations in the DNA binding and activation domains of SREBP-2 repress the apoA-II promoter activity

Similar to the other transcription factors, SREBP-2 is modular in nature and contains well-defined DNA binding and transactivation domains. It belongs to the family of bHLHZip proteins which contain a basic recognition helix and a defined leucine zipper at the end of the DNA binding domain (40). Similar to Gal4 and VP16 transcription factors (48,49), its N-terminal activation domain is highly acidic and does not contain any positively charged residues until amino acid 93.

Previous studies using reporter CAT constructs driven by two copies of repeats 2 and 3 of the LDL receptor promoter showed that deletion of the N-terminal residues 1–90 converted SREBP-1 into a dominant-negative repressor which binds to the sterol regulatory elements and represses transcription (50). On the other hand, mutations in the DNA binding domain that prevented DNA binding diminished the ability of SREBP-1 to transactivate the target promoter (50).

In the current study we observed that an SREBP-2 mutant that lacks the N-terminal activation domain (residues 1–93) binds normally to its cognate sites on the apoA-II promoter and represses the apoA-II promoter activity as expected. On the other hand, a mutant that contains seven amino acid substitutions in its DNA binding domain does not recognize any of the target SREBP binding sites and also causes dose-dependent repression of the apoA-II promoter activity. Insights into the mechanism of repression was obtained by comparison of the properties of a double mutant which has alterations in both the DNA binding and the transcriptional activation domain. This analysis showed that the double mutant that lacks the activation domain and is defective in DNA binding did not have any effect on the apoA-II promoter activity. A possible explanation could be that the SREBP-2 mutants with defects in their activation domain act as dominant-negative mutants when they heterodimerize with the endogenous SREBP factors leading to the repression of the apoA-II promoter activity. The double mutant cannot bind to its cognate site and thus does not affect the promoter activity. It is also possible that the activation domain of the SREBP-2, which is defective in DNA binding, associates with another transcription factor that is important for the activation of the apoA-II promoter. This association could titrate out (squelch) this putative factor from the promoter and thus cause a decrease in the apoA-II promoter activity. Similar squelching interference has also been observed in other systems as well (51). The possibility that SREBP-2 mutants with defects in DNA binding might heterodimerize and titrate out another bHLHZip protein, important for the activity of the human apoA-II promoter has not been supported by the limited data of this study. The identity of this putative titratable factor is unknown; however, other investigators have shown that CBP associates with the activation domain of SREBP-2 and enhances its activation (3). It has been shown that CBP associates with many other transcription factors as well as with TFIIB (52,53). Moreover, CBP itself as well as an associated factor (P/CAF) possess histone acetylase activity. Histone acetylation has been proposed recently as one of the mechanisms for increasing activity of promoters by remodeling the structure of the chromatin (35,54,55).

Overall, this study demonstrates that SREBP-2 binds to multiple sites on two types of binding motifs of the apoA-II promoter and transactivates different promoter segments in HepG2 cells. The study also establishes the DNA–protein contact points of SREBP-2 with its palindromic binding motif and the inability of SREBP-2 to heterodimerize with another bHLHZip protein, the USF. ApoA-II promoter constructs containing the SREBP binding motifs may be utilized to assess different

activation domains of SREBP-2 and possibly its interactions with potential coactivators or corepressors. Using these constructs we were able to demonstrate that SREBP-2 mutants defective in their DNA binding domain repress the apoA-II promoter activity, possibly by a squelching mechanism. Additional new studies are required to determine a potential role of SREBP-2 in the regulation of the apoA-II gene *in vivo*.

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REFERENCES

1 Hua,X., Yokoyama,C., Wu,J., Briggs,M.R., Brown,M.S., Goldstein,J.L. and Wang,X. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 11603–11607.
2 Miserez,A.R., Cao,G., Probst, L.C. and Hobbs,H.H. (1997) *Genomics*, **40**, 31–40.
3 Oliner,J.D., Andresen,J.M., Hansen,S.K., Zhou,S. and Tjian,R. (1996) *Genes Dev.*, **10**, 2903–2911.
4 Duncan,E.A., Brown,M.S., Goldstein, J.L. and Sakai,J. (1997) *J. Biol. Chem.*, **272**, 12778–12785.
5 Hua,X., Sakai,J., Brown,M.S. and GoldsteinJ.L. (1996) *J. Biol. Chem.*, **271**, 10379–10384.
6 Wang,X., Sato,R., Brown,M.S., Hua,X. and Goldstein,J.L. (1994) *Cell*, **77**, 53–62.
7 Wang,X., Pai,J.T., Wiedenfeld,E.A., Medina,J.C., Slaughter,C.A., Goldstein,J.L. and Brown,M.S. (1995) *J. Biol. Chem.*, **270**, 18044–18050.
8 Wang,X., Zelenski,N.G., Yang,J., Sakai,J., Brown,M.S. and Goldstein,J.L. (1996) *EMBO J.*, **15**, 1012–1020.
9 Briggs,M.R., Yokoyama,C., Wang,X., Brown,M.S. and Goldstein,J.L. (1993) *J. Biol. Chem.*, **268**, 14490–14496.
10 Guan,G., Jiang,G., Koch,R.L. and Schechter,I. (1995) *J. Biol. Chem.*, **270**, 21958–21965.
11 Ericsson,J., Jackson,S.M., Lee,B.C. and Edwards,P.A. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 945–950.
12 Wang,X., Briggs,M.R., Hua,X., Yokoyama,C., Goldstein,J.L. and Brown,M.S. (1993) *J. Biol. Chem.*, **268**, 14497–14504.
13 Yang,J., Sato,R., Goldstein,J.L. and Brown,M.S. (1994) *Genes Dev.*, **8**, 1910–1919.
14 Cardot P., Chambaz,J., Cladaras,C. and Zannis,V.I. (1991) *J. Biol. Chem.*, **266**, 24460–24470.
15 Ribeiro,A., Pastier,D., Kardassis,D., Chambaz,J. and Cardot,P. (1999) *J. Biol. Chem.*, in press.
16 Chambaz J., Cardot,P., Pastier,D., Zannis,V.I. and Cladaras,C. (1991) *J. Biol. Chem.*, **266**, 11676–11685.
17 Ladias,J.A.A., Hadzopoulou-Cladaras,M., Kardassis,D., Cardot,P., Cheng,J., Zannis,V.I. and Cladaras,C. (1992) *J. Biol. Chem.*, **267**, 15849–15860.
18 Vu-Dac,N., Schoonjans,K., Kosykh,V., Dallongeville,J., Fruchart,J.-C., Staels,B. and Auwerx,J. (1995) *J. Clin. Invest.*, **96**, 741–750.
19 Ogami,K., Kardassis,D., Cladaras,C. and Zannis,V.I. (1991) *J. Biol. Chem.*, **266**, 9640–9646.
20 Ogami,K., Hadzopoulou-Cladaras,M., Cladaras,C. and Zannis,V.I. (1990) *J. Biol. Chem.*, **265**, 9808–9815.
21 Viollet,B., Lefrancois-Martinez,A.-M., Henrion,A., Kah,A., Raymondjean,M. and Martinez,A. (1996) *J. Biol. Chem.*, **271**, 1405–1415.
22 Graham,F.L. and Van Der Eb,A.J. (1973) *Virology*, **32**, 456–467.
23 Porsch,P., Merkelbach,S., Gehlen,J. and Fladung,M. (1993) *Anal. Biochem.*, **211**, 113–116.
24 Edlund,T., Walker,M.D., Barr,P.J. and Rutter,W.J. (1985) *Science*, **230**, 912–916.
25 Truss,M., Chalepakis,G. and Beato,M. 1990. *Proc. Natl Acad. Sci. USA*, **87**, 7180–7184.
26 Sawadogo,M., Van Dyke,M.V., Gregor,P.D. and Roeder,R.G. (1988) *J. Biol. Chem.*, **263**, 11985–11993.
27 Sirito,M., Lin,Q., Maity,T. and Sawadogo,M. (1994) *Nucleic Acids Res.*, **22**, 427.
28 Sirito,M., Walker,S., Lin,Q., Kozlowski,M.T., Klein,W.H. and Sawadogo,M. (1992) *Gene Exp.*, **2**, 231–240.
29 Smith J., Oborne.,T., Goldstein,J. and Brown,M. (1990) *J. Biol. Chem.*, **265**, 2306–2310.
30 Gil,G., Brown,M.S. and Goldstein,J.L. (1986) *J. Biol. Chem.*, **261**, 3717–3724.
31 Hoffer,M.J., van Eck,M.M., Petrij,F., van der Zee,A., de Wit,E., Meijer,D., Grosveld,G., Havekes,L.M., Hofker,M.H. and Frants,R.R. (1993) *Biochem. Biophys. Res. Commun.*, **191**, 880–886.
32 Magana,M.M. and Osborne,T.F. (1996) *J. Biol. Chem.*, **271**, 32689–32694.
33 Mehta,K.D., Brown,M.S., Bilheimer,D.W. and Goldstein,J.L. (1991) *J. Biol. Chem.*, **266**, 10415–10419.
34 Osborne,T.F., Gil,G., Goldstein,J.L. and Brown,M.S. (1988) *J. Biol. Chem.*, **263**, 3380–3387.
35 Bennett,M.K., Lopez,J.M., Sanchez,H.B. and Osborne,T.F. (1995) *J. Biol. Chem.*, **270**, 25578–25583.
36 Yang,J., Brown,M.S., Ho,Y.K. and Goldstein,J.L. (1995) *J. Biol. Chem.*, **270**, 12152–12161.
37 Kardassis,D., Laccotripe,M., Talianidis,I. and Zannis,V.I. (1996) *Hypertension*, **27**, 980–1008.
38 Cardot,P., Chambaz,J., Kardasis,D., Cladaras,C. and Zannis,V.I. (1993) *Biochemistry*, **32**, 9080–9093.
39 Cardot,P., Pastier,D., Lacorte,J.m., Mangeney,M., Zannis,V.I. and Chambaz,J. (1994) *Biochemistry*, **33**, 12139–12148.
40 Yokoyama,C., Wang,X., Briggs,M.R., Admon,A., Wu,J., Hua,X., Goldstein,J.L. and Brown,M.S. (1993) *Cell*, **75**, 187–197.
41 Kim,J.B., Spotts,G.D, Halvorsen,Y.-D., Shih,H.-M., Ellenberger,T., Towle,H.C. and Spiegelman,B.M. (1995) *Mol. Cell. Biol.*, **15**, 2582–2588.
42 Sanchez,H.B., Yieh,L. and Osborne,T.F. (1995) *J. Biol. Chem.*, **270**, 1161–1169.
43 Yieh,L., Sanchez,H.B. and Osborne,T.F. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 6102–6106.
44 Ericsson,J., Jackson,S.M. and Edwards,P.A. (1996) *J. Biol. Chem.*, **271**, 24359–24364.
45 Jackson,S.M., Ericsson,J., Mantovani,R. and Edwards,P.A. (1998) *J. Lipid Res.*, **39**, 767.
46 Inoue,J., Sato,R. and Maeda,M. (1998) *J. Biochem.*, **123**, 1191–1198.
47 Cardot,P., Chambaz,J., Cladaras,C. and Zannis,V.I. (1991) *J. Biol. Chem.*, **266**, 24460–24470.
48 Laughon,A. and Gesteland,R.F. (1984) *Mol. Cell. Biol.*, **4**, 260–267.
49 Sadowski,I., Ma,J., Triezenberg,S. and Ptashne,M. (1988) *Nature*, **335**, 563–564.
50 Sato,R., Yang,J., Wang,X., Evans,M.J., Ho,Y.K., Goldstein,J.L. and Brown,M.S. (1994) *J. Biol. Chem.*, **269**, 17267–17273.
51 Kamei,Y., Xu,L., Heinzel,T., Torchia,J., Kurokawa,R., Gloss,B., Lin,S.C., Heyman,R.A., Rose,D.W., Glass,C.K. and Rosenfeld,M.G. (1996) *Cell*, **85**, 403–414.
52 Arias,J., Alberts,A.S., Brindle,P., Claret,F.X., Smeal,T., Karin,M., Feramisco,J., Montminy,M. (1994) *Nature*, **370**, 226–229.
53 Horvai,A.E., Xu,L., Korzus,E., Brard,G., Kalafus,D., Mullen,T.M., Rose,D.W., Rosenfeld,M.G. and Glass,C.K. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 1074–1079.
54 Ogryzko,V.V., Schiltz,R.L., Russanova,V., Howard,B.H. and Nakatani,Y. (1996) *Cell*, **87**, 953–959.
55 Yang,X.J., Ogryzko,V.V., Nishikawa,J., Howard,B.H. and Nakatani,Y. (1996) *Nature*, **382**, 319–324.