Novel mechanism of transcriptional activation of hepatic LDL receptor by oncostatin M

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Abstract In this paper we describe a sterol-independent regulation of low density lipoprotein receptor (LDLR) transcription by the cytokine oncostatin M (OM) in HepG2 cells. We show that OM-induced expression is independent of cholesterol regulation and occurs at the transcriptional level. To elucidate regulatory mechanism(s), we constructed a luciferase reporter system comprising either the native LDLR promoter including repeats 1, 2, and 3, or a synthetic promoter vector containing repeats 2+3 only, allowing us to directly examine OM effects on individual elements. Specific mutants in repeats 1, 2, and 3 were made to facilitate the mapping of the OM effect on the promoter. Wildtype and mutant constructs were assayed for cholesterol and OM regulation. The results show that mutation within the core SRE-1 element of repeat 2 totally abolished cholesterol regulation but had no effect on OM inducibility. More interesting, a mutation within repeat 1 reduced basal transcription activity to 10% of the native promoter, but OM induction was unaltered. However, the identical mutation engineered in repeat 3 significantly decreased OM induction of LDLR promoter activity. These results suggest a novel regulatory role for the repeat 3 element in LDLR transcription.—**Liu, J., R. Streiff, Y. L. Zhang, R. E. Vestal, M. J. Spence, and M. R. Briggs.** Novel mechanism of transcriptional activation of hepatic LDL receptor by oncostatin M. *J. Lipid Res.* 1997. **98:** 2035–2048.

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The hepatic low density lipoprotein receptor (LDLR) is a key regulator of human plasma LDL cholesterol (LDLc) homeostasis (1). Increased hepatic LDLR expression results in improved clearance of plasma LDLc through receptor-mediated endocytosis, which has been associated with a decreased risk of developing cardiovascular disease in humans (2). An immediate result of increased uptake of plasma LDLc through LDLR is an elevated intracellular cholesterol concentration. However, high intracellular levels of cholesterol are postulated to lead to the production of cholesterol metabolites such as 25-hydroxycholesterol (25-OHC). The accumulation of cholesterol and its toxic metabolites suppresses the transcription of genes involved in cholesterol biosynthesis, such as HMG-CoA reductase (HMGR) as well as the gene for LDLR in a well-characterized example of end-product feedback repression (3).

The *cis*-acting regulatory elements that control LDLR transcription have been localized to three GC-rich imperfect 16 bp direct repeats within 100 bp upstream of the transcriptional start site (4). Repeats 1 and 3 contain Sp1 binding sites which have been reported to support the basal transcriptional activity of LDLR (5, 6). Negative cholesterol regulation is mediated through a 10 bp sequence (5′ ATCACCCCAC 3′) within repeat 2 designated as sterol responsive element-1 (SRE-1) (7, 8). The SRE-1 enhances transcription in sterol-depleted cells through interaction with at least two SRE binding proteins, SREBP1 and SREBP2 (8–11). These proteins function as conditionally positive transcription factors. In the presence of adequate intracellular cholesterol, they exist as membrane-bound precursors embedded within the endoplasmic reticulum. Upon sterol depletion, the precursor is proteolytically cleaved, releasing mature SREBP, which then translocates to the nucleus and binds transiently to the SRE-1 sequence, thereby increasing LDLR transcription. The accumulation of intracellular oxysterol as a result of LDLR-mediated cholesterol uptake and metabolism inhibits the release of mature SREBP resulting in suppression of LDLR transcription (12). Recent evidence has shown that SREPB, when bound to the SRE-1 sequence, acti-

Abbreviations: 25-OHC, 25-hydroxycholesterol; bp, base pair(s); LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; OM, oncostatin M; LPDS, lipoprotein-depleted serum; SRE-1, sterol response element-1; SREBP, SRE-1 binding protein; FAS, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoresis mobility shift assay.

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vates LDLR transcription synergistically with the transcription factor Sp1 by enhancing Sp1 binding at the adjacent site within repeat 3 (13).

In addition to cholesterol and its metabolites, growth factors, hormones, and cytokines are also capable of modulating hepatic LDLR activity. A recent study showed that hepatocyte growth factor (HGF) increased LDLR promoter activity in HepG2 cells (14). The activity of HGF was shown to be directed through repeats 2 and 3. Inflammatory processes are often associated with elevated levels of cytokines and abnormal LDL*c* metabolism (15–20). The increased transcription of hepatic LDLR by cytokines may partially explain hypocholesterolemia associated with inflammatory states. Tumor necrosis factor-α (TNF), interleukin-1β (IL-1), and oncostatin M (OM) have been shown to increase LDL uptake and activate LDLR gene transcription. Interestingly, modulation of LDLR transcription by these cytokines appears to occur through different mechanisms. TNF and IL-1 increase LDLR promoter activity 200–400% in culture medium containing delipidated serum (21). These inductions are lost when LDL*c* is added back, suggesting that TNF and IL-1 regulate LDLR transcription by a sterol-dependent mechanism. In contrast to TNF and IL-1, OM, a cytokine predominantly produced by activated T cells and macrophages, increases LDL uptake (22) and activates LDLR transcription in HepG2 cells independent of intracellular cholesterol level (23).

To elucidate the molecular mechanism by which OM activates LDLR transcription and to localize OM responsive elements in the LDLR promoter, we took advantage of the sensitive luciferase reporter system. Our approach initially utilized a series of oligonucleotide-based promoter reporter constructs (referred to as synthetic promoter constructs) containing mutations within repeats 2 or 3. Based on the data from synthetic promoter study, analogous mutations were made in the native LDLR promoter. This approach enabled us to study the effects of mutations on both basal as well as OM-regulated transcription in a more physiological setting. Furthermore, the effects of these mutations on the DNA binding activities of transcription factor Sp1 and Sp1-like proteins were studied by performing electrophoresis mobility shift assays (EMSA).

MATERIALS AND METHODS

Cells and reagents

The human hepatoma cell line HepG2 was obtained from American Type Culture Collection (Bethesda, MD) and was cultured in minimum essential medium Eagle (EMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Summit Biotechnology, Fort Collins, CO). Cholesterol and 25-hydroxycholesterol (25-OHC) were obtained from Sigma. Secreted human recombinant oncostatin M expressed in Chinese hamster ovary cells was purified by reverse-phase high performance liquid chromatography (24). Human recombinant OM expressed in *E. coli* was purchased from R&D Systems (Minneapolis, MN). Lipoprotein-deficient serum (LPDS) was prepared as previously described (25). Actinomycin D (ACT-D) was obtained from Calbiochem (San Diego, CA). Anti-gp130 polyclonal antibodies were obtained from R&D Systems. Rabbit polyclonal antibodies against Sp1, Sp3 and Stat 3 were obtained from Santa Cruz Biotechnology, Santa Cruz, CA.

Plasmid vectors and oligonucleotides

Synthetic LDLR promoter fragments of 81 bp (consisting of contiguous repeat 2 and 3 elements from LDLR promoter in tandem separated by a six-base *Sal*I sequence) were synthesized as double-stranded oligonucleotides containing *Sac*I and *Xho*I sites at the 5′ and 3′ ends, respectively. These promoter fragments were cloned between the *Sac*I and *Xho*I sites of the pGL2TATA promoterless luciferase vector. pGL2TATA was created by insertion of a 5′ blunt 3′ *Hind*III fragment containing the adenovirus E1b TATA box and 45 bp of polylinker sequence between the *Sma*I and *Hind*III sites of the promoterless pGL2-basic luciferase reporter vector (Promega Corporation, Madison, WI). The pGL2-basic vector has no defined eukaryotic promoter or enhancer sequences. Synthetic promoter constructs were ligated and used to transform DH5α *E. coli* (Gibco, Grand Island, NY) according to standard methods. Correct clones were screened by restriction digest and verified by dideoxy sequencing using the Sequenase 2.0 kit (Amersham, Arlington Heights, IL). The plasmid pLDLR234LUC was constructed by subcloning a 177-bp fragment of LDLR promoter obtained by *Hind*III digestion of pLDLR-CAT 234 (4) into *Hind*III digested pGL2 basic vector.

Site-directed mutagenesis

pLDLR234LUC was used as a template for making repeat 1, 2, 3, and 1 and 3 mutants using the QuickChange™ Site-directed Mutagenesis Kit (Stratagene, San Diego, CA). Correct clones were screened by restriction digest and verified by dideoxy sequencing.

Transient transfection assays

HepG2 cells were transfected with plasmid DNA by the method of calcium phosphate coprecipitation.

Briefly, cells were plated one day before transfection in 48-well culture plates at a density of 1.25×10^5 cells per well in medium A (EMEM medium containing 10% FBS, 100 units/ml of penicillin G and 100 μg/ml streptomycin). One hour before transfection, fresh medium was added. Calcium phosphate precipitates containing (per well) 330 ng of LDLR reporter plasmid plus 110 ng of pRSV-βgal (to normalize transfection efficiency) were prepared. The DNA/calcium phosphate precipitates were incubated with the cells at 37°C for 3 h, at which time the cells were washed once with PBS, incubated with 15% glycerol/1× HBS for 1 min, washed twice more with PBS, and refed with either medium A or medium B (same as media A except with 10% LPDS instead of FBS). Where sterol regulation of LDLR was measured, cells received medium B with or without cholesterol (10 μg/ml plus 1 μg/ml 25-OHC). Otherwise, cells were refed with medium A. Twenty hours after replacement of media, OM at 1 nM was added. This OM concentration was chosen because it elicited the maximum response from both synthetic and native LDLR promoter luciferase constructs in studies of OM dose-dependent effects. OM expressed in Chinese hamster ovary cell supernatants produced results identical to those of the OM obtained commercially from R&D systems. Four hours after OM treatment, cells were washed twice with 1× PBS and lysed with 150 μl of 1× reporter lysis buffer (Promega Corporation, Madison, WI). Luciferase activity (40 μl of lysate/sample) was measured in a Berthold Autolumat luminometer model LB953 (Aliquippa, PA) using substrate prepared in accordance with Promega's luciferase assay system. β-Galactosidase activity (50 μl of lysate) was measured according to standard methods. Absolute luciferase activity was normalized against β-galactosidase activity to correct for transfection efficiency. Triplicate wells were assayed for each transfection condition and at least three independent transfection assays were performed for each luciferase construct.

RNA isolation and northern blot analysis

Cells were lysed in Ultraspec RNA lysis solution (Biotecxs Laboratory, Houston, TX) and total cellular RNA was isolated according to the vendor's protocol. Approximately 15 μg of each total RNA sample was separated on a 1.0% formaldehyde agarose gel. RNA was capillary transferred to a Hybond N membrane (Amersham). Nucleic acids were UV crosslinked to the membrane using a GS Gene Linker UV chamber (Bio-Rad Laboratories, Hercules, CA). Prehybridization and hybridization steps were performed under the previously described conditions (26). The blot was hybridized at 60°C to a 0.84 kb ^{32}P-labeled human LDLR probe. The probe was generated by digestion of the plasmid pEB-LDLR (generously provided by Dr. Jeff L. Ellsworth at CV-Therapeutics, Palo Alto, CA) with EcoR1 and BstX1 (14). The probe was labeled using 50 μCi [α-^{32}P]dCTP with a random primed DNA labeling kit (Boehringer Mannheim Corp, Indianapolis, IN) and was purified by G-50 Sephadex columns (Pharmacia, Piscataway, NJ). The membrane was then washed once at ambient temperature with 2× SSC, 0.1% SDS and twice at 60°C with 0.1× SSC, 0.1% SDS. The membrane was then dried and exposed to X-OMAT scientific imaging film (Kodak, Rochester, NY) with an intensifying screen for 1 to 3 days at −80°C. Where indicated, the blots were stripped and reprobed with a human GADPH probe (14) to ensure that equivalent amounts of RNA were being loaded. The plasmid pSp1-778C used for Sp1 mRNA detection was generously provided by Dr. Jim Kadonaga at the University of California at San Diego. The plasmid vector M13-hFAS containing 350 bp of human fatty acid synthase cDNA (generously provided by Dr. Timothy F. Osborne at University of California at Irvine) was utilized for detection of FAS mRNA. Differences in hybridization signals of northern blots and the nuclear run-on reactions were quantitated by a laser densitometer (Molecular Dynamics, Sunnyvale, CA). Densitometric analysis of autoradiographs in these studies as well as those discussed below included various exposure times to ensure linearity of signals.

Nuclear run-on analysis

These analyses were conducted using a procedure adapted from one that has been described previously (27). Briefly, cells were harvested with cell scrapers into PBS. The cells were pelleted by low-speed centrifugation and lysed with lysis buffer (10 mM Tris-HCl, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40). The nuclei were pelleted by centrifugation and the lysis procedure was repeated once. The nuclei were recovered by centrifugation a second time and resuspended at 10^8 nuclei/ml in glycerol storage buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA). The nuclei samples were immediately frozen under liquid nitrogen and stored at −80°C.

On the day of the experiment, the frozen nuclei were thawed and 100 μl of each sample was mixed with 100 μl 2× reaction buffer (70% glycerol, 0.02 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, 0.16 M KCl, 2 mM DTT, 0.2 mM EDTA, 2 mM rATP, 2 mM rCTP, 2 mM rGTP, 2.6 μCi/μl [^{32}P]rUTP). The reactions were incubated with shaking at 30°C for 30 min. Labeled nuclei were pelleted and resuspended with 100 μl DNase buffer (50% glycerol, 20 mM Tris-HCl, pH 7.9, 1 mM $MgCl_2$, 10 mg/ml RNase-free DNase I). The reactions were incubated with shaking at 30°C for 15 min. Samples were brought up to 125 μl with 7.5 μl 13.6 mg/ml proteinase K, 5 μl

10 mg/ml yeast tRNA, and 12.5 μl 10× SET buffer (5% SDS, 0.05 M EDTA, 0.01 M Tris-HCl, pH 7.4) and incubated at 42°C for 30 min.

Labeled RNA transcripts were extracted by addition of the following: 275 μl GCSM solution [4 M guanidinium isothiocyanate, 0.025 M sodium citrate, pH 7.0, 0.5% Sarkosyl, 0.1 M β-mercaptoethanol], 45 μl 2.0 M sodium acetate, 450 μl water-saturated phenol, and 90 μl chloroform–isoamyl alcohol 49:1. The samples were vortexed and incubated on ice for 15 min. Nuclear run-on transcripts were precipitated with isopropanol and pelleted by high speed centrifugation. Extractions and isopropanol precipitations were repeated and the samples were dissolved with 102 μl TES buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% SDS). Assays for radioactivity were conducted by liquid scintillation. Approximately 4–6.0 × 10^6 cpm of each nuclear run-on reaction was used as a probe to hybridize a Hybond N membrane (Amersham) slot blot. Each blot received the following two plasmids: 5 μg plasmid containing the human GAPDH cDNA insert, and 1 μg of the 0.84 kb fragment of the LDLR cDNA as described in the northern blot analysis. Probing the GAPDH plasmid allowed normalization of the LDLR signals measured by densitometry.

Preparation of nuclear extracts and electrophoresis mobility shift assays

HepG2 cells were seeded at 6.5–8 × 10^6 cells/100-mm dish 2 days before harvesting. Nuclear extracts were prepared by the method of Dignam, Lebovitz, and Roeder (28) except that the buffer A was supplemented with 1 mM Na_3VO_4 and 1 μg per ml of each of pepstatin and leupeptin. Nuclear extracts were quick frozen by liquid nitrogen and stored in aliquots. Protein concentrations were determined using a modified Bradford assay using BSA as a standard (Bio-Rad). Protein concentrations of nuclear extracts from different preparations were typically 3–5 mg/ml. Oligonucleotide probes were annealed and purified by electrophoresis through 20% polyacrylamide gel prior to 3′ fill-in labeling using Klenow fragment in the presence of [α-^{32}P]dATP.

Each binding reaction was composed of 10 mM HEPES, pH 7.8, 0.5 mM $MgCl_2$, 1 mM DTT, 80 mM KCL, 10% glycerol, 5 μg of poly (dI-dC), 10 μg BSA, and 1–5 μg nuclear extract or 0.5 footprint unit (fpu) of affinity-purified Sp1 (Promega Corp) in a final volume of 30 μl. After incubation for 10 min at 4°C, 0.5–1 ng of 3′ ^{32}P-labeled double-stranded synthetic oligonucleotide probe (20–40 × 10^3 cpm) was added. After incubation for 30 min at 4°C, reaction mixtures were loaded onto a 4% polyacrylamide gel and run in TGE buffer (50 mM Tris base, 400 mM glycine, 1.5 mM EDTA, pH 8.5) at 180 V for 2 h at 4°C. The gels were dried and exposed for autoradiography using Kodak X-AR film at −70°C with an intensifying screen. In competition analysis, nuclear extracts were incubated with 40-fold molar excess of unlabeled competitor DNA for 10 min prior to the addition of the labeled probe. For supershift assays, rabbit polyclonal antibodies against either Sp1 or Sp3, or irrelevant control polyclonal antibodies, were added to the binding reaction mixture and incubated for 60 min prior to loading. Synthetic LDLR repeat 2 + 3 oligonucleotides and mutant derivatives are described in **Table 1.** The sequences of EMSA probes were as follows and the consensus binding sites are underlined:

R1: 5′ TTCGAAACTCCTCCTCTTGCAGTGAGGTGAA GACATTTG 3′;

R23: 5′ TTTGAAAATCACCCCACTGCAAACTCCTCC CCCTGCT 3′;

R123: 5′ TTCGAAACTCCTCCTCTTGCAGTGAGGTG AAGACATTTGAAAATCACCCCACTGCAAACTCCTC CCCCTGCT 3′

2× consensus Sp1 oligonucleotides:

5′-CCGTACGGGGCGGGGCGCGATCGGGGCGGGGC GC-3′

Consensus Stat 3 oligonucleotides: 5′-GATCGATTTCC CCGAAATG-3′

TABLE 1. Oligonucleotides used for cloning synthetic LDLR promoter luciferase constructs

	Repeat 2	Repeat 3
R23	5′ *AAAATCACCCCACTGC*	AAACTCCTCCCCCTGC 3′
$R2_c3$	5′ *AAAATCACCCCACTTA*	AAACTCCTCCCCCTGC 3′
$R2_b3$	5′ *AAAATCACCCCTCTGC*	AAACTCCTCCCCCTGC 3′
$R2_c3$	5′ *AAAATCACGGCACTGC*	AAACTCCTCCCCCTGC 3′
$R23_a$	5′ *AAAATCACCCCACTGC*	TTTCTCCTCCCCCTGC 3′
$R23_b$	5′ *AAAATCACCCCACTGC*	AAACTCCTCCCCGTGC 3′
$R23_c$	5′ *AAAATCACCCCACTGC*	AAACTCCACCCCCTGC 3′
$R23_d$	5′ *AAAATCACCCCACTGC*	AAACTCTTTCCCCTGC 3′
R33	5′ AAACTCCTCCCCCTGC	AAACTCCTCCCCCTGC 3′

The oligonucleotides used to create the various synthetic LDLR promoter constructs are designated based on the wildtype R23LUC, which contains tandem (2+3) elements. Repeat 2 sequences are italic. For the sake of clarity, only one copy of the (2+3) element is shown, but mutants contain identical mutations in both copies of the (2+3) sequence. Constructs bearing mutations carry a letter designation following the repeat in which the mutation lies. Mutated bases are represented as bold underlined type.

Statistical analysis

Comparisons of experimental data were analyzed by one-way ANOVA and the Newman-Keuls Multiple Comparison Test. A P value < 0.05 was considered to indicate a statistically significant difference.

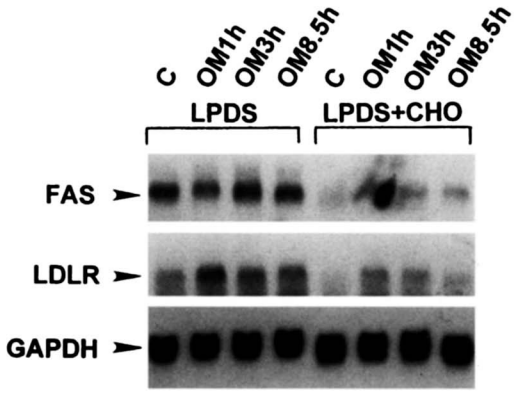

Fig. 1. Effect of OM on the level of LDL receptor mRNA. HepG2 cells were cultured in medium containing lipoprotein-depleted serum (LPDS) or in LPDS medium supplemented with 10 μg/ml cholesterol + 1 μg/ml 25-OHC (LPDS + CHO) for 24 h then stimulated with 2 nM OM for the indicated time. Total RNA was isolated and LDLR mRNA level was analyzed by northern blot as described in Materials and Methods. The membrane was stripped and reprobed with human FAS and GADPH cDNA probes.

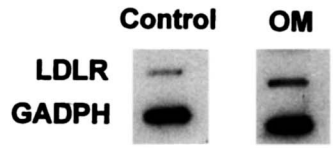

Fig. 2. Nuclear run-on analysis of LDLR transcription. Two probes were slot-blotted onto each of two nylon membrane strips. One slot received 1 μg of the 0.84 kb fragment of the LDLR cDNA. The second slot was loaded with 5 μg of the GAPDH plasmid. One nylon strip was hybridized to a ^{32}P-radiolabeled nuclear run-on reaction prepared from 1-h OM-treated HepG2 cells. The second was hybridized to a labeled nuclear run-on reaction prepared from control cells. An equal amount of radioactivity was used in each hybridization. Radioactive signals were detected by autoradiography and quantified by densitometric analysis. The figure shown is representative of two separate experiments.

RESULTS

OM increases LDL receptor transcription independent of intracellular sterols

The time-dependent effects of oncostatin M (OM) on LDLR mRNA in HepG2 cells under inducing or sterol-suppressing conditions were examined side by side by northern blot analysis. Cells were cultured in medium containing 10% lipoprotein-depleted fetal bovine serum (LPDS) or in LPDS supplemented with sterols (LPDS+CHO) (10 μg/ml cholesterol + 1 μg/ml 25-OHC). **Figure 1** shows that the basal LDLR mRNA level is lower in cells cultured in the presence of sterols relative to cells cultured in the absence of sterols, indicating that LDLR gene expression is subject to normal cholesterol feedback regulation in HepG2 cells. Addition of 2 nM OM produced a time-dependent increase in LDLR mRNA, independent of cholesterol in the culture medium. At 1 h, the OM effect had peaked at 3.0-fold, and by 8.5 h had dropped to 1.4-fold. To investigate whether OM also regulates other genes that are transcriptionally suppressed by sterols, we examined fatty acid synthase (FAS) mRNA expression, as the FAS gene promoter also contains SRE-1 and Sp1 binding sites (29). Northern blot analysis showed that the mRNA of FAS was down-regulated by sterols, but was not significantly altered by OM treatment under either inducing (LPDS) or sterol-suppressing conditions (LPDS+CHO), suggesting that the LDLR promoter has unique regulatory properties.

To investigate whether the increase of LDLR mRNA by OM occurs at the transcriptional level, HepG2 cells cultured under sterol-suppressing conditions were treated with OM for 1 h at which time nuclei from control cells and OM-treated cells were isolated. Nuclear run-on analysis (**Fig. 2**) shows that OM treatment of HepG2 cells increased the abundance of actively transcribed LDLR mRNA 2.3-fold relative to control cells after normalization with GADPH hybridization signal. These results indicate that a major component of the observed OM-induced up-regulation of LDLR mRNA occurs at the transcriptional level.

OM activates both native and synthetic LDLR promoter–luciferase reporter constructs containing repeats 2+3

A previous study from this laboratory examined the effect of OM on LDLR promoter–CAT reporter constructs (23). That report demonstrated that OM increases the CAT activity of pLDLR–CAT 234 that contains a 177 bp fragment of the LDLR promoter (−142 to +35), to a level comparable to that of the pLDLR–CAT 1563 construct that contains a longer 5′ sequence (−1471 to +35). This showed that the essential *cis*-acting elements mediating OM-regulated transcription are located within 142 bp upstream of the transcription start site. This region contains TATA-like sequences and binding sites for the transcription factors SREPB (repeat 2) and Sp1 (repeats 1 and 3). In order to further define the OM-responsive region in the LDLR promoter, a synthetic luciferace reporter vector (R23LUC) was constructed in which two tandemly arranged copies of repeats 2+3 were inserted between the *Sac*I and the *Xho*I sites of pGL2–TATA (**Fig. 3**). The effect of OM on this synthetic promoter (R23LUC) was then compared with its effect on the pLDLR234LUC vector which contains native LDLR promoter sequence from −142 to +35 (14). These reporters, along with the β-galactosi-

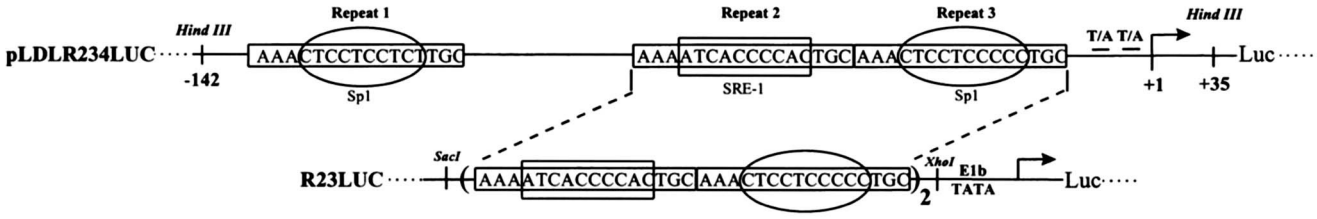

Fig. 3. LDL receptor promoter luciferase constructs. Top: pLDLR234LUC was created by inserting a 177 bp fragment containing the repeats 1, 2, and 3 elements and TATA-like sequences of the LDL receptor promoter (nt −142 to +35, with respect to the LDLR transcriptional start site) into the *Hind*III site of pGL2basic. The resulting construct drives transcription of pGL2 luciferase entirely through native LDLR promoter sequence. Repeats 1 and 3 each contain putative Sp1 binding sites. Repeat 2 contains an SRE-1 element, which binds SREBP-1. Below: R23LUC and its derivatives were created by insertion of a 81 bp oligonucleotide containing tandem copies of the repeat (2+3) region of the native LDLR sequence and the adenovirus E1b TATA box into pGL2-basic. The repeat 2+3 sequence and the E1b TATA box is separated by 8 bps.

dase expression vector pRSV-βGal, were transiently transfected into HepG2 cells and luciferase and β-galactosidase activities were measured.

Incubation of cells with 1 n*M* OM for 4 h produced a 3-fold increase in luciferase activity in R23LUC transfected cells and a 4-fold increase over control in pLDLR234LUC transfected cells with no effect on β-galactosidase activity. The effect of OM on each vector was completely abolished by cotreatment of cells with actinomycin D (**Fig. 4**), thereby confirming a transcriptional mechanism for OM-mediated up-regulation. The comparable induction by OM of R23LUC and pLDLR234LUC suggests that the activity of OM is mainly mediated through the repeat 2+3 sequence. Anti-gp130 polyclonal antibodies that have been shown to inhibit OM binding and OM receptor-mediated signaling in other cell types (30, 31) were then utilized to examine the specificity of the OM effect. Coincubation of R23LUC and pLDLR234LUC transfected cells with anti-gp130 antibodies produced a dose-dependent inhibition of OM-induced LDLR promoter activity, indicating that the observed activity of OM on the LDLR promoter is mediated specifically by the OM receptor. Additionally, we examined the effect of OM on luciferase expression from a reporter construct containing the SV40 virus early promoter (pGL2-control). The results showed that OM did not increase luciferase activity in pGL2-control transfected cells (data not shown), demonstrating that the observed effect of OM on the LDLR promoter is specific, which corroborates earlier data from the CAT-reporter system (23).

Examination of OM effects on the LDLR synthetic promoter constructs containing mutations within repeats 2 or 3

The above results demonstrate the feasibility of using the synthetic construct to rapidly generate and test re-

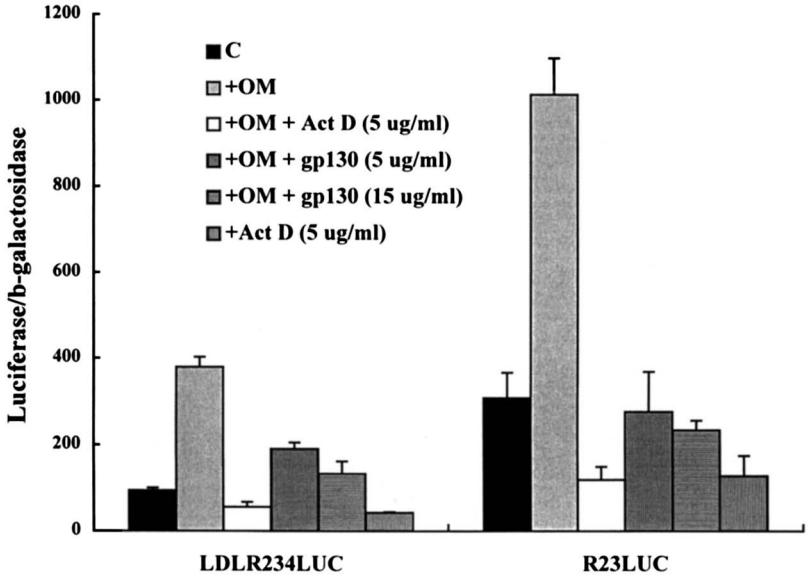

Fig. 4. OM inducibility of LDLR promoter activities are inhibited by actinomycin D and anti-gp130 polyclonal antibodies. Cells were transfected with either pLDLR234LUC or R23LUC. After transfection, cells were treated with OM for 4 h in the presence of 5 μg/ml actinomycin D, or either 5 or 15 μg/ml of anti-gp130 polyclonal antibodies added 40 min prior to OM stimulation. Luciferase expression was normalized to β-galactosidase activity to correct for variations in transfection efficiency. The data (mean ± SD) shown are representative of two separate experiments in which triplicate wells were assayed for each condition.

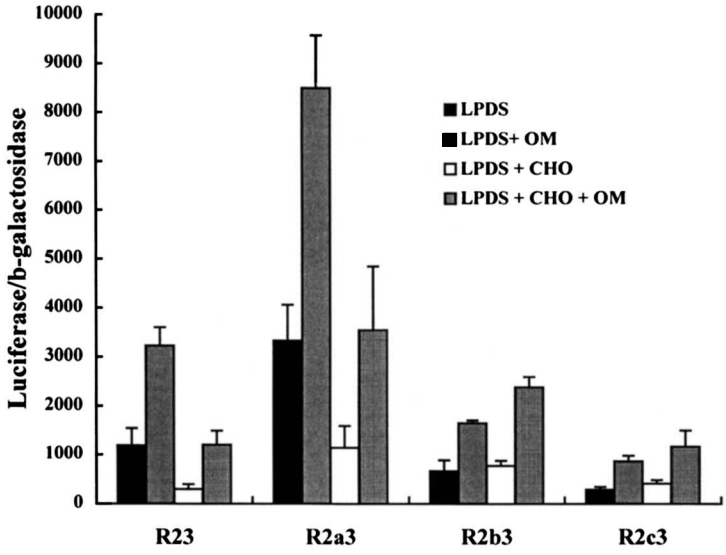

Fig. 5. Cholesterol- and OM-mediated regulation of synthetic reporter constructs containing repeat 2 mutations. Cells were transfected with wildtype R23LUC, or repeat 2 mutant $R2_a3$, $R2_b3$, and $R2_c3$. After transfection, cells were cultured in 10% LPDS or LPDS+CHO medium for 20 h and then were stimulated with OM (1 nM) for 4 h prior to lysis. The results shown are mean ± SD of two independent transfection experiments in which triplicate wells were assayed for each condition.

peat 2 and 3 mutants. In order to separately examine the role of SREBP and Sp1 in cholesterol- and OM-regulated LDLR transcription, three mutants ($R2_a3$, $R2_b3$, and $R2_c3$) having point mutations residing within repeat 2 were constructed. The oligonucleotide sequences of the mutants are illustrated in Table 1. As shown in **Fig. 5,** the mutant $R2_a3$ (AAAATCACCC CACTGC to AAAATCACCCCACT*ta*) was sterol-regulated in a manner similar to the wildtype vector (R23LUC) although basal activity was increased. As expected, the promoter activities of the two mutants ($R2_b3$ and $R2_c3$) containing mutations within the core SRE region were decreased and were not suppressed by cholesterol. However, incubation of cells with OM for 4 h increased luciferase activities to a similar extent (approximately 2.5- to 3-fold) in both the wildtype and mutant constructs regardless of sterol condition. These results provide evidence demonstrating that OM-mediated induction is not conferred through the SRE. Therefore, we focused our investigation on the repeat 3 sequence.

A previous study (3) showed that scramble mutations within repeat 3 severely reduced LDLR transcription. In addition, a report by Dawson et al. (5) tested four repeat 3 mutants that affected promoter activity and cholesterol suppression to different extents. To characterize the repeat 3 sequence for its function in OM-regulated LDLR transcription, we designed several constructs containing point mutations across repeat 3 in a manner complimentary to the mutations designed by Dawson (Table 1). The mutant R23a, containing a 3-base substitution (AAA to TTT) did not significantly change the basal promoter activity from that of wildtype R23 (**Fig. 6**). R23b, containing a C to G mutation of the 3′-most nucleotide of the Sp1 binding site, slightly increased basal (and regulated) promoter activity. However, the mutant R23c, bearing a T to A mutation (pyrimidine to purine) within the Sp1 core binding site, increased the basal transcriptional activity 3- to 4-fold, presumably because the base substitution created a higher affinity Sp1 binding site as compared with the Sp1 consensus sequence. Interestingly, none of these mutations affected OM activity. These mutants responded to OM to an extent similar to the wildtype R23LUC (Fig. 6). The mutant R23d, however, which contains a CTCCTCCCCC to CTC*t*T*t*CCCC double substitution within the core Sp1 binding region, reduced promoter activity to a baseline level and OM was unable to increase the promoter activity above background. We speculated that the loss of transcriptional activity of R23d is due to the inability of Sp1 to bind to the mutated Sp1 site within repeat 3. Therefore, gel shift assays using a ^{32}P-labeled oligonucleotide containing a single copy of the repeats 2+3 sequence (37 bp) were performed. Upon incubation of ^{32}P-labeled R23 (**Fig. 7**) with HepG2 nuclear extract, two DNA-protein complexes were detected (Fig. 7, lane 2). The formation of these complexes was inhibited by competition from a 40-fold molar excess of the unlabeled oligonucleotides R23 (lane 3), R23a (lane 4), R23b (lane 5), R23c (lane 6), and an oligonucleotide containing the consensus Sp1 binding site (lane 8), but were not inhibited by oligonucleotide R23d (lane 7) and an unrelated oligonucleotide containing the consensus sequence for the signal transducer and activator of transcription factor (Stat 3) (lane 9). The slower migrating complex comigrated with the purified Sp1 protein and was supershifted by anti-Sp1 polyclonal antibodies (lane 11), indicating

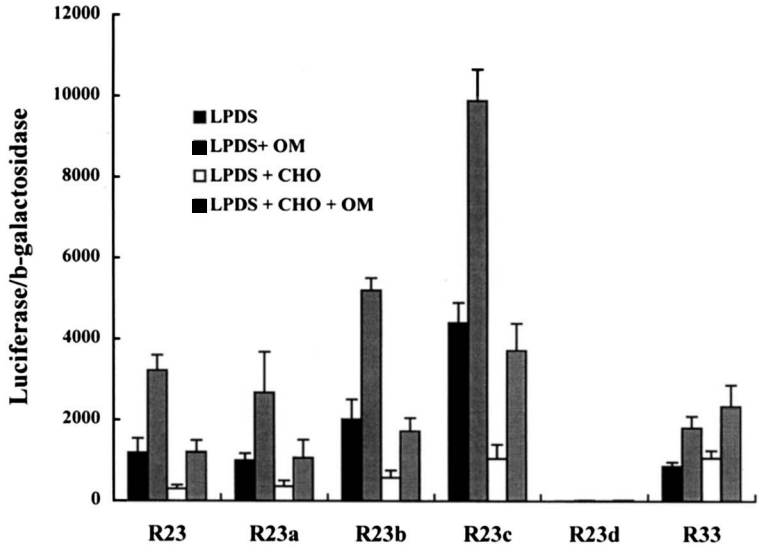

Fig. 6. Cholesterol- and OM-mediated regulation of synthetic reporter constructs containing repeat 3 mutations. Cells were transfected with repeat 3 wildtype R23LUC, repeat 3 mutant R23a, R23b, R23c, R23d, and a construct containing repeat 3 sequences only. After transfection, cells were cultured in 10% LPDS or LPDS+CHO medium for 20 h and then were stimulated with OM (1 nM) for 4 h prior to lysis. The results shown are mean ± SD of two independent transfection experiments in which triplicate wells were assayed for each condition.

that this complex contained the transcription factor Sp1. The faster migrating complex was supershifted by anti-Sp3 polyclonal antibodies (lane 12), suggesting that this complex contained Sp3, a member of the Sp1 family of transcription factors. Incubation of the nuclear extract with an unrelated polyclonal antibodies (anti-Stat 3) altered neither the intensity nor the mobility of the two complexes (data not shown). These EMSA results clearly show that the decreased transcription activity of the mutant R23d is due to the inability of Sp1 and/or Sp3 to bind to repeat 3. Concurrent loss of OM inducibility with basal transcription activity suggests that OM exerts its effect on LDLR gene transcription in a manner requiring a functional Sp1/repeat 3 interaction. Consequently, we examined the effects of OM on cells transfected with a construct containing only tandem copies of repeat 3 (R33). As expected, the promoter activity of R33 was not regulated by cholesterol, but was still moderately increased by OM (2.2 fold) (Fig. 6). These results provide additional evidence that the effect of OM on the LDLR promoter is mediated through the repeat 3 element.

Examination of OM effects on the native LDLR promoter constructs containing mutations within repeats 1, 2, and 3

The above studies, conducted in the synthetic LDLR promoter system, identified critical nucleotides involved in cholesterol- and OM-regulated, as well as basal transcription from the LDLR promoter. However, the synthetic constructs likely capture fewer of the subtleties of naturally regulated LDLR transcription than the native promoter. In addition, although OM- and sterol-regulated transcription seems to occur largely through repeats 2+3, we sought to identify the extent of the contribution of repeat 1. Therefore, we mutated repeats 1, 2, and 3 independently, and repeats 1 and 3 together by site-directed mutagenesis on the native promoter sequence in pLDLR234LUC. The vector pLDLR234-*R2*LUC contains the same base change in repeat 2 as the synthetic promoter R2c3 (AAAATCACC CCACTGC to AAAATCAC*gg*CACTGC). The vector pLDLR234-*R3*LUC contains the same mutation in repeat 3 as the synthetic promoter R23d (AAACTCCTCC CCCTGC to AAACTC*t*T*t*CCCCTGC). The vector pLDLR234-*R1*LUC contains the same 2 base substitution within the core Sp1 site as the repeat 3 mutant (AAACTCCTCCTCTTGC to AAACTC*t*T*t*CTCTTGC). In addition, we also constructed a vector, pLDLR234-*R1*/*R3*LUC, containing mutations within both repeat 1 and repeat 3. **Figure 8A** shows native and mutant promoter activities in the absence and the presence of sterols. In LPDS medium, the promoter activity of the repeat 2 mutant was reduced to 12% of that of the native promoter (pLDLR234LUC). Promoter activity was reduced to 10% and 7% of wildtype in R1 and R3 mutants, respectively. The R1/R3 double mutant fell to 3% of wildtype promoter activity. As in R23d, the decrease of basal transcriptional activity in pLDLR234-*R1*LUC is due to the inability of Sp1 to bind to the mutated repeat 1 sequence, as shown in the gel shift assay (**Fig. 9**). Figure 8B shows the effects of OM on the native promoter and the *R1*, *R2*, *R3* and *R1*/*R3* double mutants. Compared with the native promoter, OM activation is fully retained in pLDLR234-*R1* and pLDLR234-*R2*LUC, but significantly reduced in pLDLR234-*R3*LUC and pLDLR234-*R1*/*R3*LUC. Statistical analysis shows that OM induction of the native as well as the repeat 1 and repeat 2 mutants are highly significant ($P < 0.001$). However, the increases in promoter activity of the re-

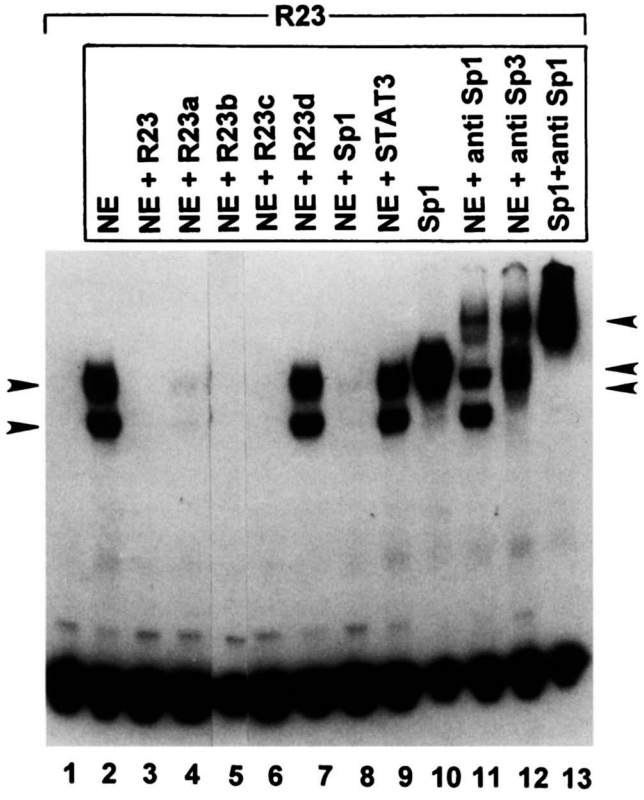

Fig. 7. EMSA competition analysis of HepG2 nuclear proteins interacting with the repeats 2+3 sequence. EMSA using no protein (lane 1), 2 μg of HepG2 nuclear extract (NE) (lanes 2–9), or purified Sp1 protein (0.5 fpu, lane 10) with radiolabeled oligonucleotide containing a single copy of the wild type repeat 2+3 sequence (R23) was conducted in the absence (lane 2) or the presence of 40-fold molar amounts of unlabeled competitors containing two copies of the wild-type or mutated repeat 2+3 sequences as described in Table 1. The arrows on the left indicate the two specific protein-DNA complexes formed with the R23 probe. Lane 1, labeled R23 without nuclear extract; lane 2, labeled R23 with nuclear extract; lane 3, excess unlabeled oligonucleotide R23; lane 4, excess unlabeled oligonucleotide R23a; lane 5, excess unlabeled oligonucleotide R23b; lane 6, excess unlabeled oligonucleotide R23c; lane 7, excess unlabeled oligonucleotide R23d; lane 8, excess unlabeled oligonucleotide containing 2X Sp1 consensus binding sites; lane 9, excess unlabeled oligonucleotide containing Stat 3 consensus binding sites. For supershift assay, nuclear extract was incubated with radiolabeled probe R23 in the presence of 4 μg of anti-Sp1 antibodies (lane 11) or 2 μg of anti-Sp3 antibodies (lane 12) for 60 min before loading onto the gels. Purified Sp1 (0.5 fpu) also was incubated with anti-Sp1 antibodies as a positive control (lane 13). The three arrows on the right side indicate the supershifted complexes.

peat 3 and repeat 1/3 mutants by OM do not reach statistical significance ($P > 0.05$). These data, coupled with previous data obtained using synthetic promoters, clearly demonstrate that the repeat 3 sequence is involved in OM induction of LDLR transcription.

Examination of the binding activities of Sp1 and Sp1-like factors on LDLR promoter

Because our data show that repeat 3 is central to the basal and OM-inducible transcription of LDLR, and as previous studies conducted by other investigators (5, 6) have shown that Sp1 binds to repeat 3 in the LDLR promoter as a positive transcription factor, we investigated the possibility that up-regulation of LDLR transcription by OM is caused by altered Sp1 expression in HepG2 cells. Northern blot analysis (**Fig. 10**) shows that incubation of OM with HepG2 cells resulted in increased expression of LDLR mRNA (approximately 3- to 5-fold), but the Sp1 mRNA levels were not changed. We further examined the Sp1 DNA binding activities of nuclear extracts prepared from control and OM-treated HepG2 cells. As shown in **Fig. 11A,** two complexes corresponding to Sp1 and Sp3 formed specifically in the presence of labeled oligonucleotide probes containing either a single copy of repeat 1 (R1) or a single copy of repeats 2+3 (R23). Treatment of HepG2 cells with OM for 15 or 60 min did not obviously change the pattern of this specific binding. By contrast, OM treatment resulted in a rapid induction of the DNA binding activity of the transcription factor Stat 3 (Fig. 11A) as reported previously (32). In the presence of labeled probe containing repeats 1+2+3 (R123), there were three additional slower migrating complexes formed in a nuclear extract concentration-dependent manner (Fig. 11B). Presumably, these complexes represent the occupancy of the Sp1 sites in repeats 1 and 3 by different combinations of Sp1 and Sp3. No changes in Sp1 or Sp3 DNA binding activities were detected between control and OM-treated cells.

DISCUSSION

Several recent studies have described LDLR regulation in HepG2 cells by non-sterol mediators (14, 21, 33–37). Many of these effectors have been shown to operate through a sterol-dependent pathway, including HGF, TNFα, and IL-1β. Others, for example, insulin, were reported to increase LDLR mRNA independent of intracellular cholesterol (33). Although sterol-independent regulation has been implied, these studies did not examine the involvement of SRE-1/SREBP in activation of LDLR transcription. Therefore, the present study provides the first detailed investigation of regulated LDLR expression that is not dependent on the sterol response element. Instead, OM regulation of the LDLR promoter appears to be conferred through the Sp1 binding site located within repeat 3. Interestingly, other genes that contain Sp1 binding sites in their promoter regions such as the SV40 virus early promoter, the rat tissue inhibitor of metalloproteinases-1 (TIMP-1) (32), and human FAS are not up-regulated by OM, sug-

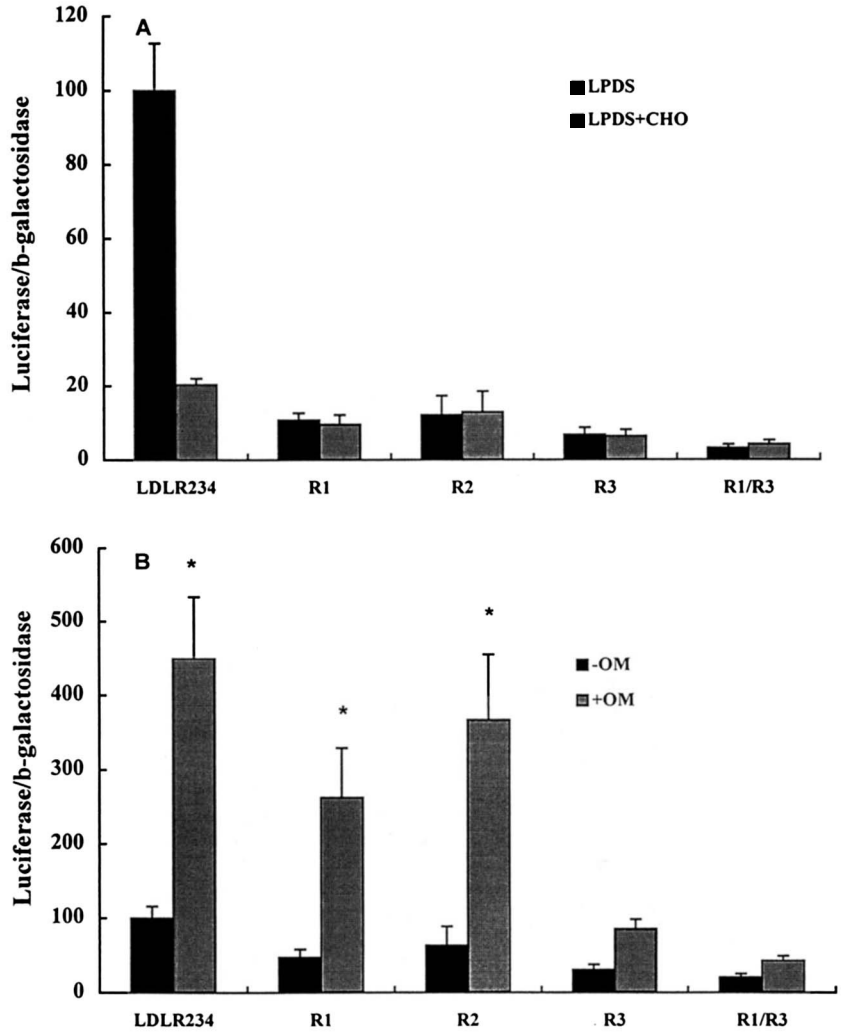

Fig. 8. Effects of OM on native promoter constructs containing mutations in repeats 1, 2, 3, and 1+3. A: Comparison of the luciferase activity in pLDLR234LUC, pLDLR234-*R1*LUC, pLDLR234-*R2*LUC, pLDLR234-*R3*LUC, and pLDLR234-*R1/R3*LUC transfected cells cultured in the absence (LPDS) and the presence (LPDS + CHO) of sterols. The normalized luciferase activity of cells that were transfected with the native promoter construct pLDLR234LUC, cultured in LPDS medium is expressed as 100%. Data are presented as mean ± SD of three separate experiments, in which triplicate dishes were transfected. B: Effects of OM on the luciferase activities driven by wildtype and mutated sequences of the LDLR promoter. Cells were transfected with pLDLR234LUC, pLDLR234-*R1*LUC, pLDLR234-*R2*LUC, pLDLR234-*R3*LUC, or pLDLR234-*R1/R3*LUC, respectively. After transfection, cells were cultured in LPDS+CHO medium for 20 h and then were stimulated with OM (1 nM) for 4 h prior to lysis. Data are presented as mean ± standard deviation of three independent transfection experiments, in which triplicate dishes were transfected. The differences in luciferase activity between control cells and OM-treated cells transfected with different promoter constructs were evaluated using the Newman-Keuls Multiple Comparison Test. Significant differences in luciferase activities were observed between OM-treated cells and control cells in pLDLR234LUC, pLDLR234-*R1*LUC, and pLDLR234-*R2*LUC transfected cells ($P < 0.001$), but were not observed in the pLDLR234-*R3*LUC and pLDLR234-*R1/R3*LUC transfected cells ($P > 0.05$).

gesting a novel regulatory role for repeat 3 in the induction of LDLR expression by OM.

Previous experiments (23) have localized the *cis*-acting sequences responsible for OM induction of LDLR transcription to a 177 bp fragment of the LDLR promoter (−142 to +35) encompassing repeats 1, 2 and 3 and TATA-like sequences. Briggs et al. (8) demonstrated that the repeat 2+3 element alone is capable of supporting basal and sterol regulated transcription in a synthetic promoter system. The present study provides evidence that the repeat 2+3 element, present in R23LUC and its derivatives, is responsible for both OM and sterol responsiveness. The approach first taken in this study was to determine the exact sequences neces-

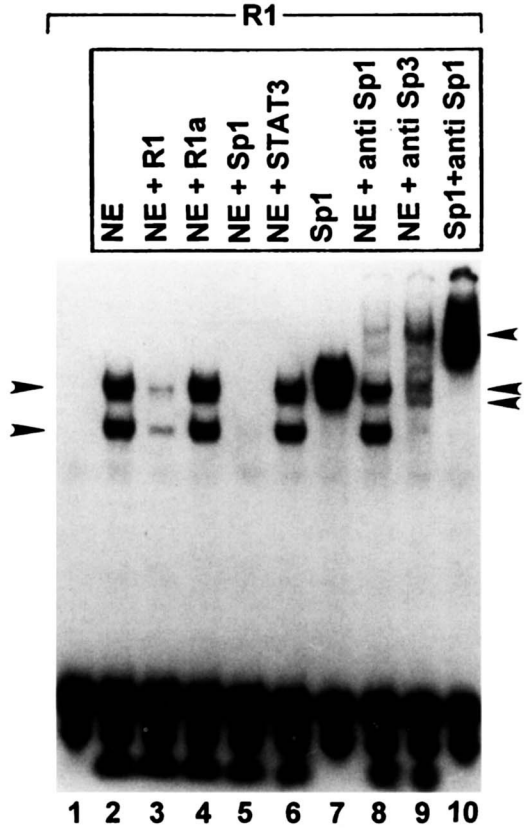

Fig. 9. EMSA competition analysis of HepG2 nuclear proteins interacting with the repeat 1 sequence. EMSA using no protein (lane 1), 2 μg of HepG2 nuclear extract (NE) (lanes 2–6), or purified Sp1 protein (0.5 fpu, lane 7) with radiolabeled oligonucleotide containing a single copy of the wild type repeat 1 sequence (R1) was conducted in the absence (lane 2) or the presence of 30-fold molar excess of unlabeled competitors. The two arrows on the left indicate the specific protein-DNA complexes formed with the R1 probe. Lane 1, labeled R1 without nuclear extract; lane 2, labeled R1 with nuclear extract; lane 3, excess unlabeled oligonucleotide R1; lane 4, excess unlabeled oligonucleotide R1a that contains 2 base substitution within the Sp1 core binding site; lane 5, excess unlabeled oligonucleotide containing 2X Sp1 consensus binding sites; lane 6, excess unlabeled oligonucleotide containing Stat 3 consensus binding sites. For supershift assay, nuclear extract was incubated with radiolabeled R1 probe in the presence of 4 μg of anti-Sp1 antibodies (lane 8) or 2 μg of anti-Sp3 antibodies (lane 9) for 60 min before loading onto the gels. Purified Sp1 (0.5 fpu) also was incubated with anti-Sp1 antibodies as a positive control (lane 10). The three arrows on the right side indicate the specific complexes containing antibodies.

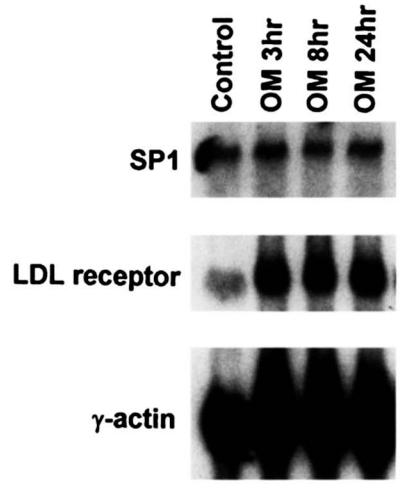

Fig. 10. Examination of Sp1 mRNA levels in HepG2 cells. HepG2 cells were incubated in 10% LPDS medium for 24 h. OM (100 ng/ml) was added to the cells for various lengths of time. Total RNA was isolated and 25 μg/lane was analyzed for LDLR mRNA, Sp1 mRNA, and γ-actin mRNA by northern blot analysis.

sary for mediating OM response by oligonucleotide-based mutagenesis across the repeat 2+3 region in pR23LUC. The promoter activity of these mutants was then assayed and compared with that of the wildtype. Although the effects of OM on LDLR mRNA expression and promoter activity are independent of sterol condition, the possibility that OM regulation is mediated through the SRE-1 element in repeat 2 needed to be addressed. Therefore, three repeat 2 mutants were designed, having mutations both within and outside the SRE-1 core sequence. The results in Fig. 5 clearly show that all three repeat 2 mutants failed to inactivate OM response, although R2b3 and R2c3, each of which contain mutations within the core SRE-1 motif, are clearly non-responsive to sterol exposure. This demonstrates that SRE-1/SREBP is not involved in OM induction of LDLR and that an OM responsive DNA element does not reside within repeat 2.

While cholesterol and OM regulation are separable phenomena in repeat 2 mutants, mutations across repeat 3 demonstrate that basal transcription, supported by Sp1, and OM regulated transcription are inseparable. The constructs R23a and R23b express luciferase activity at a level comparable to that of wildtype R23, while the overall promoter activity of the construct R23c was moderately increased as a result of a T to A substitution mutation within the core Sp1 site. Regardless of differences in basal expression level, these three constructs were fully responsive to OM in a manner similar to the wild type. The mutant R23d, which contains a two base substitution within the Sp1 binding site was unable to support basal transcription. The loss of basal promoter activity can be attributed to the inability of Sp1 to bind the mutated sequence as shown in Fig. 7. Concurrent loss of OM inducibility along with basal promoter activity suggests that OM activity is dependent on Sp1 interaction with repeat 3.

Although the repeats 2+3 region alone is capable of sustaining basal and regulated LDLR transcription, the contribution to OM-regulated LDLR transcription of each repeat element as part of the native promoter had not been previously characterized. The analysis of na-

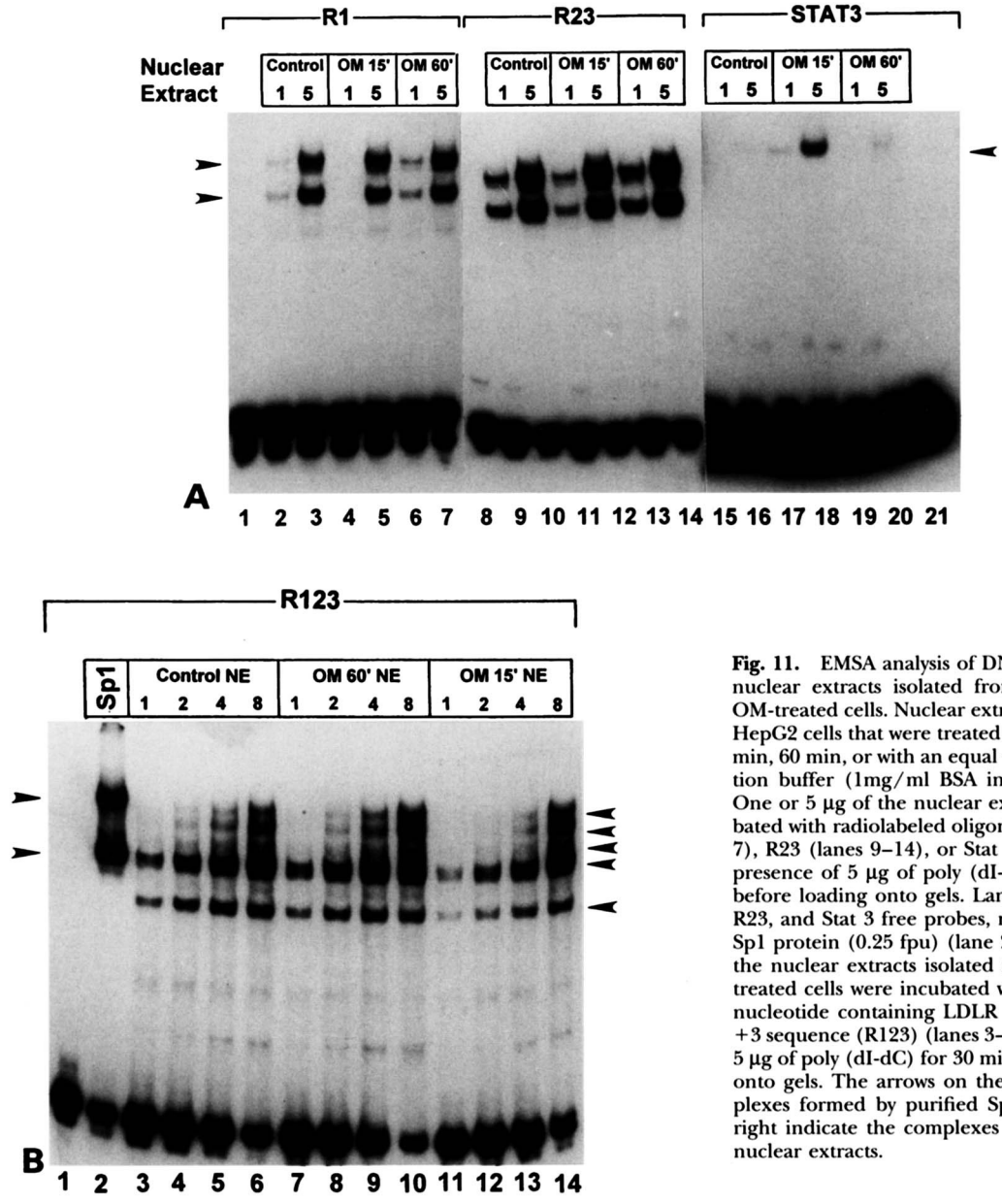

Fig. 11. EMSA analysis of DNA binding activities of nuclear extracts isolated from control cells versus OM-treated cells. Nuclear extracts were isolated from HepG2 cells that were treated with OM (1 nM) for 15 min, 60 min, or with an equal volume of the OM dilution buffer (1mg/ml BSA in PBS), respectively. A: One or 5 μg of the nuclear extract protein was incubated with radiolabeled oligonucleotide R1 (lanes 2–7), R23 (lanes 9–14), or Stat 3 (lanes 15–20) in the presence of 5 μg of poly (dI-dC) for 30 min at 4°C before loading onto gels. Lanes 1, 8 and 21 are R1, R23, and Stat 3 free probes, respectively. B: Purified Sp1 protein (0.25 fpu) (lane 2) or 1, 2, 4, or 8 μg of the nuclear extracts isolated from control and OM-treated cells were incubated with radiolabeled oligonucleotide containing LDLR promoter repeats 1+2 +3 sequence (R123) (lanes 3–14)) in the presence of 5 μg of poly (dI-dC) for 30 min at 4°C before loading onto gels. The arrows on the left indicate the complexes formed by purified Sp1. The arrows on the right indicate the complexes formed by the HepG2 nuclear extracts.

tive promoter repeats 1, 2, and 3 or repeats 1 and 3 mutants in this study provides insight into the functioning of the proximal region of the LDLR promoter as a transcriptional unit. As demonstrated in Fig. 8A, regulation by sterols was abolished not only in the repeat 2 mutant, but in the repeat 1 and repeat 3 mutants. This result indicates that cooperative interactions between SREBP and Sp1 seem to be necessary for sterol regulation as previously described (13). OM induction of pLDLR234LUC and its derivatives, as shown in Fig. 8B, was reduced only in the repeat 3, and repeat 1 and 3 mutant constructs. These results clearly show that OM exerts its effect through the proximal Sp1 binding site in repeat 3, adjacent to the TATA-like sequences, but not through the distal Sp1 binding site in repeat 1, although both sites are critical to basal transcriptional activity. Residual OM activity seen with the repeat 3 mutant constructs may be a result of minimal Sp1 binding to the mutant sequence in the context of an active transcription complex formed on the native promoter. It was previously shown that SREBP cooperatively interacts with Sp1 and thus may stabilize its binding to the mutant sequence. Alternatively, additional sequences in the native promoter may contribute slightly to the residual OM effect.

Gel shift experiments using either repeat 1 or repeats 2+3 as labeled probes revealed two specific DNA/protein complexes. Furthermore, supershift experiments

utilizing anti-Sp1 and anti-Sp3 antibodies demonstrated that these two complexes contain Sp1 and the Sp1-related transcription factor Sp3, respectively. At present the role of Sp3 in LDLR transcription is not clear. Sp3 has been reported to repress Sp1-mediated transcription in certain promoter reporter constructs (38, 39). However, treatment of HepG2 cells with OM altered neither the intensities nor the mobilities of the Sp1-containing complex and the Sp3-containing complex as compared to that of control cells. Therefore, it is unlikely that OM activation on repeat 3 involves deregulation of Sp3 activity. It will be interesting to investigate the functional role of Sp3 in LDLR transcription.

The current study suggests that OM does not directly regulate Sp1 DNA binding activity, but Sp1 binding to repeat 3 is necessary to observe the OM effect on LDLR transcription. How OM exerts its effect through repeat 3 is not clear at present. There are several possible mechanisms to explain OM's effect on LDLR transcription mediated through repeat 3. First, we speculate that OM may induce an Sp1-dependent transcriptional coactivator which enhances the interaction between Sp1 and the general transcription machinery at the TATA-like sequences. As repeat 3 is adjacent to the TATA element in both the LDLR promoter and in our synthetic constructs, an Sp1-coactivator interaction may result in increased transcription. In this case, the position of the Sp1 site with respect to the general transcription machinery becomes critical. This may explain the fact that OM does not activate FAS gene transcription wherein the position of the SRE and Sp1 sites in the promoter are reversed as compared with the LDLR promoter. Alternatively, post-translational modification of Sp1 such as tyrosine or serine/threonine phosphorylation at specific residues through an OM-regulated signal transduction pathway may be responsible for increased LDLR transcription.

In summary, our studies have demonstrated that the sterol-independent activation of LDLR transcription by OM occurs via a novel regulatory pathway dependent on repeat 3 and clearly distinct from the SRE-1/SREBP pathway. Elucidation of the mechanism by which OM exerts its actions on repeat 3 may provide a novel avenue to up-regulate LDLR gene expression. Whether such a mechanism would be synergistic with the SRE-1/SREBP activation remains to be shown. In addition, we describe a model LDLR luciferase reporter system which will be useful for evaluation of LDLR gene regulation by other nonsterol modulators.

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