Binding and functional effects of transcription factors Sp1 and Sp3 on the proximal human lecithin:cholesterol acyltransferase promoter

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Abstract **Human lecithin:cholesterol acyltransferase (LCAT) circulates in plasma bound to high density lipoproteins (HDL) and modulates the rate by which cholesteryl ester is transported to the liver. So far, little is known about the regulation of the expression of the LCAT gene. In this study we have defined the *cis*-elements, identified the *trans*-acting factors and demonstrated their functional effects and significance in determining transcriptional activity of the proximal LCAT promoter. Using deletion mutants having 5′ variable ends (from nucleotides −72 to −27), we have identified the presence of two non-consensus GC-rich regions that stimulate transcription in HepG2 and HeLa cells. These regions designated sites A (−29 to −47) and B (−49 to −65) contain the CCTCC core sequence which in electromobility shift analysis is critical for the formation of two DNA–protein complexes designated I and II. Site-directed mutagenesis suggests that both sites are equally important in promoter activity, and that cooperative interactions between both sites are not required for activity. Electromobility shift and supershift experiments using oligonucleotides spanning sites A and B identified Sp1 and Sp3 as the transcription factors interacting at these sites. To determine the significance and functional effects that Sp1 and Sp3 have in regulating LCAT promoter activity, we performed transfection experiments in *Drosophila* SL-2 cells as they lack endogenous Sp1 and Sp3. Sp1 but not Sp3 activates the human LCAT promoter and when Sp1 is co-transfected along with Sp3, Sp3 functions as a dose-dependent repressor of Sp1-mediated activation. These findings indicate that Sp1 is capable of *trans*-activating a reporter gene linked to the LCAT promoter containing Sp binding sites and suggests that the levels of Sp3 or the nuclear Sp1/Sp3 ratio may play an important role in determining the transcriptional activity of the LCAT promoter in vivo.—**Hoppe, K. L., and O. L. Francone. **Binding and functional effects of transcription factors Sp1 and Sp3 on the proximal human lecithin: cholesterol acyltransferase promoter. *J. Lipid Res.* 1998.** 39: **969–977.**

Supplementary key words LCAT • transcriptional regulation • Sp1 • Sp3.

Lecithin:cholesterol acyltransferase (E.C. 2.3.1.43) (LCAT) is a central enzyme in the plasma metabolism of cholesterol. LCAT is secreted mainly by the liver, and to a much lower extent by the brain and testis (1). The secreted 416-amino acid glycoprotein circulates in plasma associated with lipids and apolipoproteins in the high density lipoprotein (HDL) fraction (2). The principal molecular reaction catalyzed by this enzyme is the hydrolysis and transesterification of the *sn*-2 fatty acid of phosphatidylcholine and other glycerophospholipids to the 3-hydroxyl group of cholesterol. This reaction occurs preferentially on the surface of HDL, where the reaction is activated by apolipoprotein A-I (3). LCAT, like other lipases has an active site serine (4).

The physiological consequences of the esterification of cholesterol by LCAT are profound and illustrated by the biochemical and clinical characteristics of patients with either partial or total deficiency in LCAT (5). In addition, the expression of human LCAT in transgenic animals has provided further evidence for its role in plasma cholesterol homeostasis (6–8), reverse cholesterol transport (9), and atherosclerosis (10).

Significant changes in LCAT mRNA levels were reported at various stages of fetal and postnatal life suggesting a developmental and apparently sex-specific pattern of LCAT gene expression (1, 11). In addition, endotoxin administration, which mimics infection, has a profound effect on LCAT mRNA levels suggesting a role for cytokines, including tumor necrosis factor, in the regulation of LCAT plasma levels (12). On the other hand, manipulations affecting lipid metabolism such as hormones, including estrogens, l-thyroxine, and hydrocortisone, do not alter LCAT mRNA abundance (13).

The developmental and tissue-specific expression of eukaryotic genes involves the regulated assembly of nucle-

Abbreviations: DTT, dithiothreitol; EMSA, electrophoretic mobility shift assays; HDL, high density lipoproteins; HeLa, human cervix epitheloid carcinoma; HepG2, human hepatocellular carcinoma; LCAT, lecithin:cholesterol acyltransferase; nt, nucleotides; PCR, polymerase chain reaction.

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oprotein complexes comprising the basal and upstream transcriptional machinery (14). So far, little is known about the specific DNA elements and *trans*-acting factors responsible for tissue-specific expression and transcriptional activity of the LCAT gene. The 5′-flanking region of the human LCAT gene has several striking structural features including a number of putative binding sites for transcription factors at conserved sequences (15). A minimal promoter sequence extending 71 nucleotides (nt) before the transcriptional initiation site has been proposed to determine the transcriptional activity of the LCAT promoter in Hep3B (16). However, the DNA–protein binding sites and the transcription factor(s) acting through these sequences as well as their functional effects and significance in regulating LCAT promoter activity are presently not known.

In the present study, we have focused on the proximal region of the LCAT promoter and defined the *cis*-elements, identified the interacting *trans*-acting factors, and demonstrated their functional effects and significance in determining transcriptional activity.

EXPERIMENTAL PROCEDURES

Plasmid construction

The −72LCATLuc, −42LCATLuc, and −27LCATLuc were constructed using polymerase chain reaction (PCR)-generated fragments from the pUCLCATBamNsi (8), digesting with Kpn I and Sac I (+154), and cloning the desired fragment into the Kpn I and Sac I sites of pGL2 Basic (Promega, Madison, WI). In each case the predicted DNA sequence was confirmed by dideoxynucleotide sequencing. The 5′-primers contained an engineered Kpn I site, indicated by underlined bases. Oligonucleotides (Oligo Therapeutics Inc., Wilsonville, OR) used in the PCR reactions were: −72, 5′-CCCCAGGTACCAGGCCGTCCCT CCCACT-3′; −42, 5′-CCACTGGCCCTAGGTACCCTCCCACTC-3′; −27, 5′-TCCCACTCCGGTACCAGATAAGGACAGCCC-3′, 3′-primer 5′-CTCTGGATTCCCCCCGTGACCCTTAC-3′.

Sp1 expression plasmid driven by the *Drosophila* actin 5C promoter (pPacSp1) has been described previously (17) and was a generous gift of Dr. Tjian. Sp3 expression plasmid (pBKCMVSp3) driven by the cytomegalovirus promoter was constructed by inserting the Sp3 cDNA EcoRI fragment (18) from the pSP72-hSp3 plasmid (ATCC, Rockville, MD) into the EcoRI site of pBKCMV (Stratagene, La Jolla, CA). Beta-galactosidase expression plasmids pSV-βgal (Promega, Madison, WI) and pCMV-βgal (Clontech, Palo Alto, CA) were purchased from respective manufacturers.

Site-directed mutagenesis

Site-directed mutagenesis was conducted using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. Synthetic oligonucleotides 24–27 bases long carrying two mismatched bases (underlined nucleotides) were used to mutagenize nucleotides −41/−40 and −62/−61 with the resulting plasmids containing the desired mutations named −72mA and −72mB, respectively. Briefly, 5 ng of −72LCATLuc plasmid was annealed to 125 ng of each primer and extended during temperature cycling through 12 cycles of 95°C, 30 sec; 55°C, 1 min; 68°C, 11 min 36 sec in the presence of Pfu DNA polymerase. After completion of cycles, parental wild-type DNA was digested with DpnI. Plasmids containing the desired mutations were transformed into Epicurian Coli XL-1 Blue supercompetent cells. Mutations were confirmed by dideoxynucleotide sequencing. The following synthetic oligonucleotides (Oligo Therapeutics) and oligonucleotides of complementary sequence were used: −41/−40 (5′-CCCTAGGGCCTTTCCCAC TCCCACACC-3′) and −62/−61 (5′-CCAGGCCGTCTTTCCCA CTGGCCC-3′).

Cell culture and transfections

Plasmids were transfected into HepG2 (human hepatocellular carcinoma) and HeLa (human cervix epitheloid carcinoma) cells and assayed for their ability to promote transcription of the promoterless luciferase gene. Cells were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum and supplemented with 40 μg/ml gentamicin sulfate. All transfections were performed in 6-well plates seeded with 2.5×10^5 cells per well. The transfection mixtures containing 5 μg of promoter reporter plasmid DNA along with 200 ng pSV-βgal fusion plasmid were transfected into cultured cells using Lipofectin (Gibco BRL). Cells were harvested 48 h later in Reporter Lysis Buffer (Promega) and lysed by freeze-thawing. Cell debris was pelleted and the supernatant was used in assays. Luciferase activities were measured in a Dynatech ML1000 Model 2.4 luminometer with the luciferin reagent from Promega. The β-galactosidase activity of the cell lysates was determined as previously described (19) and the values were used to normalize variabilities in the efficiency of transfection.

Schneider's *Drosophila melanogaster* cell line 2 (SL-2) (20) was maintained at 26°C in Schneider's Insect Medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (insect cell tested) (Sigma) and gentamicin sulfate. Cells were seeded in 6-well plates at 2×10^6 cells per well 20 h prior to transfection. Transfections were carried out using the calcium phosphate co-precipitation method as described (21). Varying amounts of pPacSp1 and pBKCMVSp3 as indicated in figures were transfected along with 0.5 μg of reporter construct −72LCATLuc, 500 ng pCMV-βgal and using pGEM 7zf as filler DNA to a total of 5 μg. After addition of DNA, plates were left undisturbed and harvested 48 h later. Cells were harvested by pipetting up and down, pelletted, washed with PBS, resuspended in 200 μl of Reporter Lysis Buffer and lysed by freeze/thawing. Lysed cells were centrifuged for 10 min to pellet debris and the supernatant was assayed for luciferase activity as described above. The β-galactosidase activity of lysates was determined as described above and values were used to normalize variabilities in efficiency of transfection. Expression of the Sp1 and Sp3 plasmids was detected using antibodies specific to human Sp1 and Sp3. Nuclear extracts of SL-2 cells were prepared from either non-transfected cells or cells cotransfected with 100 ng of pPacSp1 and 2500 ng of pBKCMVSp3 expression plasmids, electrophoresed on an 8% SDS-PAGE, and transferred to a nitrocellulose membrane. Bound antibodies were detected by Enhanced Chemiluminescence Western Blotting Detection Kit (Amersham, Arlington Heights, IL) according to instructions of the manufacturer and exposed to autoradiography film.

Nuclear extracts

HepG2 nuclear extract was prepared as previously described (22), except that buffers A and C were supplemented with 1 mm dithiothreitol (DTT), 1 mm phenylmethylsulfonyl fluoride, 1 mg/ml of pepstatin A, and 1 mg/ml of leupeptin (Sigma., Saint Louis, MO). Aliquots of nuclear extract were snap frozen on dry ice and stored in liquid nitrogen. A rapid micropreparation method was used for preparing nuclear extracts from transfected SL-2 cells (23). The protein concentration of the extract was de-

termined by the Bradford method (24) using bovine serum albumin as a standard.

Electrophoretic mobility shift assays (EMSA)

EMSA were performed in a 20-μl reaction volume containing 25 mm HEPES at pH 7.5, 100 mm KCl, 10 μm $ZnSO_4$, 0.2% (v/v) NP-40, 20% glycerol, 1 mm DTT, 2 μg of poly (dIdC), 3 μg of nuclear extracts, and 0.2 ng ^{32}P-labeled double-stranded oligo probe. After a 15-min incubation on ice, the reaction mixture was loaded onto a 6% polyacrylamide gel in 0.5 × TBE (45 mm Tris-borate, 45 mm boric acid, 1 mm EDTA) and electrophoresed at 200 V for 2–3 h at 4°C. After electrophoresis, gels were fixed, dried and analyzed by autoradiography. For competitive DNA binding assays, extracts were incubated with an excess (see figures) of unlabeled synthetic double-stranded oligonucleotides for 15 min at 4°C prior to the addition of the labeled probe. Binding reactions with Sp1 protein (Promega) were carried out in above binding buffer and 2 μg of BSA. When indicated, 1 μl of polyclonal antiserum to Sp1(PEP2), Sp2(K-20), Sp3(D-20), or Sp4(V-20) proteins (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with the nuclear extract for 30 min on ice before the addition of the probe. Each antiserum is non-cross-reactive with the other Sp proteins. Double stranded Sp1 Consensus oligonucleotide (5′-ATTCGATCGGGGCGGGGCGAGC-3′) was purchased from Promega. The non-specific competitor oligo for EMSA had the following sequence: 5′-GATCACTGAACCCGG GACCCCTGCCCT-3′.

RESULTS

Identification of a functional region in the proximal LCAT promoter

We first wanted to identify key regions that confer transcriptional activity to the proximal 72 nt on the human LCAT promoter. We constructed a series of promoter deletion mutants having 5′ variable ends (from nt −72 to −27) and a common 3′ end at nt +154 (relative to the transcription initiation site at nt +1). The promoter fragment was inserted into a promoterless reporter plasmid pGL2 in the sense orientation at the 5′ end of the luciferase gene which was used as a general marker for promoter activity. LCAT promoter/reporter fusion plasmids were transfected into HepG2 and non-expressing HeLa cells (derived from liver and epithelium, respectively) and the level of luciferase activity in the cell extracts was measured. As shown in **Fig. 1**, luciferase expression was observed, with promoter activity being greatest in HepG2. Removal of the region located between nt −72 and −42 resulted in an approximate 2-fold reduction in luciferase activity in both cell types. A further 2-fold drop in activity was obtained after removing sequences extending from nt −42 to −27 in HepG2. The shortest promoter fragment tested (5′ end at nt −27), although having only 27% of the activity of the construct with its 5′ end at nt −72, exhibited 20-fold greater activity than the promoterless pGL2 plasmid. Thus, these findings suggest the existence of two regions that stimulate promoter activity between nt −72 and −27. These regions contain two direct repeats consisting of non-consensus GC-rich sequences that may be bound by proteins that recognize GC-rich sequences (Fig. 1B).

The −72 to −27 promoter region contains two DNA-protein binding sites

EMSA were used in an attempt to identify where the nuclear protein(s) interact with the LCAT regulatory regions identified by deletion studies. Synthetic oligonucleotides

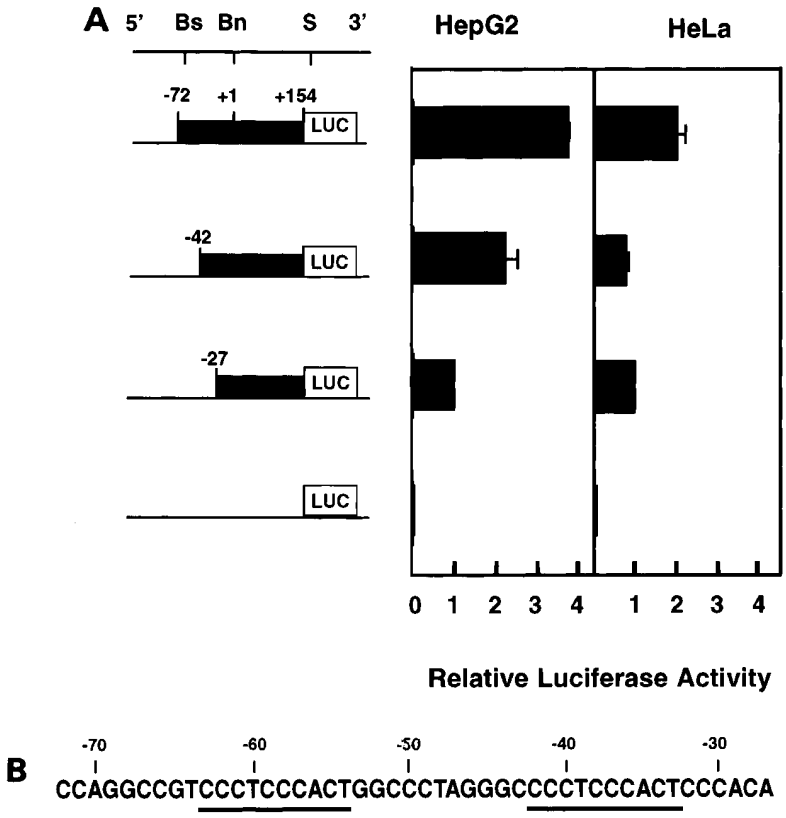

Fig. 1. Effect of 5′ deletion mutations on transcriptional activity of the human LCAT gene promoter. Panel A, a restriction map of the −72/+154 LCAT promoter is shown at the top left. The indicated plasmid constructs (left) were transfected into HepG2 and HeLa cells. The right represents the luciferase activity of each reporter construct as the mean of five separate experiments relative to −27LCATLuc construct (activity: 1) ± standard deviation. Nucleotide positions are indicated relative to the transcriptional start site, +1. Bs, Bst NI; Bn, Ban I; S, Sac I. Panel B, the DNA sequence from −72 to −27 with the two direct repeated sequences underlined.

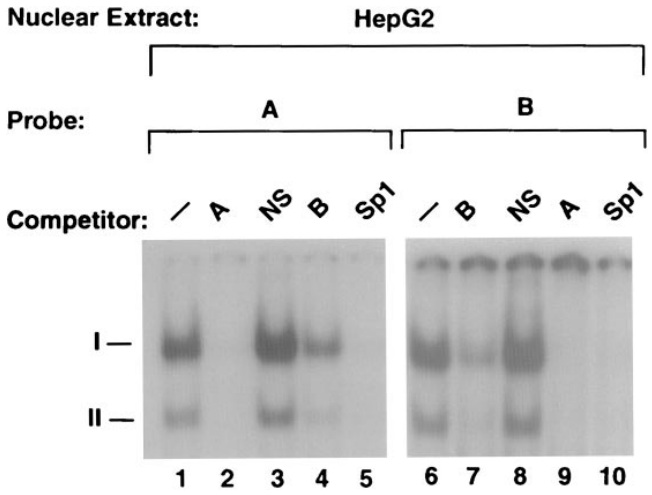

Fig. 2. Binding of HepG2 nuclear proteins to LCAT site A and site B. EMSA showing binding of HepG2 nuclear extracts to end-labeled wild-type LCAT site A oligo (lane 1) and site B oligo (lane 6), in the presence of 100-fold molar excess of unlabeled site A (lane 2) or site B (lane 7), non-specific competitor oligonucleotide (lanes 3 and 8), oligo B or A (lanes 4 and 9), or consensus Sp1 oligonucleotide (lanes 5 and 10). I indicates the slower migrating and II indicates the faster migrating complex. NS, nonspecific oligonucleotide.

spanning −51 to −29 (site A) and −74 to −52 (site B) or oligonucleotides containing mutations in these regions were used in EMSA as indicated in **Fig. 2** and **Fig. 3**. Incubation of labeled oligonucleotide A with HepG2 nuclear extracts resulted in the formation of two bands designated I and II (slower and faster migrating, respectively) (Fig. 2). Both bands were specifically competed by a 100-fold molar excess of unlabeled oligonucleotide A (Fig. 2, lane 2) but not by an unrelated oligonucleotide (Fig. 2, lane 3). Sites A and B contain the identical 10-bp sequence 5′-CCCTCCCACT-3′, which is a non-consensus Sp sites. Because of this identical sequence in sites A and B, we used 100-fold molar excess of unlabeled oligo B and competed most of both bands (Fig. 2, lane 4). We then used a 100-fold molar excess of unlabeled Sp1 consensus oligonucleotide in EMSA that resulted in both complexes being competed (Fig. 2, lane 5). The same results were obtained with oligonucleotide B (Fig. 2, lanes 7 through 10). These results indicate that the interaction of nuclear proteins with oligonucleotides A and B resulted in the formation of two sequence specific DNA-protein complexes.

To determine the minimal sequence requirements of sites A and B to bind nuclear proteins, a series of point

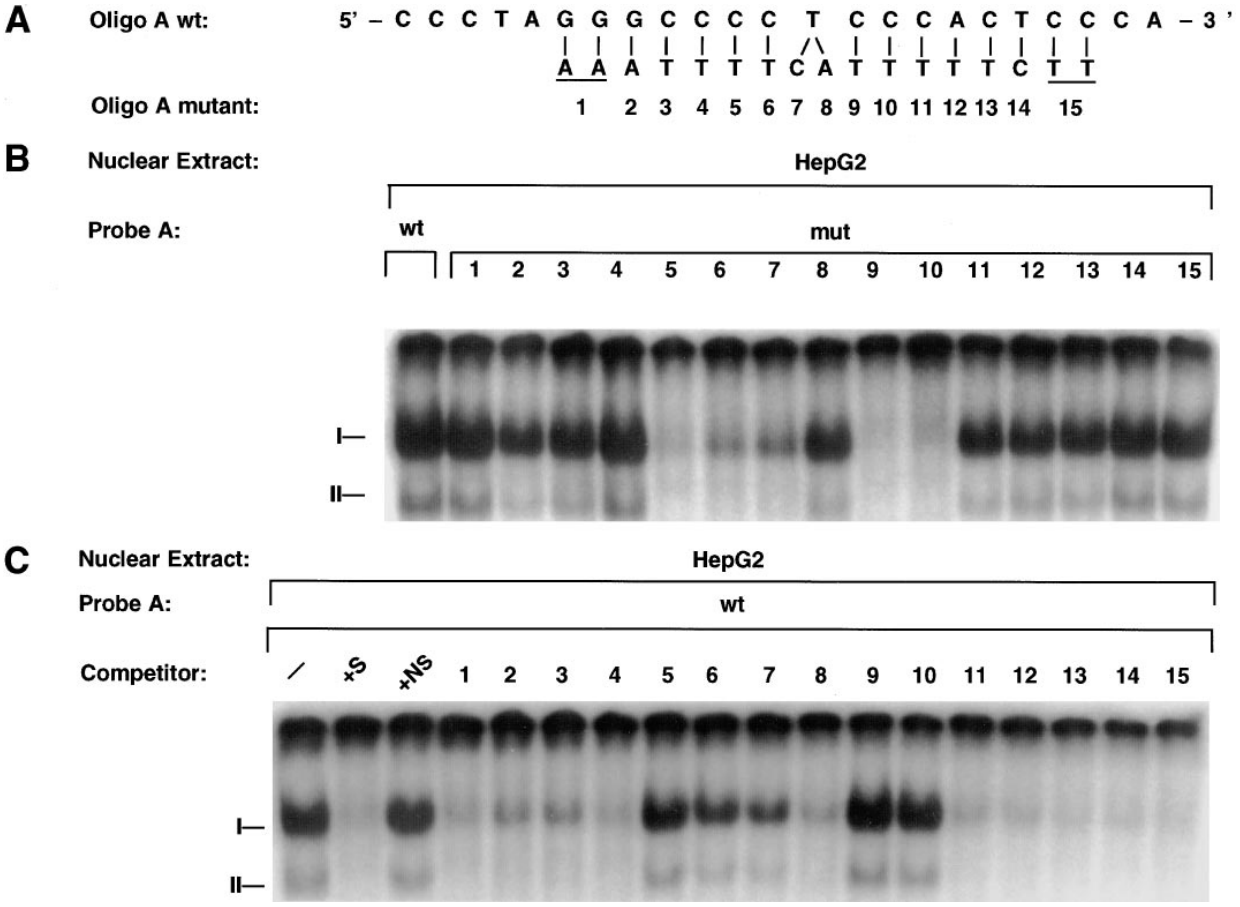

Fig. 3. Relative binding affinities of HepG2 nuclear proteins to selected site mutations of the LCAT site A. Panel A, nucleotide sequences of the wild-type LCAT site A oligo (−51 to −29) and of the oligo A mutants 1 to 15 used in EMSA. Mutations introduced are as indicated. Panel B, EMSA showing binding of HepG2 nuclear proteins to end-labeled wild-type LCAT site A oligo (wt), or labeled oligo A mutants (mut) 1 to 15 as indicated. Panel C, EMSA showing binding of HepG2 nuclear proteins to labeled wild-type LCAT site A oligo (wt) in the presence of 100-fold molar excess of unlabeled site A mut 1 to 15, respectively. S, specific; NS, nonspecific. I and II as described in legend of Fig. 2.

mutations were introduced into the oligonucleotides corresponding to sites A and B, and the resulting mutated oligonucleotides were used as probes or competitors in EMSA (Fig. 3). The five bases from −41 to −37 and repeated at −62 to −58, consisting of nucleotides CCTCC, are most critical for complex formation. When used as labeled probes, mutations outside this repeated sequence partially decreased the formation of DNA-protein complexes but did not eliminate its formation. Interestingly, mutation of nucleotide T_{39} to A_{39}, which results in the formation of an intact consensus binding site for the Sp family of transcription factors, increased the formation of complexes I and II (Fig. 3B, mut 8). Competition experiments using the oligo A wild type as labeled probe and 100-fold molar excess of unlabeled mutant oligonucleotides resulted in specific competition with those mutant oligos that formed complexes when used as probes (Fig. 3C). Similar results were seen when oligo B wild-type was used as labeled probe and 100-fold molar excess of unlabeled mutant A oligonucleotides was used as competitors (data not shown).

Sp1 and Sp3 proteins bind the −72 to −27 region of the human LCAT promoter

Sites A and B contain a mismatched consensus sequence recognized by several members of the Sp family (18, 25, 26). To study the relationship between Sps and sites A and B, we performed EMSA under several conditions, using purified Sp1 protein, in vitro translated Sp3, and crude nuclear extracts. Purified Sp1 can indeed bind to sites A and B and the resulting complex migrates with a molecular weight size identical to complex I (**Fig. 4**, lane 7). The relationship between Sp1 and the nuclear proteins binding to sites A and B was further investigated by using a polyclonal antibody specific for human Sp1. The antibody against human Sp1 recognized and supershifted part of complex I, indicating that sites A and B are bound by at least two different proteins, one of which is Sp1 (Fig. 4, lane 2). As several GC binding-proteins have been identified as members of the Sp multigene family of transcription factors, EMSA was performed in the presence of antibodies specific for human Sp2, Sp3, or Sp4 transcription factors (Fig. 4). The formation of the lower half of complex I and all of complex II was abolished by incubation of nuclear extracts with an antibody specific to human Sp3 (Fig. 4, lane 4), whereas antibodies to either Sp2 or Sp4 did not affect the formation of complexes I and II (Fig. 4, lanes 3 and 6). When antibodies to Sp1 and to Sp3 were incubated together with site A oligonucleotide, both complees I and II were supershifted or disrupted (Fig. 4, lane 5). When in vitro translated Sp3 protein was used in EMSA, the only complex formed migrated similar to complex I (Fig. 4, lane 8). However, as noted above, when antibodies to Sp3 were used in EMSA, part of complex I was disassociated. Sp3 has been reported to be present in cells as two isoforms of 115 and 80 kDa (27). Similar results were seen when oligo B was used in the same EMSA conditions (data not shown). These results strongly suggest that the two GC-like boxes in the proximal promoter of the LCAT are occupied by Sp1 and Sp3.

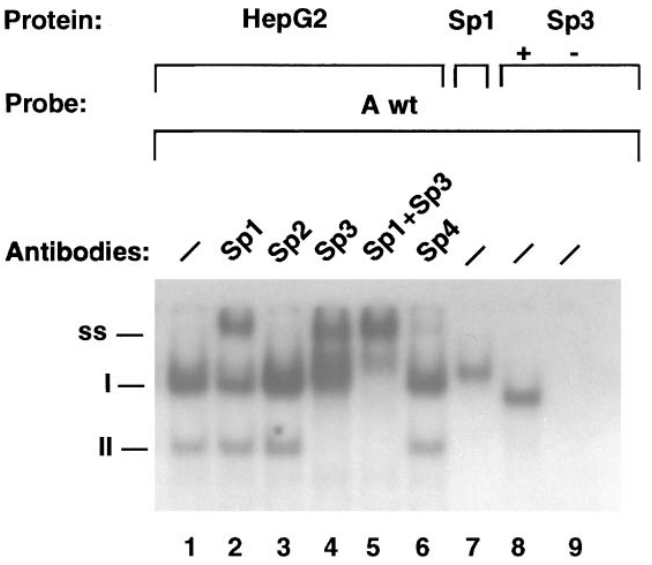

Fig. 4. Characterization of the factors binding to LCAT site A. Panel A, EMSA showing binding of HepG2 nuclear extracts to labeled LCAT site A oligo incubated in the presence of antibodies specific for human Sp1 (lane 2), Sp2 (lane 3), Sp3 (lane 4), Sp4 (lane 6). Both anti-Sp1 and anti-Sp3 antibodies were incubated together (lane 5) (see Experimental Procedures). Affinity-purified recombinant human Sp1 was incubated with labeled site A (lane 7). In vitro translated Sp3 was incubated with labeled site A (lane 8) or unprogrammed extract (lane 9). I and II as described in legend of figure 2. ss, shows the supershift complex in lanes 2, 4 and 5.

Sites A and B are equally important in determining promoter activity

To examine the functional relevance of sites A and B, site-directed mutants were engineered in the Sp binding consensus sequences present in sites A and B that abolish the formation of DNA–protein complexes in EMSA. Compared to wild-type −72LCATLuc plasmid, −72LCATLuc plasmid containing mutations in sites A or B resulted in an ~50% reduction of promoter activity, respectively (**Fig. 5**). This suggests that both sites are equally important in promoter activity and cooperative interactions between both sites are not required for activity.

Sp1 can trans-activate the human LCAT proximal promoter whereas Sp3 acts as repressor of Sp1-mediated transcription

Sp1 and Sp3 are ubiquitously expressed transcription factors. Various reports differ on whether Sp3 is a transcriptional activator or repressor, suggesting that the function of Sp3 may be context or cell type dependent. We wanted to determine the significance and functional effects that Sp1 and Sp3 have in regulating the LCAT proximal promoter activity. We performed transfection experiments in *Drosophila* SL2 cells as they represent the preferred system in which to study in vivo the functional effects of the Sp transcription factors. SL-2 cells are the only higher eukaryotic cells in which the basal transcription machinery is conserved, yet endogenous Sp1 and Sp3 are absent (17, 28). In these experiments, a constant amount of −72LCATLuc promoter/reporter plasmid was

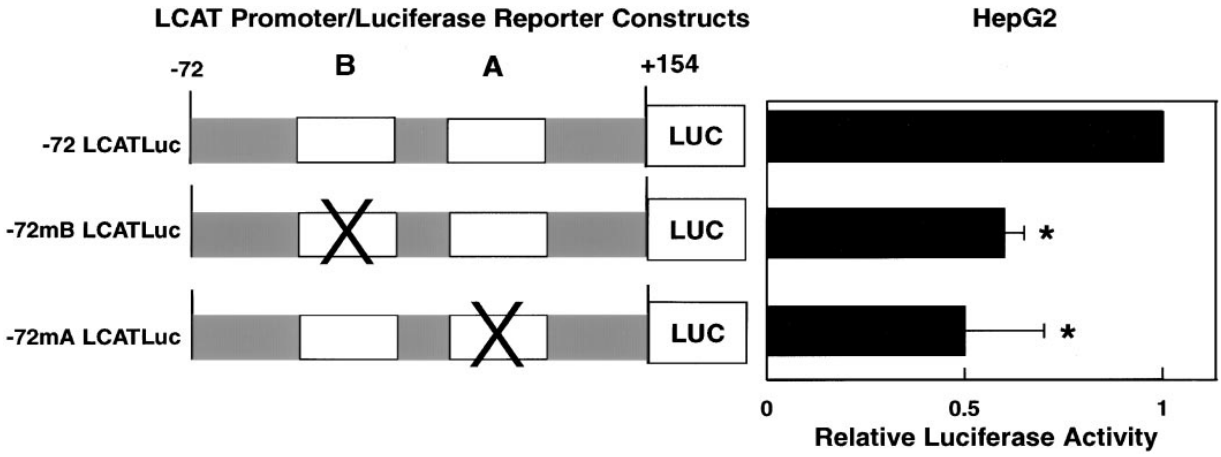

Fig. 5. Activation of the LCAT proximal promoter in the absence of functional site A or B. Wild-type and mutant constructs containing the LCAT proximal promoter region between −72 and +154 fused to the luciferase gene were transfected into HepG2 as described under Experimental Procedures. Wild-type and mutant versions of the site A or B constructs are indicated at the left. The right panel represents the luciferase activity of each reporter construct as the mean of three separate experiments relative to −72LCATLuc construct (activity: 1) ± standard deviation. An asterisk denotes statistical significance (*$P < 0.0001$).

transfected along with 10, 20, 50, 100, 200 and 300 ng of the Sp1 or Sp3 expression vectors. The luciferase activity of the resulting extracts was plotted as a function of the amount of Sp1 and Sp3 expression plasmids (**Fig. 6A**). Activation of the LCAT promoter increased rapidly between 10 and 200 ng of transfected expression plasmid pPacSp1. Higher amounts of the Sp1 expression plasmid further increased luciferase activity, but luciferase gene expression reached a plateau. These findings confirm that Sp1 is capable of strongly *trans*-activating a reporter gene linked to the LCAT promoter containing Sp binding sites. On the other hand, co-expression of the −72LCATLuc reporter plasmid along with increasing amounts of Sp3 did not affect luciferase gene expression (Fig. 6A), even when 1 μg and 4 μg of the Sp3 expression plasmid were used (data not shown).

As Sp3 is believed to repress Sp1-mediated transcriptional activation of many cellular and viral gene promoters (29), we determined the functional contribution of Sp3 to the transcriptional activity of the LCAT promoter. In these experiments, constant amounts of −72LCATLuc promoter/reporter and Sp1 expression plasmids were cotransfected along with an increasing amount of Sp3 expression plasmid. As shown in Fig. 6B, when the LCAT promoter is activated upon expression of Sp1, Sp3 represses transcription in a dose-dependent manner suggesting that the nuclear Sp1/Sp3 ratio may determine the transcriptional activity of the LCAT promoter. Western blot analyses have confirmed that Sp3 suppresses the action of Sp1 and does not affect its expression. The expression of Sp1 and Sp3 plasmids was demonstrated by immunoblot analyses of nuclear extracts isolated from either untransfected or transfected cells. As shown in Fig. 6C, Sp1 and Sp3 proteins are absent in untransfected cells whereas cells cotransfected with Sp1 and Sp3 expression plasmids clearly demonstrated the presence of Sp1 and Sp3. Although the exact concentration has not been determined, the level of Sp1 protein appears to be higher than Sp3. These results are likely due to a more efficient expression of the Sp1 plasmid (driven by the *Drosophila* actin 5C promoter) and could explain the concentrations of Sp3 expression plasmid required to repress Sp1-transcriptional activation.

DISCUSSION

In the present study, we focused on the proximal region of the LCAT promoter and defined the *cis*-elements, identified the interacting *trans*-acting factors, and demonstrated their functional effects and significance in determining transcriptional activity. The proximal 72 nt region of the human LCAT promoter is GC-rich and contains non-consensus sites that could potentially bind nuclear proteins that recognize GC-rich sequences. Using site-directed mutagenesis, we demonstrated that sites A and B are functional and equally important in activating transcription. These results support the hypothesis that *trans*-acting factors can bind simultaneously and independently to adjacent sites if the center-to-center distance of these sites is more than 10 bp (30). Comparison of the different sequences recognized by the Sp family of transcription factors suggests that the basic recognition unit is a 10 nt motif with a consensus sequence 5′-(G/T)GGGCGGPuPuPy-3′ known as the GC box. However, subsequent analyses of GC boxes from several hundred RNA polymerase II promoters revealed that the consensus sequence contains additional degeneracies such as a C to T transversion of the central C (GT box) (32). The LCAT promoter contains two identical 5′-CCCTCCCACT-3′ motifs spaced by 11 bp. In this study, in vitro binding assays have demonstrated that the central T to A transversion had little effect on the formation of complexes I and II and the CCTCC core sequence (or its inverted form, GGAGG) is critical for the formation of DNA–protein complexes as unlabeled oligonucleotides with mutations in this region failed

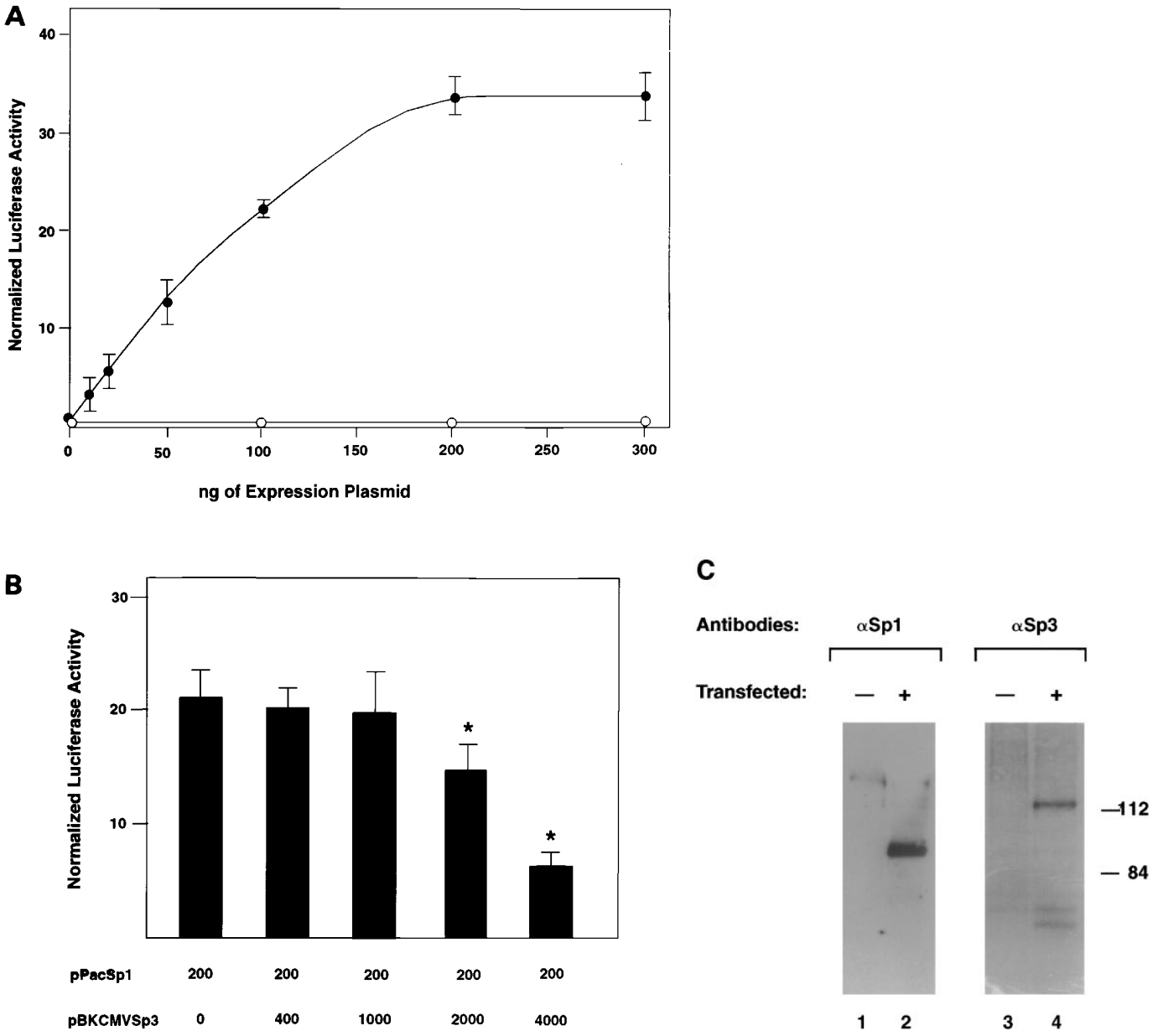

Fig. 6. *Trans*-activation of the LCAT proximal promoter by Sp1 but not Sp3. Panel A, the LCAT proximal promoter/luciferase plasmid −72LCATLuc was co-transfected with various amounts of the expression plasmid for Sp1 (closed circles) or Sp3 (open circles) in *Drosophila* SL-2 cells and the luciferase activity was measured. Normalized luciferase activity is as described in Experimental Procedures. Panel B, co-transfection of the −72LCATLuc reporter plasmid with 200 ng of Sp1 expression plasmid pPacSp1 and with varying amounts of Sp3 expression plasmid pBKCMVSp3. Mean of six experiments ± standard deviation. An asterisk denotes statistical significance (*$P < 0.005$). Panel C, Western blot of *Drosophila* SL-2 nuclear extracts that were not transfected (−) or co-transfected with 100 ng of pPacSp1 and 2500 ng of pBKCMVSp3 (+). Immunoblot analyses were performed with polyclonal antibodies to human Sp1 (lanes 1 and 2) and Sp3 (lanes 3 and 4). Five μg of nuclear extract was loaded on an 8% SDS-PAGE in lanes 1 and 2 and 10 μg of nuclear extract was loaded in lanes 3 and 4. Immunoblot of lanes 1 and 2 was exposed for 10 min and the immunoblot of lanes 3 and 4 was exposed 2 h. The migration of molecular mass markers (kDa) is indicated at the right of the autoradiogram.

to compete the complexes formed by incubation of labeled site A or B consensus sequences with HepG2 nuclear extracts. This binding site sequence, which we call the GA box, is also present in the context of several natural promoters (33).

We have demonstrated that Sp1 and Sp3 bind to sites A and B on the LCAT promoter, both containing a GA box, and form complexes I and II in EMSA. Complex I migrates similar to the complex formed with purified human Sp1, and the upper half of complex I is recognized and supershifted by an antibody specific to human Sp1. The formation of the lower half of complex I and all of complex II results from binding of Sp3 as anti-Sp3 antisera interfered with the formation of the complexes. All complexes were supershifted when both antisera to Sp1 and to Sp3 were used together with site A or site B. The use of in vitro

transcribed and translated Sp3 in EMSA resulted in a single complex that migrated similar to complex I. Analysis of the ^{35}S-methionine transcribed Sp3 protein by SDS-PAGE resulted in a single band near the 110 kDa range, as expected, and similar to the 109 kDa of Sp1 (data not shown). This discrepancy may be explained by the report of internally initiated translation of Sp3 mRNA that resulted in an 80 kDa product (27).

It is generally accepted that Sp1 stimulates transcription and Sp3 represses Sp1-mediated transcriptional activation, suggesting that Sp3 is an inhibitory member of the Sp family of transcription factors (28). Our studies in Schneider's *Drosophila* SL2 cells demonstrated that Sp1, but not Sp3, activates the human LCAT promoter. Also, when Sp1 is co-transfected along with Sp3, Sp3 functions as a dose-dependent repressor of Sp1-mediated activation. These data suggest that the levels of Sp3 or the nuclear Sp1/Sp3 ratio may play a role in regulating the transcriptional activity of the LCAT promoter.

The presence of several regulatory proteins interacting at sites A and B raises the intriguing question as to what determines the transcriptional level and tissue specificity of LCAT in vivo. LCAT mRNA has been detected in liver and the human hepatoma cell line HepG2. However, more recent studies, performed in mouse, rhesus monkey and chicken tissues indicate that LCAT expression is not restricted to liver but also occurs in testis and brain (1, 11, 34). In addition, recent experiments in transgenic mice have demonstrated that the expression of a human LCAT genomic fragment containing 561 bp of 5′ untranslated region occurs primarily in the liver, and indicated that the liver is the major and possibly the only significant cellular source of plasma circulating LCAT (8).

Although ubiquitous, Sp1 and Sp3 could influence transcriptional levels and possibly tissue specificity of the LCAT gene in several ways. Sp1 levels vary greatly in different cell types and during development (35, 36). This could, in part, explain the LCAT mRNA expression pattern observed in rat and chicken as well as in human fetal liver (1, 11). Similarly, Sp1 is expressed at different levels in gastric (35) and hematopoietic development (37) and influences gene expression in these cells (37, 38). Because Sp3 functions as a transcriptional repressor, developmental and cell type-specific regulation of LCAT gene expression could also be influenced by a dynamic positive and negative regulation exerted by Sp1 and Sp3.

It is becoming increasingly clear that Sp1 binding and *trans*-activation is regulated by a variety of stimuli that are critical in the regulation of cellular growth and differentiation. These stimuli result in various post-translational modifications of the Sp1 protein. One mechanism is the glycosylation of Sp1 with O-linked sugars which appears to play a role in transactivation (39, 40). A second mechanism for Sp1 regulation is phosphorylation, which activates or represses Sp1 depending on the specific kinase (41).

There is growing evidence of other mechanisms through which Sp1 may influence differential gene expression. It is known that Sp1 interacts with the transcriptional machinery by binding to $TAF_{II}110$ (42). However, it has been reported that the C-terminal domain of Sp1 interacts with other transcription factors and is involved in synergistic activation. When bound to distant sites in *cis*, it can interact with itself, thus looping out the intervening DNA (43). This suggests that Sp1 may establish interactions between promoters and distant regulatory elements in vivo through such a looping mechanism. Sp1 binding sites appear in numerous promoters and other regulatory sequences of tissue-specific genes. Sp1 interaction with C/EBPβ enables transcriptional activation of the liver-specific gene CYP2D5, a member of the cytochrome P450 superfamily of enzymes (44). The activation potential of HNF-4 on the apoC-III gene is dependent on the binding of multiple Sp1 molecules (45). Interaction of cholesterol-sensitive sterol regulatory element-binding proteins (SREBPs) and Sp1 stimulates binding of Sp1 to the low density lipoprotein (LDL) receptor promoter, followed by enhanced stimulation of transcription (46, 47).

It may be that interaction with tissue-specific factors, perhaps bound to elements upstream of the region analyzed in this study of the LCAT promoter, interact with Sp1 at the critical sites that we have identified. This hypothesis is in agreement with the genomic sequence in the junction region between LCAT and its upstream neighboring gene (15, 48). This intergenic region contains putative binding sites for transcription factors that could be involved in the transcriptional activity and tissue-specific pattern of expression. This hypothesis is also supported by the presence of a sequence located in the proximal 500 bp of the LCAT promoter which is responsible for high level and tissue-specific expression of the LCAT gene (K. L. Hoppe and O. L. Francone, unpublished data). So far, it is unclear how this region communicates with the proximal promoter region and whether the Sp factors are involved in this interaction. Further investigations will be required to unravel the mechanisms and interplay between *cis*-elements and *trans*-acting factors controlling the gene expression of this key enzyme in plasma cholesterol metabolism.

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