Identification of Nucleotides Responsible for Enhancer Activity of Sterol Regulatory Element in Low Density Lipoprotein Receptor Gene*

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Sterol-dependent regulation of the low density lipoprotein (LDL) receptor promoter has been localized previously to a 16-base pair sequence, designated repeat 2, in the 5'-flanking region of the gene. In the current study, we show that the central 10 nucleotides of repeat 2 are crucial for the sterol regulatory activity. This sequence includes an octamer, designated sterol regulatory element 1 (SRE-1), which was identified previously in the promoter of the gene for 3-hydroxy-3-methylglutaryl coenzyme A synthase, a sterol-regulated enzyme of cholesterol biosynthesis. We made a series of single-base substitutions within a 1471-base pair fragment of the intact LDL receptor promoter, introduced the mutant plasmids into hamster cells by transfection, and measured mRNA levels in the absence and presence of sterols. Substitutions within the 10-base pair sequence in repeat 2 largely prevented the induction of transcription which occurs in the absence of sterols. None of these point mutations affected transcription in the presence of sterols. Like an enhancer, the SRE-1 in repeat 2 functioned in an orientation-independent manner. We interpret these findings to indicate that the SRE-1 of the LDL receptor promoter is a conditional positive element that cooperates with other elements to enhance transcription in the absence of sterols and loses its function in the presence of sterols.

Sterol-regulated genes are transcribed actively when animal cells require cholesterol and are repressed when sterols accumulate. Sterol-mediated repression of transcription has been demonstrated for the genes encoding two sequential enzymes in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase (1) and HMG-CoA reductase (2), and for the gene encoding the cell surface receptor for low density lipoprotein (LDL) (3, 4). Through this regulatory process, cells obtain cholesterol by endogenous synthesis and uptake from exogenous lipoproteins during periods of cholesterol demand, and they avoid overaccumulation of cholesterol when the demand has been satisfied (5).

Sterol regulatory elements have been identified in the 5'-flanking regions of all three sterol-regulated genes through in vitro mutagenesis of promoter-bearing plasmids that were transfected into cultured cells (1-4, 6, 7). In the LDL receptor promoter, sterol responsiveness was localized to a 16-base pair sequence, designated repeat 2, which is one of three imperfect repeats (3, 4, 7). The other two 16-base pair repeats, designated 1 and 3, bind the positive transcription factor Sp1 in vitro (4). Repeat 2 differs from repeats 1 and 3 at six and four nucleotide positions, respectively, and it does not bind Sp1 (4). When repeat 2 was inserted into the herpes simplex virus (HSV) thymidine kinase promoter, it elicited a high level of expression of a reporter gene only in the absence of sterols; its enhancing effect was lost when sterols were present. On the other hand, insertion of repeat 1 or 3 into the HSV thymidine kinase construct enhanced transcription even in the presence of sterols (3, 4, 7). Mutation of several individual nucleotides within repeat 2 prevented sterol regulation of the chimeric promoter (4), suggesting that a sequence-specific DNA-binding protein is involved. The precise sequence that is recognized by this protein is as yet unknown.

The hamster HMG-CoA synthase promoter contains two sequences that show a 7/8- and 8/8-base pair match with a sequence in repeat 2 of the human LDL receptor promoter (1). Point mutations in either of these two repeat sequences lessened the induction of transcription which normally occurs in the absence of sterols, leading to a constitutively low level of transcription (1, 6). The similarity in sequence between the apparent regulatory elements in the HMG-CoA synthase and LDL receptor promoters led to the designation of this octameric sequence as sterol regulatory element 1 (SRE-1). In the HMG-CoA synthase promoter each SRE-1 appears to act as a conditional positive element, mediating high level transcription only in the absence of sterols and losing this effect when sterols are present (1).

The hamster HMG-CoA reductase promoter contains a sequence that shows a 7/8-base pair match with the SRE-1 of the human LDL receptor promoter but is situated in the opposite orientation (1, 2). A 10-base pair scramble mutation that alters six out of eight nucleotides in the SRE-1 sequence of the reductase promoter led to a constitutively high level of transcription which was not repressed by sterols (2, 6). In the HMG-CoA reductase promoter the SRE-1 is located within a cluster of binding sites for nuclear factor 1 (8, 9), and in this context the sterol regulatory element may act as a conditional negative element that actively represses transcription in the presence of sterols. This conclusion must be guarded, however.
because point mutations in the SRE-1 have not been made, and it remains possible that the 10-base pair scramble mutation affected binding sites for other regulatory proteins in addition to the putative sterol regulatory protein.

In order to evaluate proteins that bind specifically to the SRE-1 as candidates for regulatory proteins, it is necessary to determine precisely which nucleotides within the SRE-1 are required for sterol-dependent regulation. It is also important to know whether the SRE-1 resembles an enhancer element that can function in either orientation in the native promoter. The current mutagenesis experiments were undertaken to answer these questions for the SRE-1 of the LDL receptor promoter.

**EXPERIMENTAL PROCEDURES**

**Materials**—[γ-32P]ATP (>5000 Ci/mmol) was obtained from ICN. Polynucleotide kinase was obtained from Pharmacia LKB Biotechnology Inc. Enzymes used in plasmid constructions were obtained from New England BioLabs, Boehringer Mannheim, and Bethesda Research Laboratories. Reverse transcriptase was purchased from Life Sciences (AMV 007). G418 sulfate (Geneticin) was purchased from Gibco. newborn calf lipoprotein-deficient serum (d > 1.215 g/ml) was prepared by ultracentrifugation (10). Plasmid pSV3-Neo, which contains a bacterial gene that confers resistance to G418 (11), was obtained from Bethesda Research Laboratories. Plasmid pSV0-CAT (12) was kindly provided by Bruce Howard (NIH). Plasmid pTK-CAT-1 contains the HSV thymidine kinase promoter (13) extending 109 base pairs upstream of the mRNA cap site fused to the chloramphenicol acetyltransferase (CAT) gene (3). Materials for construction of LDL receptor promoter-CAT plasmids were obtained from previously reported sources (3, 4, 7). Oligonucleotides were synthesized on an Applied Biosystems model 380A DNA synthesizer.

**Plasmid Constructions**—Standard protocols were used for all recombinant DNA technology (14). Plasmid p1471, previously referred to as pLDDL-CAT 1653 (3), contains a 1507-base pair EcoRI-AluI restriction fragment encompassing the full-length human LDL receptor promoter (3) inserted into pSV0-CAT. The promoter extends 1471 base pairs upstream from the transcription start site. Plasmid pSV0-CAT is a recombinant plasmid that contains the β-lactamase gene (amP), the pBR322 origin of replication, and the coding sequences for CAT (12). Plasmid pSV0-CAT has no defined eukaryotic promoter or enhancer sequences.

To construct point mutants A–R, oligonucleotide-directed mutagenesis (15) was performed with oligonucleotides of 20 nucleotides in length containing a mismatch 10 nucleotides from the 5′ end. The inversion mutant pZ was made by oligonucleotide-directed mutagenesis using a 138-mer that hybridized to 30 nucleotides of the wild-type sequence on either side of a 78-nucleotide target sequence. The 138-mer was then purified on a denaturing polyacrylamide gel. All mutant sequences were then annealed to a complementary bridging 20-mer. The 138-mer was then purified on a denaturing polyacrylamide gel. All mutant promoters were reintroduced into the HindIII site of pSV0-CAT.

**DNA Transfection**—Chinese hamster ovary (CHO-K1) cells were grown in monolayer culture and transfected either with 0.5 μg of pSV3-Neo, 22 μg of the test plasmid, and 3 μg of pTK-CAT-1 (Protocol 1) or with 0.5 μg of pSV3-Neo, 20 μg of the test plasmid, and 5 μg of pTK-CAT-1 (Protocol 2) by the calcium phosphate coprecipitation technique as described previously (3, 7). After 2–3 weeks of selection for resistance to G418 (1 mg/ml), resistant colonies were pooled (300–500 colonies/transfection) and expanded in mass culture in the presence of 1 mg/ml G418 and 10% fetal calf serum in the absence or presence of sterols (10 μg/ml cholesterol plus 0.5 μg/ml 25-hydroxycholesterol) and harvested for RNA analysis as described previously (3, 7).

**Transcription Assays**—Primer extension assays for measurement of mRNA were carried out as described previously with minor modifications (7, 16). Primer was hybridized to 40 μg of total cellular RNA for 45 min at 68 °C, and the extension reaction was carried out for 1 h at 42 °C. Reaction products were subjected to electrophoresis on a 6% polyacrylamide gel, 40 cm in length, 1.5 mm in thickness at the negative electrode, and 0.4 mm in thickness at the positive electrode. To detect transcriptors containing CAT sequences (derived from pTK-CAT-1 or from a test plasmid), an oligonucleotide primer of 40 nucleotides complimentary to bases 400–439 of the CAT mRNA (17) was used. Two extension products of ~315 and 316 nucleotides in length are generated from pTK-CAT-1, owing to two transcription initiation sites within the HSV thymidine kinase promoter (13). These transcripts serve as an internal control for transcription efficiency and RNA quantification. A 290-nucleotide extension product is generated from p1471 and the various mutant derivative plasmids. MapT restriction fragments of 32P-labeled pBR322 DNA were used as size standards. Quantitative densitometry was performed with a Hoefer scanning densitometer (model GS300).

**RESULTS**

Transcription assays were carried out in CHO cells that were transfected with plasmids containing the 5′-flanking region of the LDL receptor gene fused to the CAT coding sequence. All constructions used an LDL receptor fragment that extended from +36 (relative to the start site for transcription) to −1471 (Fig. 1, upper half). The amount of mRNA in the permanently transfected cells was measured by a primer extension assay. As an unregulated control, we cotransfected a plasmid containing the HSV thymidine kinase promoter fused to CAT. The mRNA produced by this construct gives a longer primer extended product than does the LDL receptor promoter constructs, thus allowing simultaneous measurement of the mRNA produced by the control and test plasmids. To test for sterol-mediated regulation, the cells were incubated for 24 h either in the absence of sterols or in the presence of a mixture of 25-hydroxycholesterol plus cholesterol for 24 h prior to mRNA assay.

Cells transfected with p1471 (containing the wild-type LDL receptor promoter) produced relatively large amounts of mRNA when grown in the absence of sterols (Fig. 1, right lower panel, lane 1). For quantification, the autoradiograms were scanned densitometrically, and the amount of mRNA

**Fig. 1.** Sterol-mediated regulation of chimeric genes containing the LDL receptor promoter upstream regulatory sequences in forward and reverse orientations. The 1507-base pair EcoRI/AluI restriction fragment from the 5′ end of the LDL receptor gene used to construct p1471 is shown at the top. Nucleotide positions −1471 to +36 are numbered in relation to the transcription initiation site, indicated by the arrow at +1. A putative TATA box is designated T/A. The two regions that bind Sp1 in vitro (repeats 1 and 3) are shown, as is the SRE-1 within repeat 2. The CAT coding sequence is denoted by the cross-hatched box. To construct p2, the region from −33 to −110 of p1471 was inverted by M13 mutagenesis (15). Relative transcription was determined by a primer extension assay of the amount of CAT mRNA in stably transfected CHO cells incubated for 24 h in the absence or presence of sterols. LDL receptor promoter test plasmid expression was normalized to pTK-CAT-1. Fold induction was calculated as the ratio of normalized test plasmid expression in the absence of sterols to that in the presence of sterols for each experiment. u.t., wild type; TK, thymidine kinase; LDL, LDL receptor.
produced by the LDL receptor promoter was expressed relative to the amount produced by the HSV thymidine kinase promoter. The amount of mRNA produced by the p1471 plasmid in the absence of sterols was arbitrarily designated as 1.0. When sterols were added, the amount of mRNA from p1471 was reduced by 4.2-fold (Fig. 1, right lower panel; compare lanes 1 and 2). Stated another way, there was a 4.2-fold induction of transcription when sterols were removed from the culture medium.

To test for the orientation dependence of the sterol regulatory function, we prepared a plasmid, designated pZ, in which the segment from −33 to −110 was inverted (Fig. 1, upper half). This segment contains the SRE-1. As shown in Fig. 1 (lower right panel, lanes 3 and 4), pZ behaved similarly to the wild-type LDL receptor promoter. There was a 3.8-fold induction of transcription when sterols were removed from the culture medium.

During the course of these experiments, we noted some variability from one transfection to another. To be certain that the observed regulation was not due to random fluctuations, we conducted multiple experiments with each plasmid. The left lower panel of Fig. 1 shows the average of four experiments in which p1471 and pZ were compared. From these data, it is clear that the inversion mutant led to a slight decrease in transcription both in the absence and presence of sterols, but that the fold induction in the absence of sterols occurred independently of the orientation of the sterol-regulated element.

To test for the effect of nucleotide substitutions in the SRE-1, we prepared a series of LDL receptor promoter plasmids in which a single nucleotide within repeat 2 was altered (Fig. 2). All of these substitutions were made within the sequence extending from −53 to −68, which includes the sterol regulatory element. Each plasmid was transfected into CHO cells on two separate occasions, and for each a pool of transfectants was studied on multiple occasions to assure statistical reliability of the results. A sample of the raw data from these experiments is shown in Fig. 3, and the results are summarized in Fig. 2.

Based on the mean data from 23 experiments, the wild-type LDL receptor promoter gave approximately a 5-fold induction in the absence of sterols. Mutation of the first three nucleotides in repeat 2 of the LDL receptor promoter (−68 to −66) did not alter this regulation (Fig. 2). All of these plasmids showed slightly higher transcription rates than the plasmid containing the wild-type promoter both in the absence and presence of sterols (Fig. 2A), but the fold induction was not impaired (Fig. 2B). A major disruption of regulation was seen when any one of the nucleotides in the TCAC sequence (−64 to −61) was altered by a transversion (change from purine to pyrimidine or vice versa). A similar decrease in regulation was seen with a transition mutation in which the A (−62) of the TCAC was changed to G (Fig. 2B). Mutation of the C at −60 had less of an effect (Fig. 2B). The next four nucleotides CCAC (−59 to −56) were each crucial for regulation. Transversion mutations at any of these four positions essentially abolished the induction of transcription in the absence of sterols. A single transition mutation was also made in this region (A to G at −57). This mutation also abolished regulation. The final three nucleotides in repeat 2 (TGC at −55 to −53) were not apparently important for regulation since transversion mutations had little effect (Fig. 2B).

It is significant to note that none of the point mutations in Fig. 2 significantly reduced transcription in the presence of sterols (Fig. 2A). The mutations that abolish regulation did so by preventing the induction in the absence of sterols.
definitive evidence was available to show that the eight nucleotides comprising the SRE-1 encompassed the essential element for sterol regulation. The current study shows that the 8-base pair SRE-1, situated within repeat 2, carries most of the information necessary for this control. In addition, the two adjacent upstream nucleotides at positions -64 and -65 appear to be involved.

Substitution of any single nucleotide within the 8-base pair core SRE-1 markedly limited inducibility in the absence of sterols with the sole exception of the C at position -60. When this pyrimidine was changed to a purine (A), transcription remained inducible by nearly 4-fold. It is noteworthy that in the Syrian hamster LDL receptor gene, this position is occupied by an A. All other mutations within the SRE-1 reduced inducibility below 2.5-fold, nucleotides at these positions are all conserved in the human and hamster promoters.

In general, mutations in the tetramer at the 3’ end of the core SRE-1 (CCAC) had a more profound effect than mutations in the 5’ tetramer (CACC) (Fig. 2). The T at position -64 immediately adjacent to the core SRE-1 also played a role in regulation since substitution of a G reduced the induction. In the current study we did not alter the adjacent A at position -65. In a previous study in which the repeat 2 sequence was inserted into the HSV thymidine kinase promoter, this A was changed to a C (mutant L in Ref. 4), and this substitution reduced the amplitude of sterol regulation by 75%. These studies thus localize the regulatory region of the 16-base pair repeat 2 to the central 10 base pairs. The 3 base pairs at either end of repeat 2 are not important for sterol-dependent regulation since substitutions in these positions did not impair regulation.

None of the point mutations in the SRE-1 or other regions of repeat 2 led to a constitutively high level of transcription from the LDL receptor promoter. The only result was a failure of induction following the removal of sterols from the culture medium. These data strongly suggest that in the intact LDL receptor promoter, the SRE-1 acts as a binding site for a conditionally positive activator protein that enhances transcription in the absence of sterols and loses this effect in the presence of sterols. We found no evidence that the SRE-1 acts as the binding site for a repressor protein. This interpretation agrees with the conclusion drawn from studies of point mutations in both SRE-1 sequences of the HMG-CoA synthase promoter (1). A comparative level of detail is not yet available for the putative SRE-1 in the HMG-CoA reductase promoter.

The data from this study should be compared with the conclusions from an earlier study from this laboratory in which repeat 2 was inserted into the HSV thymidine kinase promoter at position -60, which is between two Sp1-binding sites (4). The insertion of repeat 2 into the HSV thymidine kinase promoter led to a somewhat elevated level of transcription in the absence of sterols (1.7- and 1.4-fold in forward and reverse orientations, respectively). When sterols were present, the level of transcription fell to one-half of that of the native thiimidine kinase promoter (4). This latter finding could be interpreted to suggest that the SRE-1 binds a sterol-dependent repressor protein that counteracts the function of the nearby Sp1-binding sites. An alternative explanation is that sterols removed the positive effect of repeat 2, and this left a slight negative effect owing to the disruption caused by 16 exogenous base pairs at this position. The current results with the intact LDL receptor promoter support the latter interpretation.

On the basis of the current experiments with the native LDL receptor promoter, we conclude that the SRE-1 functions as an enhancer only in the absence of sterols. Our working model suggests that cells contain a protein that binds to the SRE-1 and activates transcription in the absence of sterols and that this function is inactivated as sterols accumulate within the cell. Current efforts are directed toward the identification of such a sterol-sensitive, trans-acting enhancer protein that demonstrates binding specificity for the SRE-1.

The current studies define the nucleotides that are required for the function of the SRE-1 in the context of the intact LDL receptor promoter. Certain substitutions that are not tolerated in the LDL receptor promoter might be tolerated in the SRE-1 sequence of other genes. In the putative SRE-1 of the HMG-CoA reductase promoter, the C at the 5th position in the octamer (corresponding to position -59 in the LDL receptor promoter) is replaced by a G (1, 2). Although this substitution destroys SRE-1 function in the LDL receptor promoter (Fig. 2), it might not have that effect in the HMG-CoA reductase promoter because of compensatory substitutions at other nucleotides that flank the SRE-1. In addition, the interaction of a protein with the SRE-1 in the HMG-CoA reductase promoter might be stabilized by protein-protein interactions with neighboring DNA-binding proteins so that...
binding of the sterol regulatory protein is not abolished by this single nucleotide change. In order to clarify this point, it will be necessary to make point mutations in the HMG-CoA reductase promoter and to test their effect in transfected cells.

Rajavashisth et al. (18) recently reported the cDNA cloning of a mRNA encoding a 19-kDa protein with seven zinc fingers which binds specifically to an octamer whose sequence is consistent with the sequence requirement for sterol regulation mediated by the SRE-1. The mRNA for this protein was present in a variety of tissues, and its concentration was increased 4-fold when cultured cells were incubated with sterols. In preliminary transfection studies, the authors did not observe any effect of overexpression of this protein on sterol-dependent regulation. It is possible that this 19-kDa protein constitutes the recognition protein for the SRE-1, but further studies will be required to substantiate this conclusion.

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