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A Silencer Element for the Lipoprotein Lipase Gene Promoter and Cognate Double- and Single-Stranded DNA-Binding Proteins

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Transfection experiments with constructs containing various 5′-deleted fragments of the human lipoprotein lipase (LPL) promoter and the chloramphenicol acetyltransferase reporter gene revealed an LPL silencer element (LSE) in the region of nucleotides −225 to −81 of the LPL gene that functioned in Chinese hamster ovary (CHO) and HeLa cells. Gel retardation competition analysis showed the presence of a nuclear factor(s) capable of binding to the sequence of nucleotides −169 to −152 of LSE (LSE-6) in a single-stranded (opposite-strand) and double-stranded specific fashion, the binding affinity being almost the same in the two binding forms. Site-directed mutagenesis indicated that almost the entire sequence of LSE-6 was necessary to form the complexes and also critical for silencing activity in CHO cells. The amounts of this binding factor(s) in CHO and HeLa cells were closely associated with transcriptional silencing activity. Photochemical cross-linking experiments indicated that the single- and double-stranded elements recognized the same binding factor(s) with molecular masses of 54 to 63 kDa and 109 to 124 kDa. The 109- to 124-kDa DNA binding factor(s) was found to be a doublet of that of the 54- to 63-kDa factor by isoelectric focusing or by increasing the time of exposure to UV irradiation. When inserted upstream of another gene such as that of the simian virus 40 enhancer/promoter of pSV$_2$CAT, the sequence of nucleotides −190 to −143 (LSE-1) also suppressed transcription of the reporter gene in CHO cells. These results strongly suggest that the LSE plays a role in regulation of LPL gene expression by suppressing its transcription.

Lipoprotein lipase (LPL) is located on the luminal surface of capillary endothelial cells, where it hydrolyzes triglycerides in chylomicrons and very-low-density lipoproteins, thereby liberating fatty acids for storage in adipose tissue. Any factors that inhibit LPL activity cause impairment of plasma lipoprotein processing. In fact, individuals with a genetic deficiency of LPL activity exhibit extreme postprandial hypertriglyceridemia (47). Cachexia caused by severe infection or malignancies is a typical case of this condition, because this fatal syndrome has been shown experimentally to be induced in animals by intraperitoneal injection of certain cytokines that strongly suppress LPL activity (11, 24, 29, 42, 48). LPL, therefore, plays a crucial role in lipid metabolism in general. This enzyme is known to be regulated at both transcriptional and posttranscriptional levels during differentiation and in response to nutritional and hormonal changes (17).

The human LPL gene is approximately 30 kb long and consists of 10 exons interrupted by 9 introns (6, 18). Within the 730-bp region upstream of the transcriptional start site, several potential binding sites for known transcription factors have been identified (32): a TATA box at nucleotide −27, two CCAAT motifs at −65 and −506, homologous binding sites for C/EBP at −68 and −509, a glucocorticoid-responsive element at −644, a cyclic AMP-responsive element at −306, and three octanucleotide motifs at −580, −186, and −46 bp. There is evidence that the TATA, Oct-1, and CCAAT sites modulate the promoter function of the LPL gene as proximal positive *cis*-acting elements (14). However, no information is yet available on the *cis*-acting elements that negatively regulate expression of the LPL gene.

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In the present study, we tested for such elements. Our results revealed the presence of a silencer sequence in the upstream region of the LPL gene. We also studied the corresponding nuclear binding proteins of LPL-producing CHO cells (34) and LPL-nonproducing HeLa cells.

MATERIALS AND METHODS

Cell culture. The human epitheloid carcinoma cell line HeLa S3 (HeLa) was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml. The Chinese hamster ovary (CHO) cell line was a gift from Lawrence A. Chasin (Columbia University) and was maintained in α minimum essential medium with the same supplements as listed above.

Plasmid construction. The promoter region of the LPL gene (nucleotides −730 to +15) was amplified by PCR with reference to the published human LPL sequence (18) and was subcloned into the *Sma*I site of pUC0CAT (28). A series of 5′ deletions were generated by exonuclease III digestion. Site-directed mutagenesis was performed by synthesizing oligonucleotides which change a 5-base sequence from the wild-type sequence at position −162 to −166, and the resulting sequence was amplified by PCR. Mutated plasmid LSE-6-A was constructed by insertion of the mutated PCR products into the *Kpn*I-*Hin*dIII sites of pLPL-183CAT. Plasmids pLSE-1SV$_2$CAT and p1-ESLSV$_2$CAT were constructed by insertion of oligonucleotide LSE-1 (nucleotides −190 to −143) into the *Acc*I site of pSV$_2$CAT in the same orientation and reverse orientation, respectively, relative to the LPL promoter.

Transfection and CAT assay. Preconfluent monolayers of HeLa cells in 35-mm-diameter dishes were transfected with 5 μg of plasmid DNA with the use of Lipofectin (GIBCO Laboratories, Grand Island, N.Y.) (8). CHO cells were transfected with 5 μg of plasmid DNA by the modified calcium phosphate coprecipitation method (36). For construction of an internal control, 2 μg of β-galactosidase-encoding construct pCH110 (Pharmacia LKB Biotechnology, Uppsala, Sweden) was cotransfected with chloramphenicol acetyltransferase (CAT) constructs. Cell extracts were prepared by three cycles of freeze-thawing for determinations of levels of CAT (13) and β-galactosidase (25) activities. Each transfection experiment was carried out in triplicate. The values of CAT activities were normalized against those of β-galactosidase.

Gel retardation analysis. Nuclear extracts were prepared by the procedure described by Dignam et al. (7). A double-stranded LSE-1 probe was generated by digestion of pLSE-1SV$_2$CAT with *Acc*I. Single-stranded LSE-1 and LSE-2 (nucleotides −121 to −71) probes and competitor oligonucleotides were synthe-

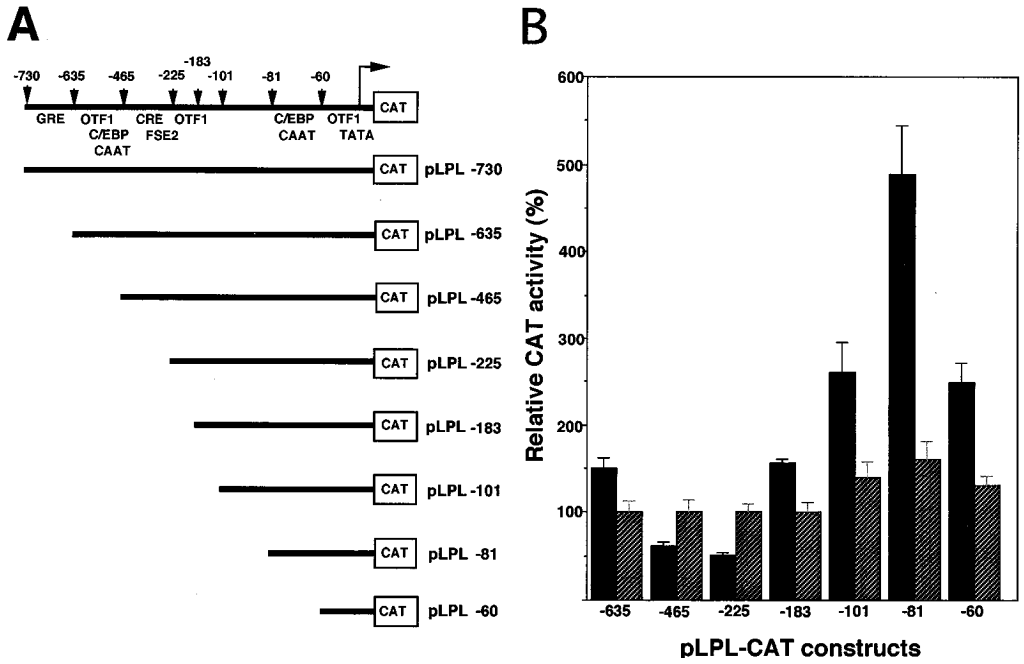

FIG. 1. CAT plasmids constructed with the 5′-flanking regions of the LPL gene and their promoter activities in CHO and HeLa cells. (A) The pLPL-CAT constructs contained nucleotides (specified in parentheses) of the 5′-flanking region of the LPL gene: pLPL-730 (−730 to +15), pLPL-635 (−635 to +15), pLPL-465 (−465 to +15), pLPL-225 (−225 to +15), pLPL-183 (−183 to +15), pLPL-101 (−101 to +15), pLPL-81 (−81 to +15), and pLPL-60 (−60 to +15). Other elements indicated and their nucleotide positions include OTF-1 at −580, −186, and −46; C/EBP at −68 and −509; a glucocorticoid responsive element at −644; a cyclic AMP-responsive element at −306; a TATA box at −27; and CCAAT boxes at −65 and −506. (B) CAT activity expressed as a percentage of the activity obtained with pLPL-730. Transfections were performed in duplicate in three separate experiments. T-bars indicate standard deviations. Solid boxes, CHO cells; hatched boxes, HeLa cells.

sized with a Applied Biosystems 394 DNA synthesizer. Gel retardation analysis (10) was conducted by using 5 μg of extracted nuclear protein and 0.1 pmol of ^{32}P-end-labeled DNA.

Biochemical analysis of LPL silencer element (LSE) binding protein. Photochemical cross-linking in situ was performed as described previously (40, 45). The gel slices containing 54- to 63-kDa and 109- to 124-kDa protein-DNA complexes were completely digested with 4 mg of *N*-tosyl-L-phenylalanyl chloromethyl ketone–trypsin (Sigma Chemical Co., St. Louis, Mo.) per ml at 37°C for 12 h, and the peptide cross-linked to the probe DNA was analyzed by isoelectric focusing in the presence of urea as described previously (15).

RESULTS

Recognition of the LSE by CAT assay. To determine the possible location of negative regulatory elements within the LPL promoter (nucleotides −730 to +15), we subcloned a series of 5′ deletions of the promoter in pUC0CAT (Fig. 1A) and transfected these CAT constructs into CHO and HeLa cells. The CAT activities observed with the various plasmids normalized against that of the pLPL-730 CAT construct are shown in Fig. 1B. With CHO cells, which constitutively produce LPL (34), mutants with deletions from nucleotides −730 to −635 showed a moderate increase and those with deletions from −635 to −225 showed a moderate decrease of CAT activity, whereas those with deletions from −225 to −81 showed a 9.6-fold increase in expression. Further deletion from −81 to −60 caused a 50% decrease from that of the −81 construct. These results suggest the presence of a strong negative *cis*-acting element between nucleotides −225 and −81 and positive *cis*-acting elements between nucleotides −635 and −225 and −81 and −60.

The functions of these putative *cis*-acting elements in LPL-nonproducing cells were assessed by transfecting the pLPL-CAT constructs into HeLa cells (Fig. 1B). In contrast to the results obtained with CHO cells, the pLPL-730 CAT construct led to very low levels (one-fifth the CAT activity) in both these cell lines, and deletion from nucleotides −225 to −81 resulted in slight up regulation of CAT activity in HeLa cells. These results suggest that the inhibitory effect of the putative negative *cis*-acting element (nucleotides −225 to −81) is functional in LPL-producing cells but has little if any function in LPL-nonproducing cells. We named this element LSE and next characterized it in more detail.

Nuclear factors binding to single- and double-stranded LSE-1. The specific nuclear factors that bind to the LSE were examined by gel retardation analysis with synthetic double-stranded oligonucleotides (LSE-1, nucleotides −190 to −143; and LSE-2, nucleotides −121 to −71) included in the LSE. Incubation of the LSE-1 oligonucleotide with nuclear extracts from CHO cells resulted in two major (M1 and M2) and one minor (m) gel shift complexes (Fig. 2, lane 1). Similar, but much weaker, signals of complexes were observed with nuclear extracts from HeLa cells (Fig. 2, lane 5). Hence, the amount of the LSE-1 binding factor (LSE-BF) appeared to be larger in CHO cells than in HeLa cells, and the amount (Fig. 2) is considered to reflect the silencing activities as shown in the 5′ deletion analysis (Fig. 1). These complexes appeared to be specific, since their formation was competitively prevented by unlabeled LSE-1 oligonucleotide (Fig. 2, lanes 2 and 6) but not by an unlabeled irrelevant (CTF/NF-1) oligonucleotide (Fig. 2, lanes 4 and 8). On incubation with the LSE-2 oligonucleotide, no complexes were detected (data not shown).

Next, we examined the ability of single-stranded oligonucleotide sequences to compete with the binding of the LSE-BF to the double-stranded LSE-1 probe. Results showed that opposite single-stranded oligomers of LSE-1 (the lower strand of the LSE-1 site, designated LSE-1-L) competed specifically with binding of the double-stranded probe (Fig. 2, lanes 3 and 7; Fig. 3A, lane 2) but that its complementary single-stranded

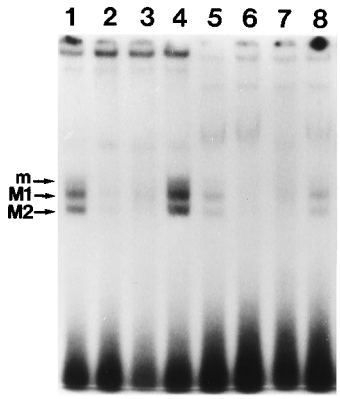

FIG. 2. Binding of double-stranded LSE-1 to nuclear extracts from CHO and HeLa cells. Radiolabeled double-stranded LSE-1 (0.1 pmol) was incubated with 5 μg of nuclear extracts from CHO cells (lanes 1 to 4) and HeLa cells (lanes 5 to 8). Either no competitor (lanes 1 and 5) or the competitor DNAs double-stranded LSE-1 at a 100-fold molar excess (lanes 2 and 6), single-stranded LSE-1 with the lower strand at a 100-fold molar excess (LSE-1-L) (lanes 3 and 7), and an unrelated oligonucleotide, CTF/NF-1, at a 100-fold molar excess (lanes 4 and 8) were added to the labeled probe. The arrows at M1 and M2 denote the major protein-DNA complexes, and that at m denotes the minor complex.

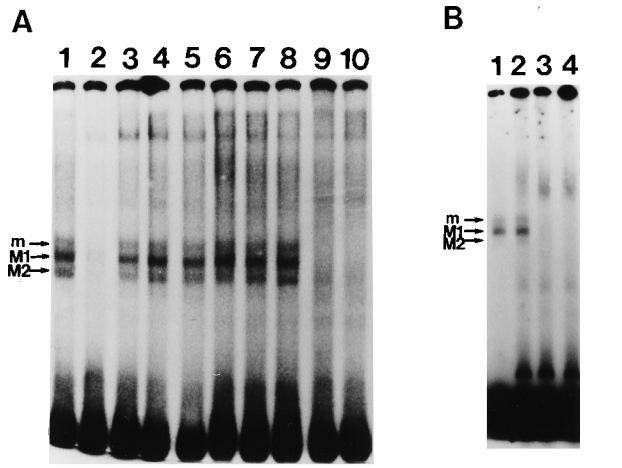

FIG. 3. Competitive gel retardation analysis to characterize asymmetric binding of LSE-BF to the double- and single-stranded LSE-1 sites. (A) Radiolabeled double-stranded LSE-1 (0.1 pmol) was incubated with 5 μg of nuclear extracts from CHO cells (lanes 1 to 7). Either no competitor (lane 1) or the competitor DNAs single-stranded LSE-1 with the lower strand (LSE-1-L) in a 100-fold molar excess (lane 2); single-stranded LSE-1 with the upper strand (LSE-1-U) in 100-fold (lane 3), 200-fold (lane 4), 400-fold (lane 5), and 800-fold (lane 6) molar excesses; and unrelated single-stranded CTF/NF-1 in an 800-fold molar excess (lane 7) were added. Radiolabeled single-stranded LSE-1 (0.1 pmol), LSE-1-L (lanes 8 and 9), and LSE-1-U (lane 10) were incubated with 5 μg of nuclear extracts from CHO cells. As the competitor DNA, single-stranded LSE-1-L was added in a 100-fold molar excess (lane 9). (B) Radiolabeled single-stranded LSE-1-L (0.1 pmol) was incubated with 5 μg of nuclear extracts from CHO cells (lane 1). The double-stranded LSE-1 oligonucleotide was obtained by annealing the labeled LSE-1-U oligonucleotide with an equimolar amount of unlabeled LSE-1-L oligonucleotide. This radiolabeled double-stranded LSE-1 (0.1 pmol) was incubated with 5 μg of nuclear extracts from CHO cells (lanes 2 to 4). Either no competitor (lane 2) or the competitor DNAs single-stranded LSE-1-L (lane 3) and double-stranded LSE-1 (lane 4) in a 100-fold molar excess were added. The arrows at M1 and M2 denote the major protein-DNA complexes, and those at m denote the minor complex.

oligomers (upper strand of the LSE-1 site, designated LSE-1-U) (Fig. 3A, lanes 3 to 6) and unrelated single-stranded oligomers (Fig. 3A, lane 7) had no effect even at an 800-fold molar excess compared with the labeled probe. To confirm asymmetric binding of the LSE-BF, the labeled single-stranded LSE-1-L and LSE-1-U were used as the probe for the gel retardation analysis. Incubation of the LSE-1-L oligonucleotide with nuclear extracts from CHO cells resulted in the formation of the sequence-specific M1, M2, and m gel shift complexes (Fig. 3A, lanes 8 and 9), and the double-stranded LSE-1 also competed with the binding completely (data not shown). However, the LSE-1-U oligonucleotide could not form these complexes (Fig. 3A, lane 10). It is important to exclude the possibility that the observed binding of the double-stranded DNA reflects binding to a small population of single-stranded DNAs present in the double-stranded DNA molecules. The double-stranded probe prepared by labeling LSE-1-U oligonucleotide and annealing it to unlabeled LSE-1-L oligonucleotide formed complexes identical to those of the single-stranded LSE-1-L oligonucleotide probe (Fig. 3B, lanes 1 to 4). Since the LSE-1-U oligonucleotide probe did not bind to the LSE-BF, the observed complexes were generated by double-stranded DNA. It is thus likely that the LSE-BF binds both double- and single-stranded LSE-1 probes specifically to the LSE-1-L but not to the LSE-1-U.

We assessed the relative binding affinities of the single-stranded LSE-1 and double-stranded LSE-1 to the LSE-BF by competitive stoichiometric analysis. For this purpose, increasing amounts of competitor DNAs, consisting of either single (LSE-1-L)- or double-stranded LSE-1 sequences, were mixed with the radiolabeled double-stranded LSE-1 probe and nuclear extracts from CHO cells and the bound complexes formed were quantitated by gel retardation analysis. The double-stranded LSE-1 complex competed to the same extents with the single- and double-stranded forms (Fig. 4). Thus, the LSE-BF bound to both single-stranded LSE-1 and double-stranded LSE-1 with the same affinity.

Sequence required for formation of an LSE-1–LSE-BF complex. For further identification of the DNA sequence involved in LSE-1–LSE-BF binding, competition experiments were performed with single- or double-stranded forms of shorter fragments designated LSE-3 (nucleotides −184 to −149), LSE-4 (−179 to −153), LSE-5 (−174 to −156), LSE-6 (−169 to −152), LSE-7 (−180 to −161), LSE-8 (−182 to −172), LSE-9 (−172 to −162), and LSE-10 (−162 to −151) spanning different regions of LSE-1 (Fig. 5A). Double-stranded forms of fragments LSE-1, LSE-3, LSE-4, and LSE-6 effectively competed with the binding of the LSE-BF to the double-stranded LSE-1 probe (Fig. 5B, lanes 2 to 4 and 6). In contrast, the other truncated fragments, LSE-5, LSE-7 (Fig. 5B, lanes 5 and 7),

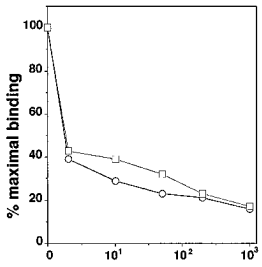

FIG. 4. Competition analysis using double- and single-stranded LSE-1 competitors. The indicated amounts of unlabeled competitor double-stranded LSE-1 (○) or single-stranded LSE-1 (□) were added to the binding reaction of a double-stranded probe and nuclear extracts from CHO cells. The radioactivity of bound materials was quantitated by Bio-Image Analyzer (BAS 3000; Fuji Film, Kyoto, Japan) after gel retardation analysis and plotted as percentages of the maximal binding (counts per minute bound in the presence of competitor/counts per minute bound in the absence of competitor × 100).

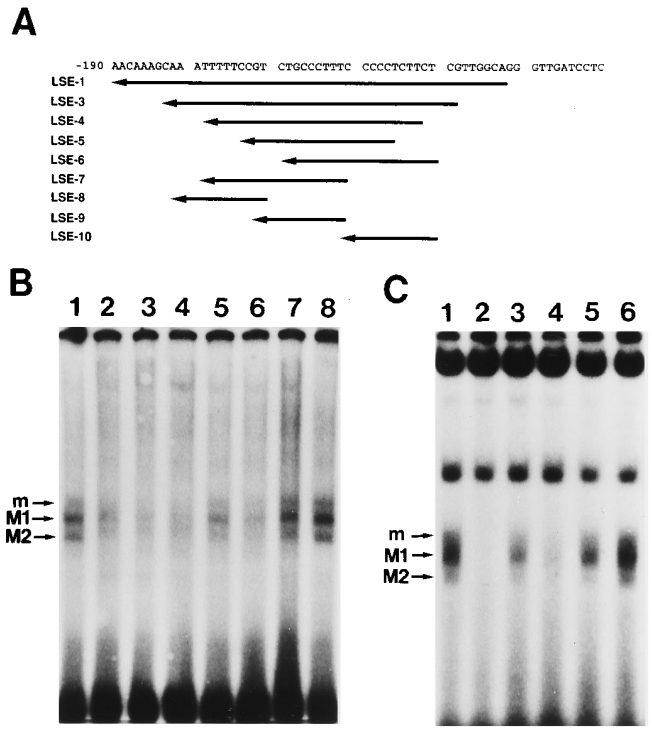

FIG. 5. Competitive gel retardation analysis to characterize the core sequence of the LSE. (A) The probe and competitor DNA segments used in the analysis are shown on the map of the 5′-flanking regions of the LPL gene from nucleotides −190 to −143. (B) Gel retardation analysis using CHO nuclear extracts and labeled double-stranded LSE-1 was performed in the absence (lane 1) or presence of the double-stranded competitor DNAs LSE-1 (lane 2), LSE-3 (lane 3), LSE-4 (lane 4), LSE-5 (lane 5), LSE-6 (lane 6), LSE-7 (lane 7), and CTF/NF-1 (lane 8) at a 100-fold molar excess compared with the labeled probe. (C) Gel retardation analysis using CHO nuclear extracts and labeled single-stranded LSE-1 (LSE-1-L) was performed in the absence (lane 1) or presence of the single-stranded competitor DNAs LSE-4 (lane 2), LSE-5 (lane 3), LSE-6 (lane 4), LSE-7 (lane 5), and CTF/NF-1 (lane 6) at a 100-fold molar excess compared with the labeled probe. The arrows at M1 and M2 denote the major protein-DNA complexes, and those at m denote the minor complex.

LSE-8, LSE-9, and LSE-10 (data not shown), were not competitive. Single-stranded forms of shorter fragments such as LSE-1, LSE-3, LSE-4, and LSE-6 also competed with the binding of the LSE-BF to the double-stranded LSE-1 probe (data not shown). We investigated the nature of the LSE-BF binding to the single-stranded probe by similar competition experiments using the single-stranded LSE-1 probe. As shown in Fig. 5C, single-stranded forms of fragments LSE-4 and LSE-6 effectively competed with the binding of the LSE-BF to the single-stranded LSE-1 probe (lanes 2 and 4), but LSE-5, LSE-7 (lanes 3 and 5), LSE-8, LSE-9, and LSE-10 (data not shown) did not. Thus, the LSE-BF bound to the single- and double-stranded LSE-1 probes with the same sequence specificity, and we deduced that the core sequence of the silencer element was 3′-ACGGGAAAGGGGGAGAAG-5′ (LSE-6).

To confirm that LSE-6 was the core sequence of the silencer element, we produced a mutation in this sequence and examined the binding of the LSE-BF to the mutated LSE-6 and its silencing activity. As shown in Fig. 6A, the four mutant oligomers examined have a replacement of the LSE-6 sequence by the sequence CGGCCGC at four different positions, resulting in four or five base substitutions introduced into the wild-type LSE-6. The blocking activity against formation of the LSE-1–LSE-BF complex was analyzed by gel retardation analysis and normalized to a value of 100% for wild-type LSE-6 (Fig. 6A). Any series of four mutant oligomers indicated very low blocking activities. Therefore, it is likely that almost the entire sequence of LSE-6 is necessary to form the complexes. This result is consistent with those of competitive gel retardation analysis using LSE-9 and LSE-10: half portions of LSE-6 were not competitive. One of the LSE-6 mutants, LSE-6-A, introduced into the pLPL-183 CAT construct containing 183 bp of LPL promoter upstream sequence yielded twofold increases of the transient expression in CHO cells (Fig. 6B). This observation implies an effect similar to that of the 5′ deletion analysis shown in Fig. 1. Thus, the core sequence required for formation of the complex was critical for silencing activity.

Characterization of LSE-BF by UV cross-linking. The molecular mass of the LSE-BF was determined by photochemical cross-linking using 5′-end-labeled double-stranded LSE-1 probe. Two major (M1 and M2) and one minor (m) gel shift

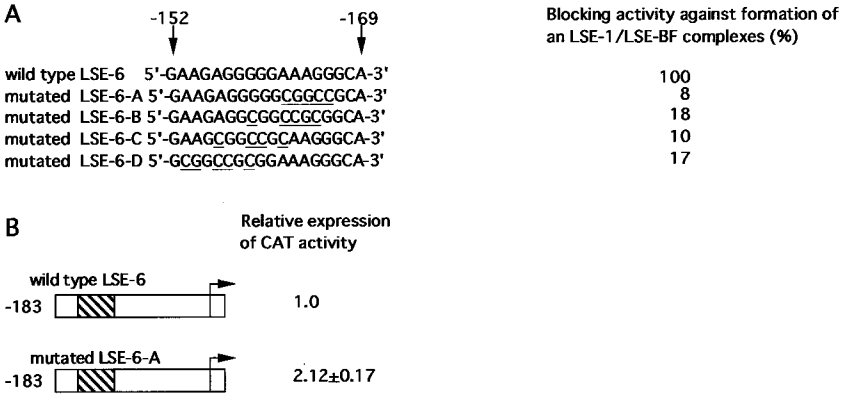

FIG. 6. Effects of mutation of the LSE-6 sequence on complex formation with LSE-BF (A) and transient CAT gene expression in CHO cells (B). (A) Competitive gel retardation analysis was performed in the presence of unlabeled competitors at a 100-fold molar excess compared with the labeled probe as described in the legends of Fig. 2 to 5. The blocking activity was determined by measuring the radioactivity of bound materials as described in the Fig. 4 legend (counts per minute bound in the absence of competitor was termed cpm-A [9.2×10^3 cpm], counts per minute bound in the presence of double-stranded wild-type LSE-6 as competitor DNA was termed cpm-W [6.8×10^2 cpm], and counts per minute bound in the presence of double-stranded mutated type LSE-6 as competitor DNA was termed cpm-M) and defined as (cpm-A − cpm-M)/(cpm-A − cpm-W) × 100. (B) The mutations shown in panel A (underlined sequences) were introduced into the indicated pLPL-183CAT construct (in the nucleotide positions indicated by hatching), and transient expression was assayed. CAT activity is expressed as the mean ± standard deviation of the ratio of mutant CAT activity to wild-type activity. Transfections were performed in duplicate in three separate experiments.

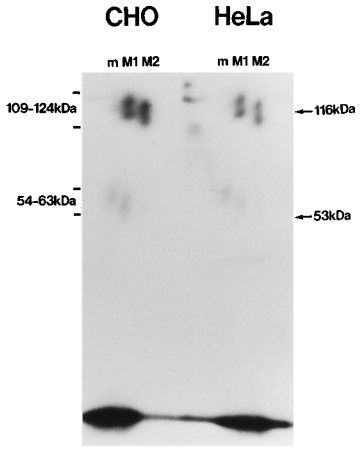

FIG. 7. Photochemical cross-linking of the LSE-BF in CHO, and HeLa cells. End-labeled double-stranded LSE-1 probe was incubated with nuclear extracts from CHO and HeLa cells. The products were analyzed by gel retardation analysis, and then the gel was UV irradiated for 30 min. The gel was autoradiographed, and specific protein-DNA complexes were excised and electrophoresed in SDS–8% polyacrylamide gel. The positions of molecular mass markers are shown on the right (by arrows). M1 and M2 denote the major protein-DNA complexes, and m denotes the minor complex detected by gel retardation analysis in Fig. 2, lanes 1 and 5.

complexes obtained with extracts of CHO and HeLa cells (as shown in Fig. 2) were excised, and the gels were cross-linked by UV irradiation for 30 min and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. As illustrated in Fig. 7, the factor(s) in both cell lines bound to the double-stranded LSE-1 comigrated at approximately 54 to 63 kDa and 109 to 124 kDa. The results demonstrated that the same LSE-BF formed a complex with the double-stranded LSE-1 probe in CHO and HeLa cells and that the LSE-BF was present in greater abundance in CHO cells than in HeLa cells. The differences in molecular masses of the three complexes m, M1, and M2 were slight, so the mobilities of these three complexes on gel retardation electrophoresis may reflect the molecular mass of LSE-BF.

As shown in Fig. 8A, the LSE-BF of CHO cells bound to the single-stranded LSE-1 probe also comigrated at 54 to 63 kDa and 109 to 124 kDa. This result suggests that the same DNA binding factor(s) is involved in complex formation with the single- and double-stranded LSE-1 sequences. With increases in the time of UV irradiation, the complexes with both single-stranded (Fig. 8A) and double-stranded (Fig. 8B) probes shifted gradually from 54 to 63 kDa to 109 to 124 kDa. Therefore, we concluded that the 109- to 124-kDa band might be a doublet of the 54- to 63-kDa LSE-BF.

The 54- to 63-kDa and 109- to 124-kDa LSE-BFs were excised from the gel after SDS-PAGE and completely digested with *N*-tosyl-L-phenylalanyl chloromethyl ketone–trypsin, and the peptide cross-linked to the DNA was analyzed by isoelectric focusing in the presence of urea. Figure 9 shows that the apparent isoelectric points of the peptides derived from the 54- to 63-kDa (lanes 1 and 3) and 109- to 124-kDa (lanes 2 and 4) LSE-BFs in CHO cells had the same isoelectric point. Thus, the binding regions of the 54- to 63-kDa and 109- to 124-kDa LSE-BFs were the same. The series of three complexes (m, M1, and M2) which showed broad bands and slightly different mobilities in UV cross-linking may indicate the presence of multiple proteins in the complex or partial proteolysis during isolation. The peptides derived from the 54- to 63-kDa and 109- to 124-kDa LSE-BFs in the m, M1, and M2 bands showed a single isoelectric point. From these results, we conclude that the 54- to 63-kDa and 109- to 124-kDa LSE-BF complexes constituting m, M1, and M2, each containing multiple components, may be derived from a single protein which has a unique binding region.

Effect of LSE-1 on the expression of a heterologous promoter. To determine whether LSE-1 represses transcription of another gene, we inserted the LSE-1 oligonucleotide into the 5′ end of the simian virus 40 enhancer/promoter region of pSV_2CAT in two orientations: 5′→3′ in $pLSE\text{-}1SV_2CAT$ and 3′→5′ in $p1\text{-}ESLSV_2CAT$. LSE-1 with the same orientation as in the LPL promoter (5′→3′) decreased pSV_2CAT activity to 62.5% ± 2.5% in CHO cells, whereas LSE-1 with the reverse orientation had no effect (Fig. 10). Therefore, LSE-1 of the LPL gene promoter partly but significantly suppressed transcription directed by the simian virus 40 enhancer/promoter in CHO cells. These results show that LSE-1 can suppress tran-

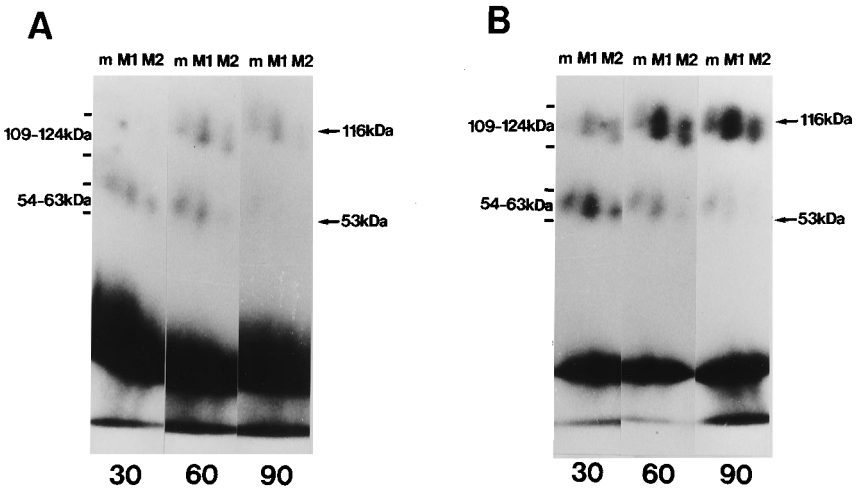

FIG. 8. Photochemical cross-linking of the LSE-BF from CHO cells, with various times of exposure to UV irradiation. End-labeled single-stranded (A) or double-stranded (B) LSE-1 probe was incubated with nuclear extracts from CHO cells. Products were analyzed by gel retardation analysis and then UV irradiated for 30, 60, or 90 min. The gel was autoradiographed, and the specific protein-DNA complexes were excised and electrophoresed in SDS–8% polyacrylamide gel. The positions of molecular mass markers are shown on the right (by arrows).

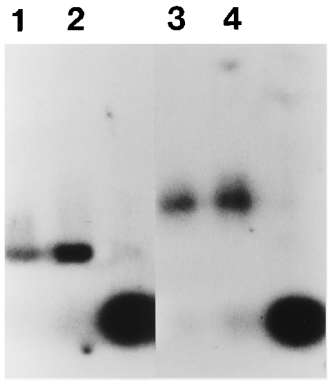

FIG. 9. Isoelectric focusing of tryptic peptides of the 54- to 63-kDa and 109- to 124-kDa LSE-BFs from CHO cells bound to single- or double-stranded LSE-1. The LSE-BF is derived from the complexes at 54 to 63 kDa (lanes 1 and 3) and 109 to 124 kDa (lanes 2 and 4), which were completely digested to peptides with *N*-tosyl-L-phenylalanyl chloromethyl ketone–trypsin. The peptides cross-linked to the probes of single-stranded LSE-1 (lanes 1 and 2) and double-stranded LSE-1 (lanes 3 and 4) were analyzed by isoelectric focusing from pH 3.5 to 10.

scription of a strong viral promoter in an orientation-dependent manner.

DISCUSSION

Severe impairment of LPL, a key enzyme of fat metabolism, is involved in marked loss of adipose tissue, leading to the well-known state of cachexia. The present study was undertaken to analyze the molecular basis of this phenomenon from the aspect of down regulation of LPL gene expression. For this purpose, we used CHO cells, which produce LPL constitutively (34), and HeLa cells, which do not produce LPL. Transfection experiments with CHO and HeLa cells revealed a negative *cis*-acting element (nucleotides −225 to −81) within the LPL promoter. The sequence of LSE is well conserved between human and murine species and is thought to be an important element in transcription regulation (14). Moreover, no homology between our GA-rich core sequence of LSE (LSE-6) and any of those of the many *cis*-acting silencer elements identified until now could be seen. Therefore, the silencer found in this study may be a novel one. Especially noteworthy is our finding of strong silencing activity in CHO cells but weak activity in HeLa cells. This finding implies that the LSE functions in LPL-producing cells where the LPL promoter would be positively active. Thus, the balance between silencer- and enhancer-accessible binding elements and available transcriptional factors could be a key point in regulation of LPL production.

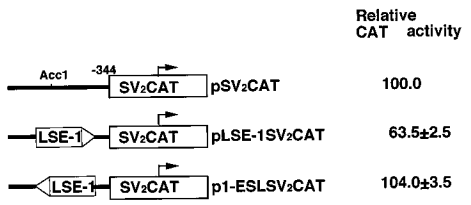

FIG. 10. Effects of LSE-1 on transcription directed by the simian virus 40 early promoter/enhancer. A single copy of the LSE-1 oligonucleotide was subcloned in the 5′→3′ (pLSE-1SV$_2$CAT) or 3′→5′ (p1-ESLSV$_2$CAT) orientation into the *Acc*I site of the 5′-flanking region of pSV$_2$CAT. CAT activities after transfection of the plasmids into CHO cells were measured and are expressed as percentages of the activity of pSV$_2$CAT. The mean values ± standard deviations of the relative activities determined in duplicate in three separate experiments are shown.

Two major (M1 and M2) and one minor (m) LSE-BFs which may contain multiple proteins were detected by gel retardation analysis, but the binding region of these LSE-BFs was the same, as shown by isoelectric focusing. Thus, the apparently heterologous LSE-BFs m, M1, and M2 seem to be basically similar proteins. In addition, this result suggests that the difference between these LSE-BFs is regulated by some posttranslational modification such as phosphorylation-dephosphorylation. Further studies are required to examine this possibility.

A variety of silencers have been characterized (1–4, 12, 16, 19, 22, 23, 26, 30, 31, 35, 37, 38, 43, 46). Some appear to be promoter specific, like the 5′ upstream element regulating the γ-crystallin gene (4). This specific activity for the homologous promoter may be due to flanking regions of the silencer. However, the LPL silencer did not appear to be promoter specific, since the simian virus 40 promoter was also partially inhibited by a fragment containing LSE-1. About 40% reduction of CAT activity was demonstrated when LSE-1 was inserted in the normal orientation, but no reduction was detected when it was inserted in the reverse orientation. In contrast, a preadipocyte repressor element found in the promoter region of the stearoyl coenzyme A desaturase 2 gene is reported to cause inhibition in an orientation-independent manner (43). The mechanism of the orientation dependency of the LSE therefore remains to be solved.

Most eukaryotic sequence-specific DNA binding proteins recognize native double-stranded sequences, but there are several reports of single-stranded protein-DNA complexes that are thought to be involved in the regulation of transcription (5, 9, 20, 21, 27, 31, 33, 39–41, 44). The estrogen receptor binds to both the double- and single-stranded forms of the estrogen-responsive element, showing higher affinity for the single-stranded form (21). This affinity preference is similar to that of the sterol regulatory element and its binding factor (41). The LSE-BF bound to both single- and double-stranded LSE-1 probes in the same sequence-specific fashion and with the same affinity. This suggests that binding to the double-stranded LSE-1 requires only the opposite single strand of the LSE-1 structure and that it is not affected by binding with the complementary single-stranded LSE-1 with low affinity.

The mechanism by which silencers repress transcription in vivo is still unclear. There are some reports that a negative regulatory factor exerts its influence by direct interaction with an activator protein (1, 16, 23, 26) or with a specific DNA sequence located either adjacent to or overlapping the site of interaction of an activator (2, 30, 46). Our results suggested that the silencer of the LPL gene is produced as a 54- to 63-kDa nuclear protein in CHO and HeLa cells and that it interacts as a monomer or a dimer with the 18-bp sequence, which interferes with the function of an activator interacting adjacently. The purification of this silencer protein or cloning of its cDNA is necessary for clarifying the precise mechanism of its repression of the LPL gene.

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