Identification of a Novel Sterol-independent Regulatory Element in the Human Low Density Lipoprotein Receptor Promoter*

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The cytokine oncostatin M (OM) activates human low density lipoprotein receptor (LDLR) gene transcription through a sterol-independent mechanism. Previous studies conducted in our laboratory have narrowed the OM-responsive element to promoter region –52 to +13, which contains the repeat 3 and two TATA-like sequences. We now identify LDLR promoter region –17 to –1 as a sterol-independent regulatory element (SIRE) that is critically involved in OM- transcription factor CCAAT/enhancer-binding protein (C/EBP)-, and second messenger cAMP-mediated activation of LDLR transcription. The SIRE sequence overlaps the previously described TATA-like element and consists of an active C/EBP-binding site (–17 to –9) and a functional CAMP-responsive element (CRE) (–8 to –1). We demonstrate that (a) mutations within either the C/EBP or CRE site have no impact on basal or cholesterol-mediated repression of LDLR transcription, but they completely abolish OM-mediated activation of LDLR transcription; (b) placing the repeat 3 sequence that contains the Sp1-binding site with a yeast transcription factor GAL4-binding site in the LDLR promoter construct does not affect OM inducibility, thereby demonstrating that OM induction is mediated through the SIRE sequence in conjunction with a strong activator bound to the repeat 3 sequence; (c) electrophoretic mobility shift and supershift assays confirm the specific binding of transcription factors C/EBP and cAMP-responsive element-binding protein to the SIRE; (d) cotransfection of a human C/EBPβ expression vector (pEF-NFIL6) with the LDLR promoter construct pLDLR234 increases LDLR promoter activity; and (e) OM and dibutyryl cAMP synergistically activate LDLR transcription through this regulatory element. This study identifies, for the first time, a cis-acting regulatory element in the LDLR promoter that is responsible for sterol-independent regulation of LDLR transcription.

Previous studies of the human low density lipoprotein receptor (LDLR) promoter identified a 177-base pair fragment of the 5′-flanking DNA from –142 to +35, relative to the major transcription start site, that is sufficient for controlling basal transcription as well as negative feedback regulation by cholesterol and its derivatives (1–3). The positive regulatory elements within this region were identified as three GC-rich imperfect 16-base pair direct repeats and two TA-rich TATA-like sequences of 7 base pairs each. Repeats 1 and 3 contain transcription factor Sp1-binding sites. Interference in Sp1 binding to either repeat severely decreases basal transcriptional activity. Sterols regulate LDLR transcription through a 10-base pair sequence within repeat 2 designated as sterol regulatory element-1 (SRE-1) (4, 5). When intracellular cholesterol is low, SRE-binding protein-1 and -2 bind to the SRE-1 sequence and interact with Sp1 in repeat 3, thereby activating LDLR transcription (6–9).

In addition to cholesterol, LDLR transcription can also be regulated by nonsterol mediators, including hormones (10), growth factors (11), cytokines (12), and second messengers such as cyclic AMP (13). However, the cis-acting elements that control LDLR transcription through sterol-independent pathways have not been identified.

To elucidate the molecular mechanisms underlying sterol-independent regulation, we previously dissected the promoter region that could be responsible for cytokine oncostatin M (OM)-induced activation of LDLR transcription (15), as OM has been shown to increase the expression of LDLR protein and mRNA independent of intracellular cholesterol (14). We have shown that mutations of the SRE-1 site or deletion of the repeat 2 sequence to completely eliminate SRE-binding protein binding has no effect on OM inducibility of LDLR promoter activity (16). Deletion analysis further narrowed down the OM-responsive element in the promoter region from –52 to +13 (17). This region contains the two TATA-like sequences and repeat 3.

In this study, we performed detailed mutagenic analysis in this promoter region to identify the cis-regulatory element that mediates the OM activity of the LDLR promoter. We provide the first evidence to demonstrate that OM activity is mediated through promoter region –17 to –1 in conjunction with the strong activator Sp1 bound to the repeat 3 sequence. We have designated this promoter region (–17 to –1) as a sterol-independent regulatory element (SIRE). The SIRE overlaps with the previously described TATA-like sequences and consists of an active CCAAT/enhancer-binding protein (C/EBP)-binding

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¶ The abbreviations used are: LDLR, low density lipoprotein receptor; SRE, sterol regulatory element; OM, oncostatin M; SIRE, sterol-independent regulatory element; C/EBP, CCAAT/enhancer-binding protein; CRE, cAMP-responsive element; CREB, CRE-binding protein; CREM, cAMP-responsive element modulator; EMSA, electrophoretic mobility shift assay; ATF, activating transcription factor; XBP, X-box-binding protein; DBD, DNA-binding domain; LPDS, lipoprotein-depleted serum; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; AP-1, activator protein-1.
Identification of a Novel SIRE in the Human LDLR Promoter

MATERIALS AND METHODS

Cells and Reagents—The human hepatoma cell line HepG2 was obtained from American Type Culture Collection (Manassas, VA) and was cultured in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum (Summit Biotechnology, Fort Collins, CO). Antibodies specific to the following proteins were obtained from Santa Cruz Biotechnology for use in supershift EMSAs: C/EBPα, C/EBPβ, C/EBPδ, CREB-1, CREB-2, CREM-1, CREB-ATF/1 (recognizes all CREB/ATF family proteins), ATF-1, ATF-2, c-Jun, JunB, c-Fos, XBP-1, CREB-binding protein, and TATA-binding protein.

Plasmid Vectors—Construction of plasmids pLDLR234, pLDLR-R3, and pLDLR-TATA has been described previously (17). To construct the mutant vectors (pLDLR-R3-m6 to pLDLR-R3-m11), double-stranded oligonucleotides containing the LDLR promoter sequence from −52 to +13 with single-base substitutions were synthesized with SacI and HindIII sites (italic) at the 5′- and 3′-ends, respectively, and inserted into pGKL3-basic vector. The mutant sequences are shown in Fig. 1.

The vector pLDLR-GAL4 was constructed by cloning a double-stranded oligonucleotide containing the LDLR promoter sequence from −36 to +15 as a GAL4-binding site with SacI and HindIII sites (italic) at the 5′- and 3′-ends (Tggaattgcgtgctgtgataacatggtgat3′) into pGKL3-basic vector. The GAL4 sequence is underlined. The vector pLDLR-GAL4-TATA2MU was constructed as described above with the exception that the TATA2 site contains a 3-base substitution (TGTTAA → Tggaat).

The plasmid pSG-GAL4-Sp1 encoding the yeast transcription factor GAL4 DNA-binding domain and the N-terminal glutamine-rich activation domain of Sp1 and pSG-GAL4 DBD encoding the GAL4 DNA-binding domain only (18) were kindly provided by Dr. Stephen Smale (UCLA). The plasmid pFAGAL4 encoding the full-length GAL4 protein was generously provided by Dr. ChaoFeng Zheng (Stratagene). The plasmid pEF-NFIL6 was a gift from Dr. Shizuo Akira (Hyogo College of Medicine, Hyogo, Japan).

Site-directed Mutagenesis—The plasmid pLDLR234 was used as a template for making mutants utilizing the QuikChange™ site-directed mutagenesis kit (Stratagene). Correct clones were screened by restriction digestion and verified by dyeoxy sequencing.

Preparation of Nuclear Extracts—Nuclear extracts were prepared by the method of Dignam et al. (19), except that buffer A was supplemented with 1 mM Na2VO4, 1 mM NaF, 1 mM Na3MoO4, 1 mM β-glycerophosphate, 10 mM 3-oxo-bisaccharide acid, 10 μg/ml o-mercaptomeratin, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, and 2 mM dithiothreitol.

Electrophoretic Mobility Shift Assays—Oligonucleotide probes were annealed and labeled by 3′-fill-in using Klenow fragment in the presence of [α-32P]dCTP. Each binding reaction was composed of 10 mM HEPES, pH 7.8, 2 mM dithiothreitol, 80 mM NaCl, 10% glycerol, 1 μg of poly(dI-dC), 1 μg of bovine serum albumin, and 6 μg of nuclear extract in a final volume of 20 μl. Nuclear extracts were incubated with 0.4–0.5 ng of 32P-labeled double-stranded synthetic oligonucleotide probe (40–80 × 10^3 cpm) for 10 min at room temperature. The reaction mixtures were loaded onto a 5% polyacrylamide gel and run in TGE buffer (50 mM Tris base, 400 mM glycine, and 1.5 mM EDTA, pH 8.3) at 180 V for 3 h at 4 °C. The gels were dried and visualized on a PhosphorImager. In competition analysis, nuclear extracts were incubated with a 2–200-fold molar excess of unlabeled competitor DNA for 5 min prior to the addition of the labeled probe. For supershift assays, antibody was incubated with nuclear extract for 30–60 min at room temperature prior to the addition of the probe.

The sequence of EMSA probes used were as follows: TATA1+2, 5′-CATGAAATTGCTGTTAATGACGTTGCGGC-3′; repeat 3, 5′-TTCGAAACCTCTCCCCCTGTGAG-3′; and p53, 5′-AGCTAGGGGGTTGCTTGAACAGGTTCT-3′. The Sp1- and p53-binding sites are underlined.

Transient Transfection Assays—HepG2 cells were transiently transfected with plasmid DNA by the calcium phosphate coprecipitation method (16). To demonstrate sterol-independent regulation, cells transfected with pLDLR234 or mutant vectors were cultured in medium containing 10% lipoprotein-depleted serum (LPDS) at 36°C to 48°C in the presence of 10 μM Na3VO4, 1 mM NaF, 1 mM Na2MoO4, 1 mM g/ml pepstatin A, and 2 mM dithiothreitol.

Solute luciferase activity was normalized against -galactosidase activities were assayed. A putative CRE (−8 to −1) that overlaps with TATA2. Fig. 3 shows that none of the mutations affected cholesterol regulation; promoter activities of all of the constructs were repressed by sterol to a similar degree. However, OM inducibility was completely abolished in the TATA2 mutant (TATA2a) and the CRE mutant (CREMU1) and only partially reduced in the TATA1 mutant (TATA1b).

Computer-aided sequence analysis suggested that the
TATA-like sequences contain a potential C/EBP-binding site (TGCTGTAAA, −17 to −9) that includes the 3′-base pair of TATA1 and the TATA2 sequence except the most 3′-base pair of TATA2, which is included in the putative CRE site (Table I).

To further demonstrate that OM activity is mediated through the putative C/EBP site and the CRE site, several reporter vectors with altered nucleotides in the 5′-region of the C/EBP site (TATA1a) and the 3′-region of the CRE site (TATAMU3d and TATAMU3e) were constructed, along with two additional mutants of the C/EBP site (TATA2b) and the CRE site (TATAMU3c). The results are summarized in Table I. The mutation in TATA1a with base substitutions in the 5′-end of TATA1 but outside the C/EBP site lowered basal activity to 59% of the wild-type level, but had no effect on OM inducibility. In comparison, the mutation in TATA1b lowered basal promoter activity to 69% of the wild-type level and also slightly decreased OM activity from 4- to 2.9-fold. This is most likely due to the A-to-G substitution immediately adjacent to the C/EBP-binding site. These data suggest that the TATA1 site contributes to basal promoter activity, but is dispensable for the OM response. In contrast, basal promoter activities were unchanged in C/EBP mutants TATA2a and TATA2b and in the
Site-directed mutagenesis was performed on the pLDLR234 vector. The wild-type and mutant vectors were transfected along with the control vector DG2-plgα into HepG2 cells. After transfection, cells were incubated in medium containing cholesterol for 24 h. OM (50 ng/ml) was added 4 h prior to lysing the cells. Results are summarized from three to six independent experiments, and triplicate wells were used in each condition. The boldface sequence is the C/EBP-binding site, and the italic sequence is the CRE site. The mutated bases are shown in underlined lowercase letters.

The major transcription start site is indicated by the astersisk.

Table I

| Construct | TATA1, TATA2, and 3′-flanking sequence (−25 to +5) | Basal Activity (-Fold of wildtype) | OM stimulated Activity (-Fold of control) |
|-----------|-----------------------------------------------|-----------------------------------|-------------------------------------------|
| Wild type (pLDLR234) | CATTGAAACTGTGTTAAAACGAGCTGGGCCCC | 1.00 ± 0.14 (n = 6) | 4.0 ± 0.88 (n = 6) |
| TATA1a | CATTGAAACTGTGTTAAAACGAGCTGGGCCCC | 0.59 ± 0.03* (n = 3) | 3.9 ± 0.75 (n = 3) |
| TATA1b | CACATCATGCTTAAAACGAGCTGGGCCCC | 0.69 ± 0.10* (n = 6) | 2.9 ± 0.45* (n = 6) |
| TATA2a | CATTGAAACTGTGTTAAAACGAGCTGGGCCCC | 1.24 ± 0.33 (n = 6) | 1.1 ± 0.15* (n = 6) |
| TATA2b | CATTGAAACTGTGTTAAAACGAGCTGGGCCCC | 0.96 ± 0.16 (n = 6) | 1.2 ± 0.14* (n = 6) |
| CREM1 | CATTGAAACTGTGTTAAAACGAGCTGGGCCCC | 1.20 ± 0.29 (n = 6) | 1.0 ± 0.11* (n = 6) |
| CREM1a | CATTGAAACTGTGTTAAAACGAGCTGGGCCCC | 0.83 ± 0.06* (n = 6) | 1.4 ± 0.11* (n = 6) |
| CREM1b | CATTGAAACTGTGTTAAAACGAGCTGGGCCCC | 0.73 ± 0.10* (n = 6) | 4.0 ± 0.51 (n = 6) |
| CREM1c | CATTGAAACTGTGTTAAAACGAGCTGGGCCCC | 0.83 ± 0.09* (n = 6) | 3.9 ± 0.32 (n = 6) |
| Consensus C/EBP site | TATA1 | TGTAAAT | |
| Consensus CRE | TATA1 | TGTAAAT | |

*p < 0.001 compared with control.

CRE mutant CREM1 and only slightly decreased in the CRE mutant TATAMU3c, but OM stimulation in these mutant vectors was completely abolished. Farther downstream mutations in the 3′-region of the CRE site slightly decreased basal promoter activity (73–83% of the wild-type level) without affecting OM-induced promoter activity. These data clearly localized a novel SIRE in promoter region −17 to −1 that is critically involved in the OM-mediated activation of LDLR transcription.

To determine whether Sp1 binding to repeat 3 is necessary for OM to function through the SIRE, the repeat 3 sequence in pLDLR-R3 was replaced by a GAL4-binding site to create the vector pLDLR-GAL4, and the repeat 3 sequence in a TATA2 mutant of pLDLR-R3 was replaced by a GAL4 site to generate pLDLR-GAL4-TATAMU2MU. These two reporter constructs were cotransfected with expression vectors that express the GAL4 DNA-binding domain only (GAL4 DBD), the full-length GAL4 protein (GAL4), or a fusion protein containing the GAL4 DNA-binding domain and the Sp1 activation domain (GAL4-Sp1).

The results in Fig. 4 show that the promoter activities of both wild-type pLDLR-GAL4 and mutant pLDLR-GAL4-TATAMU2MU were increased by cotransfection with transcription factor GAL4 or the GAL4-Sp1 fusion protein, but were not affected by cotransfection with the GAL4 DBD, which does not contain a transactivation domain. OM did not increase wild-type promoter activity with cotransfection of the GAL4 DBD. However, OM increased the pLDLR-GAL4 promoter activities to the same extent (2–3-fold) with cotransfection of either GAL4 or GAL4-Sp1. This OM activity was totally abolished in the promoter construct pLDLR-GAL4-TATAMU2, which carries a mutation in the SIRE site. These results clearly demonstrate that the OM activity of LDLR transcription is mediated through the SIRE site in conjunction with a strong activator bound to the repeat 3 sequence.

To detect transcription factors that bind to the SIRE, gel shift assays using a 32P-labeled oligonucleotide probe (TATA1+2) containing LDLR promoter sequence −25 to +5 were performed with nuclear extracts prepared from untreated and OM-treated HepG2 cells. Three DNA-protein complexes (C1, C3, and C4) were detected in control extracts, and 4 complexes (C1, C2, C3, and C4) were detected in OM-treated extracts (Fig. 5A). Formation of these complexes was inhibited by a 100-fold molar excess of the unlabeled oligonucleotide TATA1+2, but was not inhibited by a 100-fold molar excess of oigonucleotides containing binding sites for Sp1 (repeat 3) or p53, demonstrating the specificity of the binding. In contrast to the TATA1+2 probe, the binding of Sp1 to the 32P-labeled repeat 3 probe was not altered by OM treatment (Fig. 5B).

Fig. 6 shows the time-dependent induction of the C2 complex by OM. C2 was detected in OM-treated cells after 30 min, and the intensity of C2 appeared to increase with longer OM treatment. Interestingly, the OM-induced appearance of the C2 complex on the SIRE element was totally inhibited by treating cells with U0126, an inhibitor of the extracellular signal-regulated kinase (ERK) upstream kinase MEK (20). These data suggest that the binding of C2 to SIRE site depends on the ERK-induced phosphorylation of protein components of C2.

Supershift EMSA analyses with antibodies directed against several members of the C/EBP and CREB families were conducted to characterize the transcription factors present in these DNA-protein complexes. The C3 complex in both control extracts (Fig. 7A) and OM-treated extracts (Fig. 7B) was completely supershifted by anti-CREB-1 (lanes 2 and 3) and anti-CREM-1 (lane 8) antibodies. Antibodies against ATF-1 (lane 4) and ATF-2 (lane 5), two other members of the CREB family, supershifted the C1 complex and also slightly decreased the intensity of C3. Anti-C/EBPβ antibody produced a supershift band and slightly decreased the intensity of C3 in control extracts (lane 9).

In OM-treated extracts, the intensities of C3 and OM-induced C2 were both slightly decreased by anti-C/EBPβ antibody. Anti-C/EBPβ antibody produced a supershift band only in the OM-treated extracts (Fig. 7B, lane 11), whereas anti-C/EBPα antibody generated a faint supershift band in both control and OM-treated extracts (lane 10). In comparison with the control extract, the intensities of supershifted bands produced by antibodies against ATF-1 (Fig. 7B, lane 4) and ATF-2 (lane 5) with OM-treated extracts were decreased. In contrast, antibodies against CREB-binding protein (lane 6), CREB-2 (lane 7), and the TATA-binding protein and TATA-binding protein-associated factors TAF130 and TAF100 (data not shown) had no effect.

It is known that the consensus binding site (TGACT) for AP-1 is composed of a c-Jun homodimer or a c-Jun and c-Fos heterodimer, overlaps with the CRE site (TGACGTCA) (21, 22). Since OM has been shown to stimulate AP-1 activity (23), we were interested to determine whether the OM-induced C2 complex contains AP-1. Supershift.
assays with antibodies that recognize c-Jun, JunB, c-Fos, and XBP-1 were performed using OM-treated nuclear extract. XBP-1 is another member of the leucine zipper family of transcription factors that also binds to the CRE, and XBP-1 was shown to be regulated by interleukin-6 (24). Fig. 7 shows that antibodies to c-Fos, JunB, and XBP-1 had no effect on any of the four complexes. All three antibodies to c-Jun supershifted the C1 complex, but none of these anti-c-Jun antibodies affected the C2 complex. These data suggest that the C1 complex may be a heterodimer of ATF and c-Jun; c-Jun is not present in the C2 complex. Additional gel shift assays using control extract detected a weaker supershift band with anti-c-Jun antibodies as compared with that seen in OM-treated extract (data not shown), suggesting that OM stimulation may increase the c-Jun DNA-binding activity. Together, these supershift EMSA analyses suggest that 1) C3 is predominantly composed of homodimers and/or heterodimers of members of the CREB family (C3 may also contain a small amount of C/EBPβ); 2) C1 may contain heterodimers between ATF and c-Jun; 3) C4 contains transcription factors that do not appear to belong to the CREB or C/EBP family; and 4) the nature of the C2 complex is presently unknown. C2 may contain a novel factor, as none of the antibodies that recognized most, if not all, transcription factors that could bind to the SIRE sequence significantly affected this complex.

To determine the relationship between C/EBPβ binding and promoter activity, we cotransfected HepG2 cells with pLDLR234 and pEF-NFIL6, which encodes NFIL-6, a human homologue of rat C/EBPβ (25, 26). Fig. 8 shows that under cholesterol-replete conditions, pEF-NFIL6 increased LDLR promoter activity in a dose-dependent manner. At a low concentration of pEF-NFIL6, the stimulatory effect of OM was diminished, whereas at a higher concentration of pEF-NFIL6 that produced a 10-fold increase in basal promoter activity, OM stimulation was abrogated. We speculate that the loss of response to OM stimulation is through saturation of the binding site (SIRE) on the LDLR promoter construct due to the overexpression of NFIL-6. These results, together with the EMSA data presented in Fig. 7, suggest that the C/EBP site within this promoter region may be a functional cis-acting element that positively regulates LDLR transcription.

Takagi and Strauss (13) have reported that 8-bromo-cAMP increases LDLR mRNA without stimulating LDLR promoter activity. This led to the conclusion that the upstream promoter

**Fig. 4.** Examination of the OM activity of the LDLR promoter construct containing a GAL4-binding site in place of repeat 3. The GAL4-LDLR promoter constructs containing the wild-type SIRE (pLDLR-GAL4) and the mutant SIRE (pLDLR-GAL4-TATA2MU) were cotransfected with 50 ng/well expression vector pSG-GAL4 DBD, pSG-GAL4-Sp1, or pFA-GAL4 into HepG2 cells. After transfection, cells were cultured in Eagle’s minimal essential medium containing 10% fetal bovine serum for 20 h prior to treating cells with OM for 4 h.

**Fig. 5.** EMSA analyses of HepG2 nuclear proteins interacting with the SIRE sequence. A, a double-stranded oligonucleotide (designated as TATA1+2) corresponding to LDLR promoter region −25 to +5 was radiolabeled and incubated with 6 µg of nuclear extract prepared from untreated control cells (lanes 2–5) or OM-treated (1 h) cells (lanes 6–9) for 10 min at 22 °C in the absence (lanes 2 and 6) or presence (lanes 3–5 and 7–9) of 100-fold molar amounts of unlabeled competitor DNA. The reaction mixtures were loaded onto a 5% polyacrylamide gel and run in TGE buffer at 180 V for 2.5 h at 4 °C. The arrows indicate the complexes. B, EMSA was performed with the radiolabeled repeat 3 probe described in the legend to Fig. 2 and 5 µg of nuclear extract prepared from untreated control cells or OM-treated (1 h) cells. Wt, wild-type.

**Fig. 6.** Time-dependent induction of the C2 complex by OM. Nuclear extracts were prepared from control cells (lane 1) or from cells that were treated with OM for 30 min (lane 2), 60 min (lane 3), and 120 min (lane 4) alone or with OM and 10 µM U0126 (U) for 60 min (lane 5) or treated with 10 µM U0126 alone (lane 6). Nuclear extracts were incubated with the 32P-labeled TATA1+2 probe and analyzed by EMSA.
of the human LDLR gene does not contain a classical CRE. To examine the function of the identified CRE site (−8 to −1) in the regulation of LDLR transcription, HepG2 cells were transfected with the full-length LDLR promoter construct pLDLR234 (−143 to +35) or a reporter containing only the TATA-like element, pLDLR-TATA (−36 to +13) (17). Fig. 9A shows that dibutyryl cAMP by itself is a weak stimulator of LDLR transcription, only increasing the luciferase activity of pLDLR234 by 2-fold without a significant effect on pLDLR-TATA at concentrations up to 5 mM. However, in the presence of OM, luciferase activity was markedly increased up to 8-fold for the full-length promoter and up to 3-fold for the minimal promoter construct in a dibutyryl cAMP dose-dependent manner. This synergistic effect of cAMP and OM depends on the presence of both intact C/EBP and CRE sites, as mutation in either site greatly reduced the synergistic activation of the LDLR promoter (Fig. 9B). These data suggest that interaction of activated CREB through protein kinase A-induced phosphorylation with an OM-activated transcription factor(s) leads to the formation of a more robust transcriptional activator complex.

DISCUSSION

A number of studies have demonstrated the modulated expression of LDLR by nonsterol regulators that are structurally and functionally distinct from cholesterol and its derivatives. In spite of the demonstration that most of these nonsterol mediators such as cytokines and growth factors regulate LDLR expression at the transcriptional level, the molecular mechanisms for sterol-independent regulation are totally unknown, as neither the cis-regulatory elements on the LDLR promoter nor the trans-acting factors that mediate these regulations have been identified. In this study, we identified, for the first time, a SIRE in the human LDLR promoter that is responsive to the cytokine oncostatin M.

The OM-responsive element was previously narrowed down to promoter region −52 to +13. This region contains the Sp1-binding site and the two TATA-like sequences. To distinguish Sp1-mediated constitutive promoter activity from OM-stimulated promoter activity, we made single-base mutations across the core Sp1-binding site within the repeat 3 sequence. These mutations revealed that OM activity was diminished when Sp1 binding was abrogated, but was not concurrently lost (Fig. 1). One explanation would be that OM induced a DNA-binding protein to bind this region, and mutations affected this factor differently than Sp1 binding. However, failure of the detection of an OM-induced DNA-binding activity in the repeat 3 sequence excluded this possibility.

In searching for additional regulatory elements 3′ of repeat 3, a total of eight mutants that encompass the two TATA-like sequences were constructed and examined for their effects on basal and OM-induced promoter activities. The two mutants TATA1a and TATA1b, which targeted the TATA1 sequence, lowered basal promoter activity to 60−70% of the wild-type level without affecting OM activity, thereby suggesting that this region is not involved in the action of OM, but may contribute to basal promoter activity. We have conducted gel shift assays using a probe containing the TATA1 sequence (−30 to −13) to detect nuclear proteins from HepG2 cells that could bind to the TATA1 element. However, no DNA-protein complex was detected (data not shown). Therefore, at present, it is not clear how the TATA1 sequence regulates LDLR transcription. In contrast to the TATA1 mutations, the OM inducibility of LDLR promoter activity was completely abrogated by mutations within TATA2 and its 3′-flanking sequence containing a potential CRE site. Additional mutations downstream from the CRE site did not affect OM activity. Taken together, these eight mutants defined promoter region −17 to −1 as the cis-regulatory element that is responsible for OM-induced activation of
LDLR transcription. As revealed by a computer-aided sequence analysis, this promoter region consists of continuous binding sites for C/EBP (217 to 29) and CREB (28 to 21). Because this promoter region not only mediated the effect of OM, but also responded to cAMP stimulation (Fig. 9), we designated this promoter region as a SIRE.

Next, we were interested to know the relationship between the SIRE and the Sp1-binding site in repeat 3. To determine whether Sp1 binding to repeat 3 is necessary for the SIRE to respond to OM, we replaced repeat 3 in pLDLR-R3 with a GAL4-binding site to create vector pLDLR-GAL4. The basal promoter activity of pLDLR-GAL4 in HepG2 cells that did not express GAL4 or GAL4-Sp1 was very low and did not respond to OM stimulation in a way similar to pLDLR-TATA. OM stimulation was restored equally when the promoter was activated by coexpression of either full-length GAL4 protein or the fusion protein containing the GAL4 DNA-binding domain and the Sp1 activation domain in a manner similar to pLDLR-R3. The mutation in the TATA2 site of pLDLR-GAL4 totally abolished OM stimulation. The same results were obtained with coexpression of GAL4-VP16 fusion protein (data not shown).

To understand the molecular basis of the SIRE, gel shift and supershift assays were performed to characterize the transactivators that interact with this element. In HepG2 nuclear extract without OM treatment, three DNA-protein complexes (C1, C3, and C4) were formed with the TATA1 probe, which contains the SIRE site. The C1 and C3 complexes were shown to contain homodimers or heterodimers of members of the CREB and C/EBP families (Fig. 7A). CREB or C/EBP binds to the SIRE independently, as mutations abolishing the CRE site did not affect C/EBP binding, and conversely, CREB binds the SIRE probe containing a mutation within the C/EBP site (data not shown).

In OM-treated HepG2 nuclear extract, in addition to C1, C3, and C4, an additional complex (C2) was formed. We found that binding of the C2 complex to the TATA1 probe required the intact C/EBP and CRE sites because mutation in either site eliminated C2 binding to the probe (data not shown). This is in contrast to the independent interaction of CREB and C/EBP with the CRE site. Moreover, induction of C2 by OM depends on ERK activation, as treating cells with OM in the presence of U0126, an inhibitor to the ERK upstream kinase MEK, totally prevented C2 formation without an effect on the other three complexes. Previously, we have shown that U0126 abolished the OM induction of endogenous LDLR mRNA and decreased the OM-stimulated promoter activity of pLDLR-R3 (17). Thus, the inhibitory effect of U0126 on the C2 complex strongly suggests that the SIRE is a cis-acting element that

FIG. 9. Synergistic activation of LDLR promoter activity by dibutyryl cAMP and OM. A, HepG2 cells were transfected with the reporter constructs pLDLR234 and pLDLR-TATA. After transfection, cells were cultured in medium containing cholesterol (LPDS + cholesterol) for 20 h and then stimulated with dibutyryl cAMP at the indicated doses in the absence or presence of OM (50 ng/ml) for 4 h prior to lysis. B, HepG2 cells were transfected with the reporter constructs wild-type pLDLR234 (Wt), pLDLR234-CREMU1, and pLDLR234-TATA2a, respectively. After transfection, cells were cultured in medium containing cholesterol (LPDS + cholesterol) for 20 h and then stimulated with dibutyryl cAMP at 0.5 mM in the absence or presence of OM (50 ng/ml) for 4 h prior to lysis.
mediates the extracellular signal transmitted through the mitogen-activated protein kinase pathway to regulate LDLR transcription. We speculate that the C2 complex contains protein components that are substrates of ERK. The nature of the C2 complex is presently unknown, as gel shift assays with antibodies directed against members of the CREB, C/EBP, and AP-1 families; TATA-binding protein; TAF130; and TAF100 failed to supershift or abolish the C2 complex. These experiments suggest that C2 may contain a novel transcription factor. The identity of the C2 is currently under further investigation.

The involvement of AP-1 in LDLR transcription deserves further investigation. In supershift assays, although c-Jun was detected in the C1 complex, pure recombinant c-Jun could not bind to the TATA1+2 probe under similar binding conditions. Interestingly, when recombinant c-Jun was mixed with nuclear extracts prepared from control or OM-treated HepG2 cells, the intensity of C1 was increased, and anti-c-Jun antibodies completely supershifted the C1 complex (data not shown). These data suggest that the c-Jun homodimer is not able to bind to the SIRE, but c-Jun may dimerize with ATF and consequently bind to the SIRE. The relationship between phosphorylation of c-Jun and formation of the OM-induced C2 complex is currently under investigation.

In summary, we have identified the SIRE as a novel sterol-independent cis-acting regulatory element in region 17 to 1 of the human LDLR promoter. This element overlaps the previously described TATA-like sequences. It contains contiguous binding sites for the transcription factors C/EBP and CREB. The SIRE is not involved in the classical feedback regulation by cholesterol, but is critically involved in regulations mediated by oncostatin M, cAMP, and C/EBP. We hypothesize that the SIRE acts to integrate different signaling pathways controlling the transcription of the LDLR gene independent of cellular cholesterol. These novel findings provide a molecular basis for the mechanism of sterol-independent regulation of LDLR and link extracellular signaling events to promoter elements on the LDLR gene. This work opens up a new avenue for developing alternative pharmaceutical interventions to lower plasma low density lipoprotein cholesterol via a mechanism of action distinct from the classical inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase.

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REFERENCES

1. Sudhof, T. C., Van der Westhuyzen, D. R., Goldstein, J. L., Brown, M. S., and Russell, D. W. (1987) J. Biol. Chem. 262, 10773–10779
2. Dawson, P. A., Van der Westhuyzen, D. R., Sudhof, T. C., Brown, M. S., and Goldstein, J. L. (1988) J. Biol. Chem. 263, 3372–3379
3. Sudhof, T. C., Russell, D. W., Brown, M. S., and Goldstein, J. L. (1987) Cell 48, 1061–1069
4. Smith, J. R., Osborne, T. F., Goldstein, J. L., and Brown, M. S. (1990) J. Biol. Chem. 265, 2306–2310
5. Briggs, M. R., Yokoyama, C., Wang, X., Brown, M. S., and Goldstein, J. L. (1993) J. Biol. Chem. 268, 14490–14496
6. Wang, X., Briggs, M. R., Yokoyama, C., Goldstein, J. L., and Brown, M. S. (1998) J. Biol. Chem. 263, 14497–14504
7. Wang, X., Sato, R., Brown, M. S., Hua, X., and Goldstein, J. L. (1994) Cell 77, 53–62
8. Yokoyama, C., Wang, X., Briggs, M. R., Admon, A., Wu, J., Hua, X., Goldstein, J. L., and Brown, M. S. (1993) Cell 75, 187–197
9. Sanchez, H. B., Yieh, L., and Osborne, T. F. (1995) J. Biol. Chem. 270, 1161–1169
10. Rudling, M., Norstedt, G., Oliveira, R., Rehner, E., Gustafsson, J., and Angelin, B. (1995) Proc. Natl. Acad. Sci. U. S. A. 89, 6983–6987
11. Pak, Y. K., Kanuck, M. P., Berries, D., Briggs, M. R., Cooper, A. D., and Ellsworth, J. L. (1996) J. Lipid Res. 37, 985–998
12. Stopeck, A. T., Richardson, C. M., Mancini, F. P., and Hui, D. P. (1993) J. Biol. Chem. 268, 17489–17494
13. Takagi, T., andStrauss, J. F. (1988) Can. J. Physiol. Pharmacol. 67, 968–973
14. Grove, R. I., Mazzucco, C. E., Radka, S. F., Shoyab, M., and Kiener, P. A. (1991) J. Biol. Chem. 266, 18194–18199
15. Liu, J., Grove, R. I., and Vestal, R. E. (1994) Cell Growth Differ. 5, 1333–1338
16. Liu, J.,Streff, R., Zhang, Y. L., Vestal, R. E., Spence, M. J., and Briggs, M. R. (1991) J. Lipid Res. 32, 2035–2046
17. Li, C., Kraemer, F. B., Ahlborn, T. E., and Liu, J. (1999) J. Biol. Chem. 274, 6747–6763
18. Enami, K. H., Navarre, W. W., and Smale, S. T. (1995) Mol. Cell. Biol. 15, 5906–5916
19. Dignam, J. D.,Lebovitz, R. M., andReeder, R. C. (1983) Nucleic Acids Res. 11, 1475–1489
20. Favata, M. F., Horiiuchi, K. Y., Manos, R. J., Daulerio, A. J., and Stradley, D. A., Feese, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F., Copeland, R. A., Maguida, R. L., Scherle, P. A., and Trzaskos, J. M. (1998) J. Biol. Chem. 273, 18623–18632
21. Lalli, E., and Sasse-Combs, P. (1994) J. Biol. Chem. 269, 17539–17542
22. Lee, K. A. W., and Massen, N. (1993) Biochim. Biophys. Acta 1174, 221–233
23. Botelho, F. M., Edwards, D. R., and Richards, C. D. (1998) J. Biol. Chem. 273, 5211–5218
24. Wen, X. Y., Steward, A. K., Soomman, R. R., Henderson, G., Hawley, T. S., Reimold, A. M., Glime, L. H., Baumann, H., Malek, L. T., and Hawley, R. G. (1999) Int. J. Oncol. 15, 173–178
25. Ishihiki, H., Akira, S., Tanabe, O., Nakajima, T., Shimamoto, T., Hirano, T., and Kishimoto, T. (1990) Mol. Cell. Biol. 10, 2757–2764
26. Akira, S. (1997) Int. J. Biochem. Cell Biol. 29, 1401–1418
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