The Roles of Sterol Regulatory Element-binding Proteins in the Transactivation of the Rat ATP Citrate-Lyase Promoter*

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ATP citrate-lyase (ACL) is a key enzyme supplying acetyl-CoA for fatty acid and cholesterol synthesis. Its expression is drastically up-regulated when an animal is fed a low fat, high carbohydrate diet after prolonged fasting. In this report, we describe the role of sterol regulatory element-binding proteins (SREBPs) in the transactivation of the rat ACL promoter. ACL promoter activity was markedly stimulated by the overexpression of SREBP-1a and, to a lesser extent, by SREBP-2 in Alexander human hepatoma cells. The promoter elements responsive to SREBPs were located within the 55-base pair sequences from −114 to −60. The gel mobility shift assay revealed four SREBP-1a binding sites in this region. Of these four elements, the −102/−94 region, immediately upstream of the inverted Y-box, and the −70/−61 region, just adjacent to Sp1 binding site, played critical roles in SREBPs-mediated stimulation. The mutation in the inverted Y-box and the coexpression of dominant negative nuclear factor-Y (NF-Y) significantly attenuated the transactivation by SREBP-1a, suggesting that NF-Y binding is a prerequisite for SREBPs to activate the ACL promoter. However, the multiple Sp1 binding sites did not affect the transactivation of the ACL promoter by SREBPs. The binding affinity of SREBP-1a to SREs of the ACL promoter also was much higher than that of SREBP-2. The transactivation potencies of the chimeric SREBPs, of which the activation domains (70 amino acids of the amino terminus) were derived from the different species of their carboxyl-terminal region, were similar to those of SREBPs corresponding to their carboxyl termini. Therefore, it is suggested that the carboxyl-terminal portions of SREBPs containing DNA binding domains are important in determining their transactivation potencies to a certain promoter.

ATP citrate-lyase (ACL)[1] is a cytosolic enzyme that catalyzes the cleavage of citrate into oxaloacetate and acetyl-CoA (1). In liver and adipose tissue, this enzyme plays an important role in supplying acetyl-CoA for both fatty acid and cholesterol synthesis (2). As the specific inhibition of ACL in rats significantly decreases the plasma levels of triacylglycerol and cholesterol, ACL is expected to be a potential target for hypolipidemic intervention (3, 4). The activity of ACL is mainly regulated at the level of transcription by diet regimen and insulin, like other lipogenic enzymes, such as fatty acid synthase and acetyl-CoA carboxylase (5). The sequences of the 5′ flanking region of the ACL gene are highly conserved in humans and rats, whereas there is no homology in the regions of the 5′ untranslated region and the first intron, suggesting that transcription is regulated in the same manner in these two species (6, 7). Although ACL plays an important role in fatty acid and cholesterol biosynthesis and is highly controlled by diet at the transcription level, studies on the structure and function of this promoter have been very limited thus far.

SREBPs are the transcription factors that regulate the transcription of many genes involved in cholesterol and fatty acid synthesis, such as low density lipoprotein (LDL) receptor, farnesyl-pyrophosphate synthase, squalene synthase, hydroxy-methylglutary-CoA reductase, hydroxymethylglutary-CoA synthase, fatty acid synthase, and acetyl-CoA carboxylase (8–12). Nascent SREBPs reside in the endoplasmic reticulum and the nuclear envelope as precursor forms (13). The transcriptionally acitive amino-terminal segments are released from the precursor SREBPs by a sequential two-step cleavage process. Once cleaved, the amino-terminal segment translocates into the nucleus where it binds to sterol regulatory elements (SREs) in the promoters of target genes. Three isoforms of SREBPs have been identified. SREBP-1a and -1c are derived from a single gene, using different transcription start sites. SREBP-1c is known as a weak activator because of its short acidic domain. SREBP-2 is transcribed from a separate gene, and it shows about 50% sequence homology to SREBP-1 isoforms. The *in vivo* roles of the SREBP isoforms have been characterized in transgenic mice overexpressing active forms of each isoform (14–16). Those reports have shown that SREBP-1 is associated more with the genes involved in fatty acid synthesis, whereas SREBP-2 preferentially stimulates genes involved in cholesterol synthesis. For example, transgenic mice that overexpress the active forms of SREBP-1a and -1c showed more elevated expression of the mRNAs of lipogenic enzymes such as fatty acid synthase and acetyl-CoA carboxylase than those of the LDL receptor and other cholesterogenic enzymes.

Recent reports have shown that SREBP-1 might be the potential transactivator that mediates lipogenic enzyme gene regulation during the fasting/refeeding cycle. Refeeding a high carbohydrate/low fat diet following long-term fasting up-regulates the nuclear concentration of mature SREBP-1 and its mRNA in adipose tissue and liver (17, 18). Recently, insulin was reported to increase SREBP-1c mRNA in streptozotocin-induced diabetic rat liver and isolated hepatocytes, and

[1] The abbreviations used are: ACL, ATP citrate-lyase; SREBP, sterol regulatory element-binding protein; SRE, sterol regulatory element; LDL, low density lipoprotein; LDLR, LDL receptor; NF-Y, nuclear factor-Y; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

SREBP-1c was suggested as possibly mediating insulin action upon regulation of the genes involved in lipid and carbohydrate metabolism (19, 20). However, the action of the SREBPs on the promoters of lipogenic enzymes is confirmed in only a few genes, which include fatty acid synthase, acetyl-CoA carboxylase, stearoyl-CoA desaturase, and glycerol-3-phosphate acyltransferase (21–25). Thus, studies on SREBP activation of other lipogenic genes including ACL may add more data for the generalization of the hypothesis.

In this study, we demonstrate that SREBPs stimulate the ACL promoter through their binding to an upstream promoter region and that the activation requires NF-Y binding. We also show that SREBP-1a and -2 have different potencies in activating the ACL promoter derived from their carboxyl-terminal domain and not from the amino-terminal activation domain.

MATERIALS AND METHODS

Cell Culture—Alexander cells obtained from ATCC were cultured in minimal essential medium supplemented with 10% fetal calf serum, 100 unit/ml penicillin G-sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B. All cell culture materials were purchased from Life Technologies, Inc.

Construction of ACL Promoter-Luciferase Plasmids—The rat ACL promoter fragments spanning −1860 to +67, −419 to +67, −99 to +67, and −60 to +67 were amplified by polymerase chain reaction (PCR) using *Pfu* polymerase and respective primers based on the sequences reported previously (6). The amplified fragments were gel-purified with a Qiaquick gel extraction kit (Qiagen Inc.) and then subcloned into the *Sma*I site of pGL3-basic plasmid (Promega, Madison, WI). The ACL promoter-luciferase constructs were designated as pACL1860, pACL419, pACL99 and pACL60, respectively. Plasmid pACL114 construct was generated by the deletion of the *Sac*I fragment of −419 to −115 from pACL419. Mutant constructs were generated from pACL114 and pACL419 with the mutagenic oligonucleotides (20 mer) using the QuikChange™ site-directed mutagenesis kit (Stratagene). The mutated sequences are shown in Figs. 3 and 4. The sequences of all the constructs were confirmed using the T7 sequencing kit (Amersham Pharmacia Biotech). The construct of p5xSRE-tk was generated by inserting 5 copies of LDLR SRE1 sequence I into *Sac*I site and herpes simplex thymidine kinase promoter into *Sma*I and *Xho*I sites of pGL3-basic plasmid. All transfection plasmids were prepared with the Qiagen Plasmid Midi Kit (Qiagen Inc.).

Transient Transfection Assay—Alexander cells were plated at a density of 2×10^5 cells/35-mm dish. On the following day, transfection was performed with 0.4 μg of the indicated ACL promoter-luciferase constructs, 0.2 μg of pCMV-β-galactosidase plasmid (CLONTECH), and the indicated amounts of pcDNA3 (Invitrogen) or SREBP expression plasmids pCSA10 or pCS2. Plasmid pCSA10, which encodes amino acids 1–490 of SREBP-1a, and pCS2, which encodes amino acids 1–485 of SREBP-2, were provided by Dr. T. Osborne (University of California, Irvine). The chimeric SREBPs constructs (Fig. 5) were generated as follows: the *Bgl*II restriction site was introduced into pCSA10 and pCS2 at the 70th codon of SREBPs cDNA, and the *Eco*RI-BglII fragments (220 base pairs) corresponding to activation domains were exchanged between pCSA10 and pCS2. Plasmid pmYA (Δ4YA13 m29), which encodes the dominant negative form of the A subunit of NF-Y, was also used in the indicated transfection experiment; it was provided by Dr. R. Mantovani (University of Milan, Italy) (26). Transfection was performed using FuGENE6 transfection reagent (Roche Molecular Biochemicals) for 6 h according to the manufacturer's instructions. After 2 days, cells were washed with phosphate-buffered saline (Life Technologies, Inc.) and lysed in 200 μl of reporter lysis buffer (Promega). Luciferase activities were measured using the Luciferase Assay System (Promega) and normalized with β-galactosidase activities (27) to correct the transfection efficiency. Relative luciferase activity is expressed as the normalized luciferase activity per microgram of protein.

Preparation of Recombinant Human SREBPs and Nuclear Extracts—Recombinant human SREBPs were expressed in *Escherichia coli* BL21(DE3)pLysS. SREBP expression vectors pET-SREBP-1a and pET-SREBP-2 were generated by inserting the cDNA fragments from pCSA10 and pCSA2, respectively, between the *Eco*RI and *Sal*I sites of pET-21a (Novagen). The bacteria freshly transformed with each expression vector were grown to mid-log phase, and proteins were induced for 4 h with 1 mM isopropyl-β-D-thiogalactopyranoside. The bacteria were harvested by centrifugation and disrupted by sonication. The recombi-

nant proteins containing amino-terminal T7 and carboxyl-terminal polyhistidine (His$_6$) tag were purified to homogeneity by Ni-NTA-agarose (Qiagen) chromatography. The purity and concentration of the recombinant proteins were verified by SDS-polyacrylamide gel electorphoresis followed by Coomassie Brilliant Blue staining. Nuclear extracts were prepared from the livers of Harlan Sprague-Dawley rats (weighing about 200 g) according to the procedures described previously by Gorski *et al.* (28).

DNase I Footprinting—The probes corresponding to nucleotides −142 to +67 of the ACL promoter were generated by PCR using pACL419 as a template. ^{32}P-labeled sense oligonucleotides (nucleotides −142 to −116) and unlabeled antisense oligonucleotides (+36 to +67) were used to label the sense strand, whereas ^{32}P-labeled antisense oligonucleotides (−20 to +5) and unlabeled sense oligonucleotides (−142 to −116) were used as primers to label the antisense strand. The PAGE-purified probes (50,000 cpm) were incubated with 0, 1, 2, and 4 μg of purified SREBP-1a for 20 min on ice in 50 μl of reaction buffer containing 10 mM HEPES, pH 7.9, 60 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 7% glycerol, and 1 μg of poly(dI-dC). Then, DNase I (Roche Molecular Biochemicals) diluted in 50 μl of 10 mM MgCl$_2$, 5 mM CaCl$_2$ was added to the DNA-protein binding reactions. After a 2-min incubation at room temperature, the digestion reactions were stopped by adding 100 μl of stop buffer containing 1% (w/v) SDS, 200 mM NaCl, 20 mM EDTA, pH 8.0, and 0.1 μg/μl glycogen. The DNA was extracted with phenol/chloroform and recovered by ethanol precipitation. The pellets were dissolved in loading buffer and then resolved on denaturing 6% polyacrylamide gel. The footprints were compared with the G+A ladder produced by the chemical cleavage sequencing reaction of the same probe to determine the corresponding nucleotide sequences.

Gel Mobility Shift Assay—The probes corresponding to nucleotides −99 to −41 and −142 to −80 of the ACL promoter were generated by PCR using ^{32}P-labeled primers (20 mer) and pACL419 plasmid as a template. To generate mutant probes, corresponding mutant pACL419 plasmids were used as templates. The PAGE-purified probes (20,000 cpm) were incubated with purified recombinant SREBPs (1 μg) or liver nuclear extract (7.5 μg) in a final volume of 20 μl containing 10 mM HEPES, pH 7.9, 75 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 5 mM MgCl$_2$, 10% glycerol, 1 μg of poly(dI-dC), and 0.5% BSA. After a 20 min incubation at room temperature, samples were resolved on a 4% polyacrylamide gel in 1× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA) at 200 V for approximately 1 h at 4 °C. For the competition assays, unlabeled oligonucleotides indicated in the figures were added to the reactions at an approximately 100-fold molar excess. For the NF-Y supershift assay, the antibody against the B subunit of NF-Y (0.2 μg), provided by Dr. R. Mantovani, was added to the reactions. The sequences (coding strands) of the oligonucleotides used in the competition assays are as follows: NF-Y, 5′-GGGGTAGGAACCAATGAAATGAAA-GGT-3′ (29); LDL receptor-SRE1, 5′-TTTGAAAATCACCCCACTGCA-AAC-3′ (9); ACL −142/−116, 5′-AGCAGCGAATGGGGAGGAGCCTA-GAGC-3′; ACL −102/−73, 5′-GCGTGTGCCCAATCGCCAGGCTGCAT-GGCC-3′; ACL −67/−41, 5′-GCTGATGGGGGGCGGGGAGGAG-CCCG-3′.

RESULTS

SREBPs Strongly Induced ACL Promoter Activity—To determine whether SREBPs are able to activate the rat ACL promoter and to focus on the responsive regions, we prepared a series of plasmid constructs containing the various lengths of the rat ACL promoter linked to the luciferase gene. ACL promoter-reporter constructs and the putative binding sites of transcription factors are shown in Fig. 1. The nucleotide sequence is numbered based on the published sequence of the rat ACL gene (6). Promoter-reporter constructs were transiently transfected into the Alexander cells in the presence or absence of pCSA10 or pCS2 encoding the mature form of human SREBP-1a or SREBP-2, respectively (Fig. 1). The luciferase activity indicating ACL promoter activity was increased by the overexpression of SREBP-1a and SREBP-2. In most constructs tested, SREBP-1a showed higher activation than SREBP-2. The level of promoter activities of pACL1860 and pACL419 were almost the same in presence or absence of SREBPs. Nucleotide sequence analysis showed six Sp1 binding sites and one NF-Y binding site in the region from −419 to +67 (6). Deletion of five Sp1 binding sites (pACL114) markedly de-
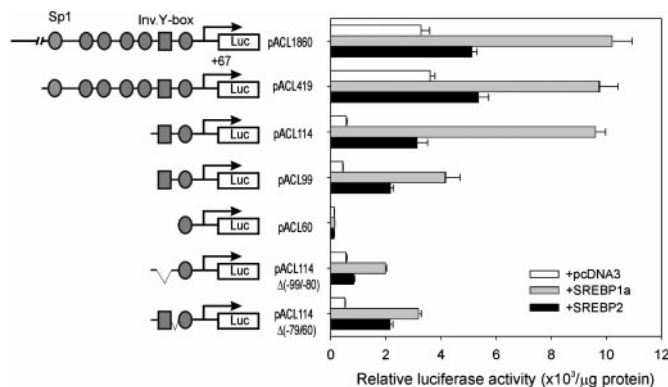
FIG. 1. **Transcriptional activation of the ACL promoter by the overexpression of SREBPs.** The ACL promoter-luciferase constructs used in the experiments are shown on the *left side* of the graph. The putative binding sites for Sp1 and NF-Y are indicated by *ovals* and *square*, respectively. Triplicate dishes of Alexander cells were transfected with 0.4 μg of the indicated ACL promoter-luciferase plasmids and 0.2 μg of pCMV-β-galactosidase plasmid to normalize the transfection efficiency. In addition, SREBP expression plasmids pCSA10 (*gray bars*), pCS2 (*black bars*) or negative control pcDNA3 (*white bars*) were also added to 0.4 μg using FuGENE6 reagent. After a 2-day incubation in medium supplemented with 10% fetal calf serum, luciferase activities and β-galactosidase activities were measured. The values indicate normalized luciferase activity/μg of protein in the cell extracts and represent the mean ± S.E. obtained from three independent experiments.

FIG. 2. **Determination of the SREBP-1a binding sites in the ACL promoter by DNase I footprinting.** The 209-bp DNA fragments of the ACL promoter (nucleotides −142 to +67) were ^{32}P-labeled at the end of the sense or antisense strand and then PAGE-purified. The radiolabeled probes and the purified human recombinant SREBP-1a expressed in *E. coli* were used for the reaction. The preparation of recombinant protein and DNase I footprinting assay was performed as described under "Materials and Methods." DNase I-treated reaction mixtures were subjected to electrophoresis on denaturing 6% polyacrylamide gel. The same radiolabeled probes were also subjected to chemical cleavage sequencing reactions ($G + A$). The reaction products in the lanes are as follows: *lanes 1* and *7*, $G + A$ reaction; *lanes 2* and *6*, reactions without protein; *lanes 3–5*, reactions with 1, 2, and 4 μg of SREBP-1a, respectively. The protected regions (*boxes*) and their relative positions in the ACL promoter are indicated.

creased the basal transcription to 16% relative to that of pACL419. However, the luciferase activity of pACL114 could be highly induced to the level of 98% and 58% of the induced activity of pACL419 by SREBP-1a and −2, respectively. Further deletion to nucleotide −100 (pACL99) reduced the enhancement by SREBP-1a and -2 to the level of 43 and 70%, respectively, of the induced activity of pACL114. Deletion to −61 (pACL60) abolished the basal transcription activity and the transactivation of SREBPs. The constructs pACL114Δ(−99/−80) and pACL114Δ(−79/−60), which were generated from pACL114 by deleting the region from −99 to −80 and from −79 to −60, respectively, significantly decreased the induction by SREBPs. These results demonstrated that the SREs responsible for the induction of ACL promoter activity by SREBPs exist in the region from −114 to −60.

SREBP Binding Regions Were Determined by DNase I Footprinting and Gel Mobility Shift Assay—To localize the SREBP-1a binding sites in the region from −114 to −60 of the ACL promoter, DNase I footprinting was performed using purified recombinant human SREBP-1a expressed in *E. coli* (Fig. 2). SREBP-1a protected two regions, from −72 to −57 and from −88 to −113, adjacent to the inverted Y-box. The inverse sequence of −71 to −62 (GTGAGCTGAT/ATCAGCTCAC) has 80% homology to SRE1 (ATCACCCCAC) identified in the promoter of LDL receptor (8). The sequence from −116 to −107 (CTCAGGCTAG) has 90% homology to SRE3 (CTCAGCCTAG) of glycerol-3-phosphate acyltransferase promoter (25).

To confirm the SREBP binding to these two footprinted regions, gel retardation assays were done. We prepared downstream (−99/−41) and upstream probes (−142/−80) covering the protected regions (Figs. 3A and 4A). SREBP-1a produced two shifted bands with −99/−41 probe (Fig. 3B), of which the upper band was the complex containing two molecules of SREBP-1a. When the LDLR-SRE1 sequence was added to the reaction as a competitor, both shifted bands disappeared completely. Although competitor −102/−73, containing inverted Y-box, slightly decreased the complex formation, neither the conserved NF-Y binding sequence reported nor the Sp1-binding sequence, which exists in the ACL promoter (−67/−41), com-
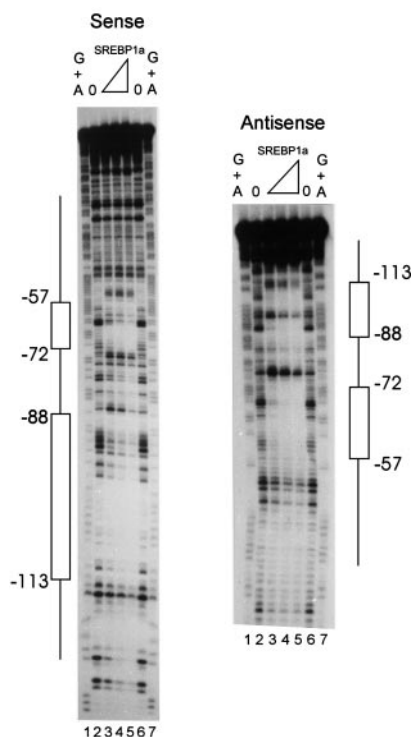
peted complex formation. To determine the precise binding sites for SREBP-1a, we introduced mutations in the probes (Fig. 3A). The substitutions of three base pairs in the conserved SRE1 region of −99/−41 probe produced the mutant oligonucleotides m1 and m2, which formed complexes with the SREBP-1a at significantly lower but still detectable levels (Fig. 3C). The mutation of the inverted Y-box (CCAAT) to CGTTT (m4) slightly decreased the formation of the complex and prevented the formation of complex containing two molecules of SREBP-1a (Fig. 3C). The mutations in both conserved SRE1 and inverted Y-box (m2+4) completely inhibited binding of SREBP-1a to the probe (Fig. 3C). Because all probes were generated by PCR using the same ^{32}P-labeled antisense primer (−60/−41) and then PAGE-purified, the differences in intensity between shifted bands were not caused by the differences in the specific activities between wild type and mutant probes. These results suggest that SREBP-1a binds not only to the conserved SRE1 sequence but also weakly to the inverted Y-box in the ACL promoter.

When the −99/−41 probe was incubated with nuclear extracts isolated from rat liver, one major shifted band was present (Fig. 3D, *lane 1*). This band disappeared when anti-NF-YB antibody was added to the reaction (Fig. 3D, *lane 2*), suggesting that this band was produced by the binding of NF-Y. The NF-Y band was not present if the mutated inverted Y-box (m4) was used as a probe (Fig. 3D, *lanes 5–8*). When SREBP-1a was added to the reaction, an additional shifted band with lower
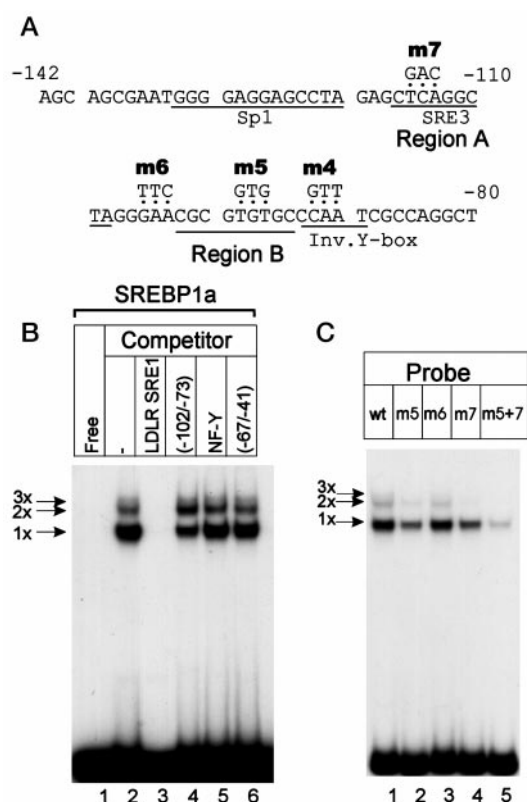
FIG. 3. **SREBPs and NF-Y bind to the proximal region of the ACL promoter.** The sequences of wild type and mutant probes (nucleotides −99 to −41) utilized for the gel retardation assay are shown in *A*. The inverted Y-box (*Inv. Y-box*), SRE1, and Sp1 binding sites are *underlined*. The mutated sequences are presented on the *top* of the strand at the respective positions. Mutants are named *m1* to *m4* and *mSp1*. Gel mobility shift assays and competition assays shown in *B* were performed with the ^{32}P-labeled wild type probe and recombinant SREBP-1a (1 μg). Unlabeled oligonucleotides LDLR-SRE1, −102/−73 of the ACL promoter, the conserved NF-Y-binding sequence, and −67/−41 of the ACL promoter were added as competitors at 100-fold molar excess where indicated. The shifted DNA-protein complexes are indicated. *C* shows the gel mobility shift assays using wild type probe (*wt, lanes 1* and *5*) or indicated mutant probes (*lanes 2–4* and *6–8*) and recombinant SREBP-1a. *D* shows the gel mobility shift assays and supershift assays for NF-Y binding. The wild type probe (*WT*) and the nuclear extracts (7.5 μg) prepared from rat liver were incubated in the absence or the presence of recombinant SREBP-1a (*lanes 1* and *3*). Anti-NF-YB (0.2 μg) was added where indicated (*lanes 2* and *4*). The shifted bands produced by DNA-protein complexes are indicated. The same assays were done with the inverted Y-box mutant m4 (*lanes 5–8*).

mobility, which was not clearly separated from the complex containing two molecules of SREBP-1a, was produced as well as the bands formed by NF-Y or SREBP-1a alone (Fig. 3*D, lane 3*). This additional band is produced presumably by the binding of both NF-Y and SREBP-1a, because this complex was supershifted by the anti-NF-YB antibody (Fig. 3*D, lane 4*), and the addition of SREBP-1a diminished the complex formed by NF-Y alone.

The upstream probe, −142−80 (Fig. 4*A*), also produced the three shifted bands with SREBP-1a (Fig. 4*B*). The binding of SREBP-1a was completely competed by the conserved SRE1 sequence of the LDL receptor promoter but was not competed by the conserved Y-box (29) or the oligonucleotide (−142/−116) that contained the Sp1 binding. The unlabeled oligonucleotide (−102/−73), containing the inverted Y-box, slightly decreased the complex formation in a manner similar to that observed in the experiment with the −99/−41 probe (Fig. 4*B, lane 4*). To determine the SREBP-1 binding sites, a gel mobility shift assay was performed with mutant probes generated by PCR using the same ^{32}P-labeled sense primer (−142/−123). SREBP-1a binding to m5 and m7 mutants was slightly decreased (Fig. 4*C, lanes 2* and *4*). Introducing a double mutation (m5+7) nearly abolished the SREBP-1a binding (Fig. 4*C, lane 5*), and the shifted bands were completely abolished by triple mutations (m4+5+7) (data not shown). However, the mutation outside the potential protein binding sites (m6) did not affect SREBP-1a binding. Mutant m5, which has mutations 3 base pairs away from CCAAT, did not affect NF-Y binding (data not shown).

These gel retardation results show that NF-Y binds to the inverted Y-box and that SREBP-1a binds to four regions, designated as regions A, B, C, and the inverted Y-box (Figs. 3*A* and 4*A*).

DNA Binding Domains of SREBPs Determine Their Potencies on Transactivating the ACL Promoter—Although both SREBP-1a and SREBP-2 have almost the same potencies to activate the transcription of p5xSRE-tk, which contains five copies of LDLR-SRE1 at the upstream of herpes simplex thymidine kinase promoter, SREBP-2 was a much less effective activator than SREBP-1a for the ACL promoter (Fig. 5*A*). Among the several possibilities that may explain the different potencies, we first compared the binding affinities of SREBP-1a and -2 with the ACL promoter SREs. Although SREBP-1a showed no difference in binding to the LDLR-SRE1 and ACL SREs, SREBP-2 bound to the ACL SREs much less efficiently than to the LDLR-SRE1 (Fig. 5*B*). Therefore, the different affinities of SREBPs to multiple SREs in ACL promoter might explain the differences in their transactivation activities. However, it is still possible that the amino-terminal activation domains of these factors might have different compatibilities to the ACL promoter. To rule out this possibility, we constructed two kinds of plasmids, p2A-1aD and p1aA-2D expressing chimeric SREBPs, of which the sequences for the amino-terminal activation domain (amino acids 1–70) were exchanged as illustrated in Fig. 5*C*. The p2A-1aD could induce the ACL promoter as efficiently as pCSA10, whereas p1aA-2D induced it to a lesser extent, similar to pCS2 (Fig. 5*D*). This finding suggests that the potencies of SREBPs in transactivating the ACL promoter depend on the carboxyl-terminal portion of SREBPs but not the amino-terminal activation domains and that the binding affinity of SREBPs to the promoter elements is an important factor in determining the potency of transactivation.

The Functional Roles of SREBP-binding Elements and the Inverted Y-box—To evaluate the functional significance of the SREBP binding elements and the inverted Y-box in the ACL promoter, a transient transfection experiment was performed.

FIG. 4. **SREBP-1a binds to the upstream region containing the inverted Y-box of the ACL promoter.** *A* shows the sequence of upstream probe (−142 to −80) used in the gel mobility shift assays. The Sp1 binding site, SRE3, and the inverted Y-box (*Inv. Y-box*) are *underlined*. The mutated sequences are shown on the *top* of the strand at the respective positions and are designated as *m4, m5, m6,* and *m7*. *B* shows the gel mobility shift assays performed with ^{32}P-labeled wild type probes and recombinant SREBP-1a (1 μg). Unlabeled oligonucleotides LDLR-SRE1, −102/−73 of the ACL promoter, the conserved NF-Y-binding sequence, and −67/−41 of the ACL promoter were added as competitors at 100-fold molar excess where indicated. *C* shows the gel mobility shift assays performed with wild type probe (*wt*) or indicated mutant probes and recombinant SREBP-1a.

The mutant clones, basically produced from pACL114, were transiently transfected into Alexander cells, and their responsiveness to SREBP-1a was tested (Fig. 6). The constructs have mutations that prevented the binding of SREBP-1a and/or NF-Y in the gel mobility shift assays (Fig. 3, 4). Any single mutation did not completely abolish the responsiveness of pACL114 to SREBPs. Mutation at conserved SRE1 in region C (m2) and at nucleotides in region B immediately upstream of the inverted Y-box (m5) significantly decreased activation by SREBPs without changing the basal transcription activity. However, the m7 mutation did not affect the activation, even though it also prevented the SREBP1a binding in gel mobility shift assay. As region A exists at the vector-promoter junction in pACL114, we could not exclude the possibility that the neighboring vector sequences altered the binding affinity to SREBP-1a and thereby made this site nonfunctional. The double mutations at regions B and C (m2+5) completely abolished the responsiveness of the ACL promoter to SREBP-1a without a decrease in basal activity. The mutation at the inverted Y-box (m4) significantly decreased the basal promoter activity as well as the responsiveness to SREBP-1a. The double mutations at region C and the inverted Y-box (m2+4) decreased the basal promoter activity to a level similar to that of m4 and decreased stimulation of the ACL promoter by SREBP-1a. The mutations at the most proximal Sp1 binding site (mSp1) did not affect the responsiveness of SREBP-1a. Because both SREBP1a and
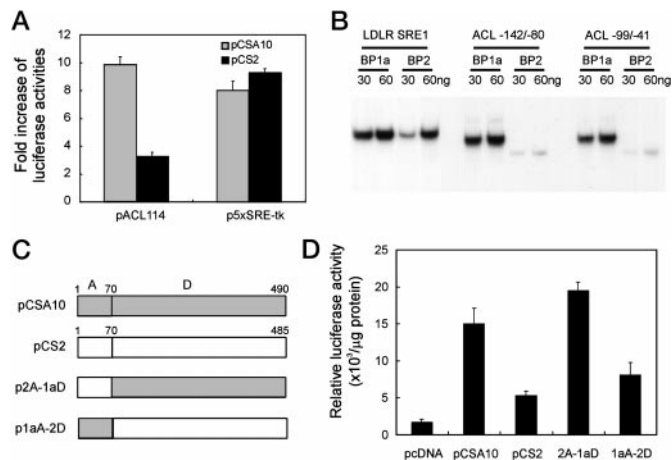

FIG. 5. **Comparison of binding affinities between SREBP-1a and SREBP-2 to the ACL promoter and activities of chimeric SREBPs.** The transient transfection assay shown in *A* was done by introducing pACL114 or p5xSRE1-tk (0.5 μg) together with 40 ng of expression plasmid. The fold increase was shown as the ratio of the luciferase activities in the presence of expression plasmid to those in the absence of expression plasmid. The gel mobility shift assay shown in *B* was done with ^{32}P-labeled LDLR-SRE1, ACL promoter fragments −142/−80, and −99/−41. The probes were incubated with the indicated amount of purified human SREBP-1a or SREBP-2 protein. Two chimeric SREBP expression plasmids, p2A-1aD and p1aA-2D, were generated by exchanging the coding sequences for amino acids 1–70 between SREBP-1a and -2, as illustrated in *C*. The ACL promoter-luciferase construct, pACL114 (0.5 μg), was transfected together with 40 ng of the expression plasmids. Two days after transfection, cells were harvested, and the luciferase activities were measured (*D*). The results were expressed as the normalized luciferase activity/μg of protein in the cell extracts. Values represent the mean ± S.E. obtained from three independent experiments performed in triplicate.
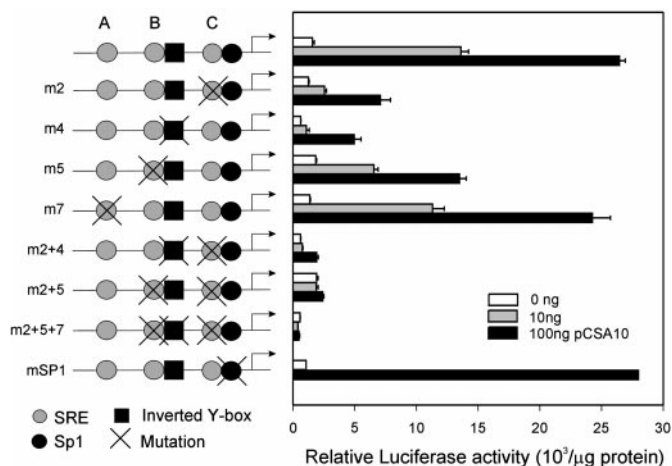

FIG. 6. **The mutations of binding sites of SREBP-1a and NF-Y in the ACL promoter attenuate the SREBP-1a-driven transactivation of the ACL promoter-luciferase construct.** The wild type and mutant pACL114 constructs were illustrated schematically on the *left side* of the graph. The mutant sequences are shown in Fig. 3 and 4. Triplicate dishes of Alexander cells were transfected with 0.4 μg of pACL114 and 0.2 μg of pCMV-β-galactosidase plasmid, together with 10 ng (*gray bars*) or 100 ng (*black bars*) of pCSA10 or pcDNA3 (*white bars*). The total amount of transfected plasmids was adjusted to 0.8 μg by adding pcDNA3. Two days after transfection, cells were harvested, and the luciferase activities were assayed. Values represent the mean ± S.E. from three independent experiments.

NF-Y can bind to the inverted Y-box of the ACL promoter, we needed to determine which was the responsible factor for the decrease of responsiveness to SREBP-1a shown in the constructs containing the mutations at the inverted Y-box (m4). By using the dominant negative mutant of NF-Y, pmYA, which encodes the mutated A subunit of NF-Y lacking an ability to
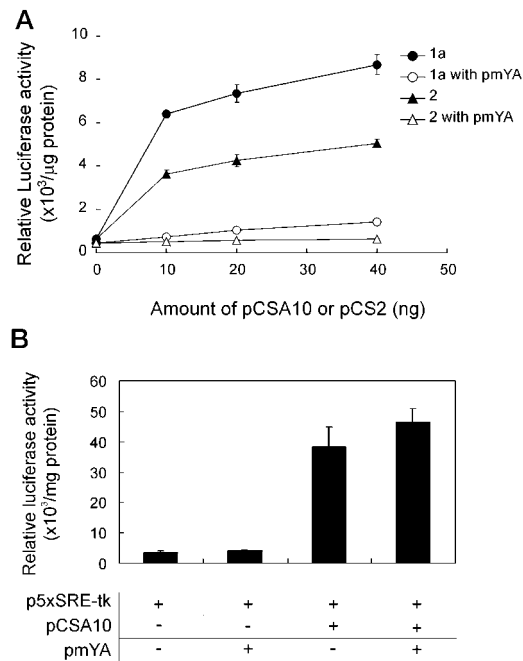
FIG. 7. **The stimulation of ACL promoter-luciferase construct by SREBPs is dependent on NF-Y.** The indicated amounts of pCSA10 or pCS2 along with 0.4 μg of pACL114 and 0.2 μg of pCMV-β-galactosidase plasmid were transfected into Alexander cells (*A*). Plasmid pcDNA3 (0.4 μg) or pmYA29 (0.4 μg) encoding a dominant negative form of NF-YA was cotransfected as indicated. The plasmid p5xSRE-tk (0.4 μg) was transfected with 40 ng of pCSA10 and 0.4 μg of pmYA as indicated (*B*). The plasmid pUC9 was used to make the total amount of transfected DNA equal. Two days after transfection, luciferase activities were measured. Normalized luciferase activities expressed as the mean ± S.E. from three independent experiments are shown.

bind to NF-Y consensus sequence (26), we could exclude the NF-Y binding effect. When pmYA was added, the stimulation of the ACL promoter activity by SREBP-1a and -2 was effectively suppressed almost to the basal level (Fig. 7*A*). However, SREBP-1a-induced activation of p5xSRE-tk was not changed by adding pmYA. We could obtain the same result when the construct containing mutations at the inverted Y-box (m4) was used (data not shown). These results suggested that NF-Y binding to the inverted Y-box is required for the effective trans-activation of the ACL promoter by SREBPs.

DISCUSSION

SREBPs are known as a family of transcription factors that regulate the genes involved in fatty acid and cholesterol synthesis. It has been suggested that SREBP-1 and SREBP-2 have different effects on target genes, that is, SREBP-1 preferentially activates genes involved in fatty acid synthesis and SREBP-2 activates genes involved in cholesterol synthesis. Recent reports have suggested that SREBPs stimulates the expression of ACL gene and that SREBP-1a would be a more specific factor. Shimomura *et al.* (30) reported that the level of mRNA for ACL was elevated by 3- to 4-fold in transgenic mice overexpressing the mature form of SREBP-1a or -1c and by 1.5-fold in transgenic mice overexpressing SREBP-2. In this study, we demonstrate that the rat ACL promoter is highly activated by the direct interaction of SREBPs with multiple SREs on the ACL promoter, and that SREBP-1a is more effective activator than SREBP-2. In addition, we show that the binding affinities of these SREBPs to ACL SREs determine their potency in this promoter, supporting previous reports that SREBPs have preferential target genes.

In the ACL promoter, the region between nucleotides −114

and −60 was the major determinant for the activation by SREBPs. The recombinant SREBP-1a strongly bound to three regions, A, B, and C, in the proximal promoter of the ACL gene. The SRE in region C (GTGAGCTGA) bears sequences homologous to SRE1, which has been found in many target genes of SREBPs. As expected, mutations at region C significantly reduced the responsiveness to SREBPs without decreasing basal activity of ACL promoter. The SRE in region B, the upstream region of the inverted Y-box, showed a novel sequence (5′-AACGCGTGTG-3′; −104 to −95) differing from known consensus SREs. The mutation at region B prevented the binding of SREBP-1a (Fig. 4*C*) without changing NF-Y binding (data not shown) and also decreased the stimulation of ACL promoter by SREBP-1a (Fig. 6). Region A has sequences highly homologous to SRE3, which was reported in glycerol 3-phosphate acyltransferase promoter. However, mutations in region A (m7) did not alter the responsiveness to SREBPs, even though SREBP-1a showed strong affinity to this region and its binding was significantly reduced to the mutant probe (m7). The fact that mutations at both SREs in region B and C resulted in complete loss of responsiveness of the ACL promoter to SREBP-1a suggested that these two SREs play important roles in SREBP-mediated regulation.

The importance of the Sp1 binding sites for the action of SREBPs has been reported in many promoters, such as LDL receptor (31), acetyl-CoA carboxylase (22), and fatty acid synthase promoter (32). But in the case of ACL, although the deletion of five upstream Sp1 binding sites decreased the basal activity of the ACL promoter, it did not alter the effect of SREBPs activating the ACL promoter (Fig. 1). Also, the mutation of the most proximal Sp1 binding site downstream of region C did not affect the activation by SREBPs (Fig. 6). On the other hand, the responsiveness to SREBPs was markedly reduced when the binding of NF-Y was disturbed by the mutation at the inverted Y-box or by the overexpression of the dominant negative form of NF-YA (Figs. 6 and 7). These results indicate that the regulation of the ACL promoter by SREBPs requires the neighboring binding of NF-Y, as was observed in farnesyl-pyrophosphate synthase, squalene synthase, hydroxymethylglutaryl-CoA synthase, glycerol-3-phosphate acyltransferase, and *SREBP-2* genes (9, 10, 12, 25, 33).

The ACL promoter activation by SREBP-1a and −2 were quite different. The amino-terminal 60 amino acid region of SREBP-1a is known to be an activation domain that interacts with multiprotein complex including CREB-binding protein (34, 35). SREBP-1c, which is derived from the same gene as SREBP-1a by using different transcription start site, is known as a weak activator because of its short amino-terminal region (36). However, the different potencies of SREBP-1a and -2 for the ACL promoter activation were not originated from their differences in activation domains. Instead, SREBP-2 showed much less affinity to SREs in the ACL promoter than SREBP-1a, and the chimeric SREBP, which has the SREBP-2 activation domain followed by remaining the carboxyl-terminal region of SREBP-1a, could activate the ACL promoter as efficiently as SREBP-1a itself. This finding implies that SREBPs can activate preferential target genes through their different affinities to SREs in the promoters. The mechanism by which SREBPs activate their preferential target genes has not been studied thus far. Therefore, it will be necessary to evaluate promoter selection by studying the binding affinity of SREBP isoforms in other promoters too.

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The Roles of Sterol Regulatory Element-binding Proteins in the Transactivation of the Rat ATP Citrate-Lyase Promoter

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