# Multiple mechanisms, independent of sterol regulatory element binding proteins, regulate low density lipoprotein gene transcription

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**Abstract** **Transcription of the LDL receptor gene is markedly enhanced in the Jurkat T cell line by stimulation with the combination of the phorbol ester phorbol 12-myristate 13-acetate (PMA) and the protein synthesis inhibitor cycloheximide (CHX). The DNA sequences necessary for this response were identified by analysis of Jurkat T cells permanently transfected with reporter gene expression vectors containing fragments of the LDL receptor promoter extending from 68 bp to 1472 bp 5′ of the major transcription start site. The magnitude of the response of this array of promoter fragments to stimulation with PMA and CHX was similar to that previously observed with a ~6.5 kb promoter fragment. However, the various promoter fragments differed with regard to the role of the sterol regulatory element-1 (SRE-1) sequence. Thus, whereas a 142 bp promoter mediated transcription stimulated by PMA and CHX independently of SRE-1, a shorter 115 bp promoter was absolutely dependent on SRE-1. Furthermore, internal deletion of promoter sequences from −142 bp to −113 bp from longer promoter constructs in which the SRE-1 was mutated prevented the induction of transcription by PMA and CHX. Electrophoretic mobility shift assays (EMSAs) demonstrated sequence-specific, stimulus-independent binding by Jurkat nuclear proteins to the novel response element mapped between −142 and −115. Even though the minimal 115 bp or 68 bp promoter fragment required an intact SRE-1 to respond to PMA and CHX, transcriptional induction persisted when nuclear levels of sterol regulatory element binding proteins (SREBPs) were made undetectable by culture in suppressive sterols. Taken together, these data indicate that non-sterol stimuli such as the combination of PMA and CHX induce LDL receptor gene transcription through at least two distinct promoter elements, neither of which requires the presence of SREBPs. However, the element proximal to the transcription start site is dependent on the SRE-1.**—Makar, R. S. J., P. E. Lipsky, and J. A. Cuthbert. **Multiple mechanisms, independent of sterol regulatory element binding proteins, regulate low density lipoprotein gene transcription.** ***J. Lipid Res.*** **2000.** 41: **762–774.**

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Elevated levels of plasma low density lipoprotein (LDL) cholesterol are associated with premature death from atherosclerotic diseases (1). Lowering plasma LDL cholesterol levels pharmacologically decreases the morbidity and mortality from atherosclerosis (1). Whereas inhibition of cholesterol synthesis can lower LDL cholesterol levels, the mechanism of this approach depends on a secondary increase in the expression of cellular receptors for LDL (2). The abundance of cellular LDL receptors is controlled primarily at the transcriptional level (3–5). Cholesterol, derived from endogenous synthesis or the uptake of circulating plasma LDL, acts via a feedback repression loop to decrease the transcription of the LDL receptor gene and also to decrease cholesterol synthesis by transcriptional, translational, and post-translational regulatory mechanisms (4). Transcriptional control of the LDL receptor gene by sterols is achieved by altering the level of transcriptional activators that bind to a distinct element in the LDL receptor promoter region, the sterol regulatory element-1 (SRE-1) (6–9). When cellular sterol levels are low, the nuclear level of sterol regulatory element binding proteins (SREBPs) increases and gene expression is enhanced (10–14). In the presence of sterols, the level of SREBPs in the nucleus falls and LDL receptor gene transcription decreases (12–14).

The potential for non-sterol regulation of LDL receptor gene transcription is intriguing. If LDL receptor activity was maintained or increased, despite the presence of sterols, then elevated plasma LDL cholesterol levels might diminish, decreasing the rate of progression of atherosclerosis. We have previously demonstrated that Jurkat T cells are an excellent model system for studying non-sterol reg-

Abbreviations: BCS, bovine calf serum; CAT, chloramphenicol acetyltransferase; CHX, cycloheximide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDL, low density lipoprotein; PMA, phorbol 12-myristate 13-acetate; SRE, sterol regulatory element; SREBP, sterol regulatory element binding protein.

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ulation of LDL receptor gene expression. LDL receptor gene transcription was induced in Jurkat cells by stimulation with the combination of the phorbol ester phorbol 12-myristate 13-acetate (PMA) and the protein synthesis inhibitor cyclohexamide (CHX) (15). The induction was not prevented by sterol down-regulation of basal transcription (15), suggesting that the response may bypass the control of gene expression by sterols. In the present studies, we have extended these observations and demonstrate that there are two distinct promoter elements regulating this non-sterol-mediated induction of LDL receptor gene transcription. Each of these elements appears to function independently of the abundance and activity of SREBPs. The proximal element between −1 and −115 bp requires an intact sequence that can bind SREBPs, i.e., SRE-1, but is operational when SREBPs are undetectable in the nucleus. The more distal element between −115 and −142 functions even when SRE-1 is mutated and cannot mediate sterol-dependent gene regulation. These results indicate that multiple sterol-independent promoter elements govern up-regulation of LDL receptor gene transcription and suggest that exploiting these elements may provide the basis for lowering of plasma LDL cholesterol in the future.

## MATERIALS AND METHODS

### Cell preparation and culture

Jurkat T cells were maintained in complete medium, consisting of RPMI-1640 (Gibco BRL Life Technologies, Gaithersburg, MD) containing gentamicin (10 μg/ml), penicillin G (200 units/ml), and 10% v/v iron-supplemented bovine calf serum (Hyclone Laboratories, Logan, UT). For experiments examining sterol regulation, cells were cultured for 48 h in lipoprotein-depleted medium, i.e., supplementation of RPMI-1640 medium with either lipoprotein-depleted serum or lipoprotein-poor plasma (16) with or without 0.5 μg/ml 25-hydroxycholesterol (Steraloids Inc., Wilton, NH) and 10 μg/ml cholesterol (Eastman Kodak Co., Rochester, NY) dissolved in ethanol. Mature (nuclear) forms of SREBPs are markedly decreased 2 h after addition of this combination of suppressive sterols (14). In some experiments measuring the abundance of nuclear SREBPs, the cysteine protease inhibitor acetyl-leucinal-leucinal-norleucinal, which prevents the degradation of nuclear SREBPs (14), was added 2 h before mitogenic stimulation. In general, for experiments examining mitogen responses, cells were stimulated for varying lengths of time (determined by the individual experiments) with 10 ng/ml PMA (Calbiochem, San Diego, CA) and 10 μg/ml cyclohexamide (CHX, Sigma Chemical Co., St. Louis, MO), or with 10 μg/ml anisomycin (Sigma) or 150 μg/ml puromycin (Sigma).

To measure CAT activity in cells treated with PMA and CHX, stimulated and unstimulated (control) cells were washed extensively to remove the protein synthesis inhibitor and then incubated in complete medium for an additional 4 h before they were harvested. Whole cell lysates were prepared and assayed for CAT activity. Preliminary experiments demonstrated that this procedure removed CHX and permitted translation of CAT mRNA. To measure CAT mRNA levels, cells were harvested after stimulation and total RNA was isolated and subjected to nuclease protection assay.

### Vector construction

The construction of the vectors 1472wt-CAT, 142wt-CAT, 1472mut-CAT, and 142mut-CAT was detailed previously (17). These vectors contain either 1472 bp or 142 bp from the LDL receptor promoter and each contains 36 bp from exon 1. Those vectors designated wt-CAT contain a wild-type SRE-1. In those designated mut-CAT, 9 of 10 bp in the central core of SRE-1 were mutated (17).

A series of truncated vectors, extending upstream 226 bp, 115 bp, 88 bp, and 68 bp from the transcription start site (see **Fig. 1**), was generated using the polymerase chain reaction (PCR). Oligonucleotide primers with flanking restriction enzyme sites permitted insertion of the resulting PCR fragments into the basic plasmid pML4 (described in reference 15). Each vector was produced with a wild-type or mutated SRE-1 sequence by using plasmids with the required SRE-1 sequence as starting material. The promoter fragments and flanking DNA in the resultant plasmids were sequenced for verification. The vector 595wt-CAT was produced by digesting 1472wt-CAT with the restriction enzyme *Bam*HI and subcloning the gel-purified fragment, which contained a portion of the LDL receptor promoter fused to the CAT gene, into *Bam*HI-digested pML1 (pML4 lacking the CAT gene). The vector 297wt-CAT was generated similarly by digesting 1472wt-CAT with *Hpa*I and subcloning the gel-purified fragment, after filling-in the ends with the Klenow fragment of *Escherichia coli* DNA polymerase, into *Bam*HI-digested and filled-in pML1. The corresponding vectors containing a mutated SRE-1 (595mut-CAT and 297mut-CAT) were assembled by digesting the wild-type vectors with *Eag*I (one restriction enzyme site in the promoter at 113 bp upstream from the transcription start and a second site in the base plasmid), gel purification and insertion of the appropriate gel-purified fragment from *Eag*I-digested 1472mut-CAT. The orientation of each of the promoter fragments was confirmed by restriction mapping. In every vector, the LDL receptor fragment continued downstream to +36 bp from the transcription start site.

The deletion mutants Δ297wt-CAT, Δ297mut-CAT, Δ226wt-CAT, and Δ226mut-CAT were produced using PCR. The 5′ oligonucleotide extended to 297 bp or 226 bp upstream from the transcription start site and contained a flanking *Kpn*I site for ligation into the polylinker sequence of pML4. The 3′ oligonucleotide contained a flanking *Eag*I site that permitted ligation with a gel-purified *Eag*I-*Hind*III derived from either 142mut-CAT or 142wt-CAT vectors. These products were inserted together into pML4 digested with *Kpn*I and *Hind*III and the sequence of the resultant plasmids was confirmed by restriction mapping and direct sequencing.

A control promoter construct, containing a fragment from the Herpes simplex virus thymidine kinase (TK) promoter driving CAT was assembled by digesting pBLCAT2 (18) with *Hind*III and *Xho*I. The resultant fragment, extending from 105 bp 5′ to 51 bp 3′ of the transcription start site in the TK gene, was filled-in with Klenow and ligated into *Hind*III-digested and filled-in pML4 to produce TK-CAT. The identity and orientation of the promoter fragment was confirmed by restriction mapping. This minimal promoter includes two consensus DNA sequences for the transcription factor Sp1 (19).

### Preparation of stable transfectants

Jurkat T cells were transfected by electroporation, as previously described (20). Briefly, cells were washed, resuspended in RPMI-1640, and mixed with 20 μg of linearized plasmid DNA in a Gene Pulser electroporation cuvette (Bio-Rad). Electroporations were conducted with a Gene Pulser apparatus (Bio-Rad) at 0.25 kV and 960 μF. G418 (Gibco Life Technologies, Grand Island, NY) was added to the transfected cells (final concentration 2 mg/ml


**Fig. 1** Structure of human LDL receptor promoter-reporter constructs. All constructs terminated at +36 (0 = transcription start site) and varied both in total length and in the presence of a wild-type or mutated SRE-1. A: 1472 bp, 595 bp, and 297 bp LDL receptor promoter fragments are depicted. Restriction enzyme sites used in vector construction are indicated in the 1472 bp promoter fragment. The sizes of the promoter fragments and regulatory repeats are shown to scale (0.5× compared with B). B: Promoter fragments varying in length from 68 bp to 297 bp and deletion constructs Δ297 and Δ226, which contain an internal deletion from 113 bp and 142 bp, are diagrammed. The sizes of the promoter fragments, deleted regions, and regulatory repeats are to scale (2× compared with A). SREBPs (sterol regulatory binding proteins) bind to SRE-1 (cross-hatched area, repeat 2). The transcriptional activator Sp1 binds to repeats 1 and 3 (stippled areas).

for the first week, 1 mg/ml thereafter) 24 h after electroporation to select for stable integrants containing the plasmid DNA. G418-resistant cells usually grew out 3–4 weeks after transfection.

### Measurement of CAT activity

CAT activity was measured as detailed previously (17). Briefly, whole cell lysates were prepared by three cycles of freeze-thawing and their protein content was determined using Bradford reagent (Bio-Rad). Aliquots (0.2–20 μg of protein) were incubated for 2 h at 37°C in a standard CAT assay (21) in a final volume of 0.15 ml containing 0.53 mm acetyl CoA and 0.35–0.7 nCi of [$^{14}$C]chloramphenicol (~60 mCi/mmol) (DuPont NEN, Boston, MA). After the incubation, the acetylated reaction products were extracted in ethyl acetate, chromatographed on Polygram Sil G silica gel plates (Brinkmann Instruments, Inc., Westbury, NY) using a chloroform–methanol 95:5 (v/v) solvent system and the fraction of radioactive acetylated versus unacetylated reaction products was quantitated using the AMBIS 100 radioanalytical scanner.

### Measurement of mRNA by nuclease protection

Levels of mRNA were quantified as described previously (15, 17). Briefly, total RNA (10–40 μg) isolated from Jurkat T cells was hybridized with single-stranded cDNA probes for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), LDL receptor, and CAT. Single-stranded $^{32}$P-labeled probes were synthesized with an oligonucleotide primer in the presence of 0.75 μm [α-$^{32}$P]dCTP (~3000 Ci/mmol, Amersham Co., Arlington Heights, IL), unlabeled dCTP (0.75 μm for CAT, 1.5 μm for LDL receptor, ⩾270 μm for GAPDH probes), 0.1 mm dATP, dTTP, dGTP, and the Klenow fragment of *E. coli* DNA polymerase. The sizes of the probes (including M13 sequences) were: GAPDH = 598 nucleotides, LDL receptor = 337 nucleotides, CAT = 319 nucleotides. Hybridizations were carried out overnight at 37°C (CAT) or 48°C (LDL receptor), after which the samples were digested with 5 units of mung bean nuclease/10 μg total RNA (Gibco BRL) and separated by gel electrophoresis. The [$^{32}$P]cDNA content was analyzed with an AMBIS 100 radioanalytical scanner (15). In the experiments described, results are presented as relative CAT or LDL receptor mRNA levels, normalized using the amount of GAPDH mRNA to correct for procedural losses, and expressed as fold induction compared to unstimulated control cells calculated by the following formula: GAPDH-normalized CAT or LDL receptor mRNA levels in mitogen-stimulated cells/GAPDH-normalized CAT or LDL receptor mRNA levels in control unstimulated cells.

### Isolation of nuclear extracts

Nuclear extracts for electrophoretic mobility shift assays (EMSAs) were generated from Jurkat cells using a modified version of the protocol described by Dignam, Lebovitz, and Roeder (22). All operations were carried out at 4°C. Proteinase inhibitors used were obtained from Boehringer Mannheim, with the exception of PMSF and aprotinin, which were obtained from Sigma. Briefly, Jurkat cells were pelleted via centrifugation for 5 min at ~700 *g*, and the cells were washed in 1 ml of DPBS (pH 7.4) supplemented with DTT (0.5 mm, Calbiochem) and proteinase inhibitors (0.5 mm PMSF, 2.2 μg/ml aprotinin, 50 μg/ml calpain inhibitor I (ALLN), 10 μg/ml leupeptin, 5 μg/ml pepstatin). The cells were repelleted by centrifugation at ~700 *g*, and then resuspended in 500 μl of chilled, hypotonic buffer A (10 mm HEPES–KOH at pH 7.9, 1.5 mm $MgCl_2$, 10 mm KCl, 1 mm sodium ortho-vanadate, 25 mm β-glycerophosphate, 0.5 mm DTT) supplemented with proteinase inhibitors (see above). The cells were allowed to swell on ice for 20 min and were then disrupted by 5 passes through a 23-gauge needle followed by 10 passes through a 27-gauge needle. The cell suspensions were left on ice another 10 min before pelleting the nuclei by centrifugation for 5 min at 3000 *g*. The resulting nuclear pellet was extracted for 1 h in 200 μl of a chilled high salt buffer C (20 mm HEPES–KOH at pH 7.9, 25% v/v glycerol, 420 mm NaCl, 1.5 mm $MgCl_2$, 0.2 mm EDTA, 1 mm sodium ortho-vanadate, 25 mm β-glycerophosphate, 0.5 mm DTT), supplemented with proteinase

inhibitors (see above). After extraction, the nuclear debris was pelleted by centrifugation at 100,000 *g* for 30 min and the resulting supernatants were dialyzed for 2 h against ~500 volumes of chilled buffer D (20 mm HEPES–KOH at pH 7.9, 20% v/v glycerol, 100 mm KCl, 0.2 mm EDTA, 1 mm sodium ortho-vanadate, 25 mm β-glycerophosphate, 0.5 mm DTT) supplemented with proteinase inhibitors (see above; all inhibitors were used except ALLN). After dialysis, the samples were briefly centrifuged at 16,000 *g* to pellet any precipitated protein. Samples were aliquoted and snap-frozen in liquid nitrogen for storage at −80°C. Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA; Cat.# 500-0116).

Nuclear extracts for immunoblot analysis of SREBP-1 and -2 were made from Jurkat cells processed as described (23, 24) with minor modifications as detailed (17). Briefly, after harvesting in the presence of protease inhibitors (50 μg/ml calpain inhibitor I, 10 μg/ml leupeptin, 2.2 μg/ml aprotinin, 5 μg/ml pepstatin, and 0.5 mm phenylmethylsulfonyl fluoride) and incubation in hypotonic buffer (10 mm HEPES–KOH at pH 7.9, 1.5 mm $MgCl_2$, 10 mm KCl, 1 mm EDTA, 1 mm EGTA, 0.5 mm DTT), cells were sheared, centrifuged, and the nuclear pellet was resuspended in high salt buffer (20 mm HEPES–KOH at pH 7.9, 25% v/v glycerol, 400 mm NaCl, 1.5 mm $MgCl_2$, 1 mm EDTA, 1 mm EGTA, 0.5 mm DTT) containing all protease inhibitors. Extracted nuclear proteins were separated by centrifugation at 100,000 *g* for 30 min to pellet other nuclear material. Protein concentration was measured using Bradford reagent (Bio-Rad).

### Electrophoretic mobility shift assays

EMSAs were conducted in which 5-μg aliquots of Jurkat nuclear extract were incubated in 30 μl binding reactions containing 10 mm HEPES–KOH at pH 7.9, 10% (v/v) glycerol, 50 mm KCl, 0.1 mm EDTA, 0.5 mm sodium ortho-vanadate, 12.5 mm β-glycero-phosphate, 0.5 mm DTT, 250 μg/ml sodium poly[d(I-C)]-poly [d(I-C)] (Pharmacia; average length, 4907 bp), ~5 fmoles of $^{32}$P-labeled probe (generated by a fill-in reaction with Klenow and [α-$^{32}$P]dCTP), and proteinase inhibitors (0.5 mM PMSF, 2.2 μg/ml aprotinin, 50 μg/ml ALLN, 10 μg/ml leupeptin, 5 μg/ml pepstatin). Specific unlabeled competitors (see **Table 1**) were added to some binding reactions and the reactions were incubated for 10 min at room temperature before the addition of labeled probe. After aliquoting the probe, the reactions were incubated another 20 min at room temperature and were then loaded directly onto a 4% native polyacrylamide gel in 0.5× TBE buffer (1× TBE buffer contains 100 mm Tris-borate and 2 mm EDTA). The reaction products were separated by electrophoresis at 400 volts, 4°C for approximately 1 h and the gel was dried and exposed to Amersham Hyperfilm MP at −80°C.

### Immunoblotting for SREBPs

Immunoblotting was carried out as detailed previously (17). Fifty μg aliquots of nuclear extract were solubilized in SDS sample buffer and then individual proteins and pre-stained molecular weight markers (Bio-Rad) were separated by SDS-PAGE (8%) and transferred to PVDF membranes (Immobilon-P, Millipore Corp., Bedford, MA). SREBP-1 was identified by immunoblotting with mouse monoclonal anti-SREBP-1 antibody (generously provided by Drs. Michael Brown and Joseph Goldstein, UT Southwestern) against amino acid residues 301–407 of human SREBP-1a (25). This antibody recognizes all splice variants of SREBP-1 as residues 301–407 are common (12). Bound antibody was detected with affinity-purified horseradish peroxidase-conjugated sheep anti-mouse IgG and enhanced chemiluminescence (ECL, Amersham International, UK). SREBP-2 was identified by immunoblotting with rabbit polyclonal anti-SREBP-2 antibody (generously provided by Drs. Michael Brown and Joseph Goldstein, UT Southwestern) against amino acid residues 48–403 of human SREBP-2 (11, 13). Bound antibody was detected with affinity-purified horseradish peroxidase-conjugated donkey anti-rabbit IgG and ECL (Amersham).

### Statistical analysis

The significance of the effect of stimulation was determined by comparison with comparably treated, but unstimulated, control cells (paired two-tail Student's *t*-test). The effects of promoter length and sequence mutations were compared using the unpaired two-tailed Student's *t*-test.

## RESULTS

Previous studies had documented that the combination of PMA and CHX up-regulated transcription of a promoter construct including ~6.5 kb of DNA 5′ of the transcription start site (15). In the current studies, we sought to define the element(s) mediating this response. Initially, Jurkat T cells were stably transfected with a promoter–reporter construct that comprised 1472 bp 5′ of the transcription start site of the LDL receptor gene (1472wt-CAT). Stimulation of these cells with PMA and CHX produced a response pattern identical to that previously described for the ~6.5 kb fragment (**Fig. 2**, mean stimulation 5.1 ± 1.2-fold; n = 11 transfectants; $P < 0.01$ compared with unstimulated cells). In contrast, PMA and CHX increased CAT activity in cells transfected with the minimal thymidine kinase

TABLE 1. Oligonucleotides used for electrophoretic mobility shift assays

| | |
|---|---|
| 142–104 | 5′ GATCCCTTCACGGGTTAAAAAGCCGATGTCACATCGGCCTTCG 3′<br>3′ GAAGTGCCCAATTTTTCGGCTACAGTGTAGCCGGCAAGCTCTAG 5′ |
| Mutant A | 5′ GATCCCTTCACttGTTAAAAAGaaGATGTCACATCGGCCTTCG 3′<br>3′ GAAGTGaaCAATTTTTCttCTACAGTGTAGCCGGCAAGCTCTAG 5′ |
| Mutant B | 5′ GATCCCTTCACGGGTTcAccctCCtATGTCACATCGGCCTTCG 3′<br>3′ GAAGTGCCCAAgTgggaGGaTACAGTGTAGCCGGCAAGCTCTAG 5′ |
| 142–115 | 5′ CTTCACGGGTTAAAAAGCCGATGTCACA 3′<br>3′ GAAGTGCCCAATTTTTCGGCTACAGTGT 5′ |
| GSmut | 5′ GAAAGCAGCTATGCTTGCCAACATTGAAAGCAGCTATGCTTGCCAACATT 3′<br>3′ CTTTCGTCGATACGAACGGTTGTAACTTTCGTCGATACGAACGGTTCTAA 5′ |

Double-stranded oligonucleotides used in EMSAs are listed. The 142–104 and 142–115 oligonucleotides contain DNA sequences from the proxiaml LDL receptor promoter. Mutants A and B are identical to the 142–104 oligonucleotide except for the nucleotide substitutions indicated in lowercase. GSmut is an irrelevant oligonucleotide.

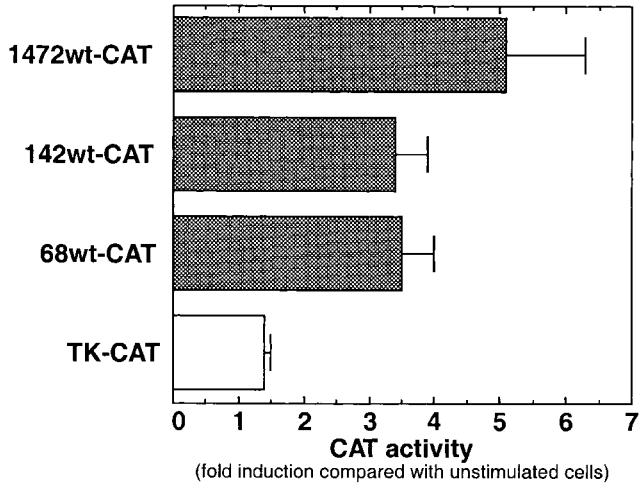


**Fig. 2** Stimulation with PMA and CHX induces reporter gene expression. Jurkat T cells were permanently transfected with reporter constructs driven either by LDL receptor gene promoter fragments of the indicated lengths (68 bp, 142 bp, and 1472 bp) containing a wild-type SRE-1 element or a reporter construct driven by the minimal Herpes simplex thymidine kinase promoter. The cells were incubated in complete medium and stimulated for 2 h with the combination of phorbol 12-myristate 13-acetate (PMA) and cyclohexamide (CHX) before harvesting and assaying for reporter gene expression as detailed in Materials and Methods. Results are mean ± SEM of experiments using 6–11 separate stable transfectants (see text).

promoter (TK-CAT) construct by only 1.4 ± 0.1-fold (n = 6 transfectants). A fragment encompassing only 142 bp 5′ of the transcription initiation site (142wt-CAT) was also able to induce CAT gene expression when stimulated with PMA and CHX. Responses were similar in magnitude to those observed with the 1472wt-CAT construct (Fig. 2, 3.4 ± 0.5-fold induction, n = 11 transfectants; $P < 0.001$ compared with unstimulated cells). Comparable results were noted when CAT mRNA levels rather than CAT enzymatic activity were quantified (data not shown). Further truncation of the LDL receptor promoter region to 68 bp (68wt-CAT) had no impact on responsiveness to PMA and CHX (Fig. 2, 3.5 ± 0.5-fold induction, n = 8 transfectants; $P < 0.001$ compared with unstimulated cells). The 68wt-CAT construct retains SRE-1 and the adjacent Sp1 site (see Fig. 1) but does not contain the more 5′ Sp1 site (repeat 1 in reference 7).

The protein synthesis inhibitor CHX not only induced LDL receptor and reporter gene transcription in combination with PMA (15) but also increased gene expression as a single agent (data not shown) as observed previously (15). Similarly, other protein synthesis inhibitors, anisomycin and puromycin, induced LDL receptor gene expression either alone (anisomycin) or when combined with PMA (puromycin) (15). Both of these responses were comparable with promoter fragments of 1472 bp and 142 bp (**Table 2**).

The next series of experiments examined the effect of mutation of SRE-1 on the response of LDL receptor promoter-CAT constructs to stimulation by PMA and CHX.

TABLE 2. Protein synthesis inhibitors induce reporter gene expression

| Stimulus | 1472wt-CAT | 142wt-CAT |
|---|---|---|
| | *CAT mRNA Levels* | |
| PMA | 1.4 ± 0.1 | 1.5 ± 0.1 |
| Puromycin | 1.5 ± 0.1 | 1.6 ± 0.1 |
| PMA and puromycin | 3.0 ± 0.6 | 2.7 ± 0.2 |
| Anisomycin | 3.1 ± 0.3 | 2.8 ± 0.3 |
| PMA and anisomycin | 3.8 ± 0.7 | 3.5 ± 0.7 |

Transfected Jurkat cells incubated in complete medium were stimulated with puromycin (150 μg/ml) or anisomycin (10 μg/ml), either alone in combination with PMA (10 ng/ml) or PMA alone for 2 h before the cells were harvested and total RNA was isolated. CAT mRNA was quantified by nuclease protection assay. Results are mean ± SEM of 3 or more experiments.

Nine of 10 base pairs within the central core sequence of SRE-1 were mutated. This effectively prevented any induction of reporter gene expression with sterol depletion (17). As shown in **Fig. 3**, the degree to which stimulation with PMA and CHX induced CAT activity in cells transfected with mutated LDL receptor promoter-CAT constructs depended upon promoter length. PMA and CHX induced 1472mut-CAT activity by 3.1 ± 0.5-fold (n = 6 transfectants; $P < 0.02$ compared with unstimulated cells). Likewise, PMA and CHX induced 142mut-CAT activity by

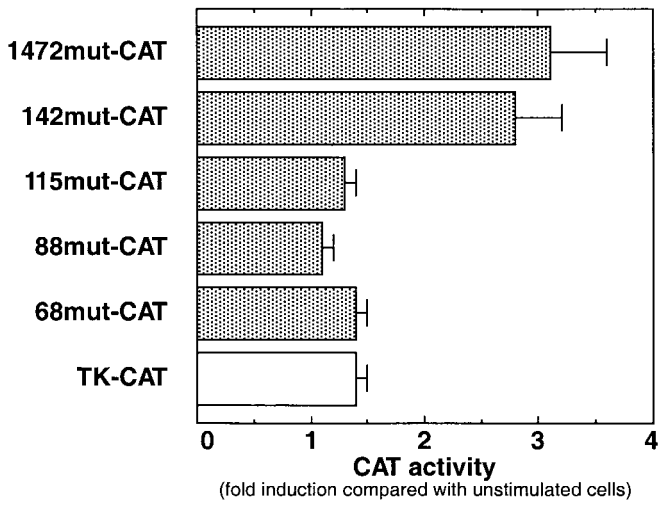


**Fig. 3** Mutation of SRE-1 in shorter promoter fragments abolishes responses to PMA and CHX. Jurkat T cells permanently transfected with reporter constructs driven by LDL receptor gene promoter fragments of the indicated lengths (68 bp to 1472 bp) containing a mutated SRE-1 were incubated in complete medium and then stimulated for 2 h with the combination of phorbol 12-myristate 13-acetate (PMA) and cyclohexamide (CHX) before harvesting and assaying for reporter gene expression as detailed in Materials and Methods. The SRE-1 was mutated by changing 9 of 10 bp in the central core sequence. This prevented sterol regulation (17). The results obtained with a reporter construct driven by the minimal Herpes simplex thymidine kinase promoter are included for comparison purposes. Results are mean ± SEM of experiments using 6–11 separate stable transfectants (see text).

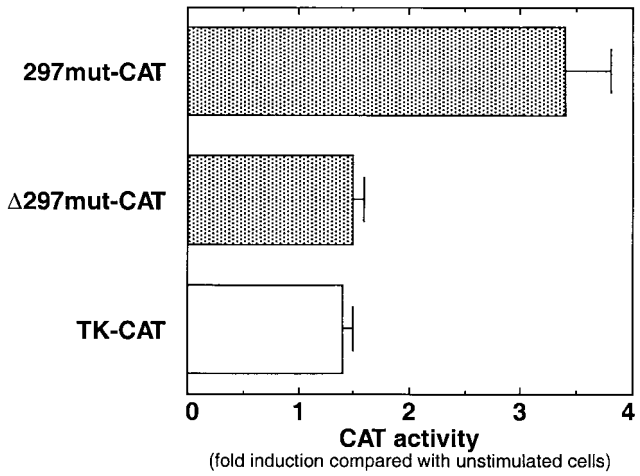


**Fig. 4** Deletion of a 30 bp fragment abolishes responses to PMA and CHX in promoter fragments with mutated SRE-1. Jurkat T cells permanently transfected with reporter constructs driven by LDL receptor gene promoter fragments extending to −297 bp from the transcription start site and containing a mutated SRE-1, either alone (297mut-CAT) or in combination with an internal deletion of 30 bp (from −113 bp to −142 bp) (Δ297mut-CAT), were incubated in complete medium and then stimulated for 2 h with the combination of phorbol 12-myristate 13-acetate (PMA) and cyclohexamide (CHX) before harvesting and assaying for reporter gene expression as detailed in Materials and Methods. The results obtained with a reporter construct driven by the minimal Herpes simplex thymidine kinase promoter are included for comparison purposes. Results are mean ± SEM of 7 experiments using separate stable transfectants.

2.8 ± 0.4-fold (n = 11 transfectants; $P < 0.001$ compared with unstimulated cells). Thus, mutation of SRE-1 did not significantly affect the response of either promoter to stimulation with PMA and CHX ($P > 0.2$ when compared with responses of the cells transfected with wild-type SRE-1 promoter-CAT constructs). In contrast, mutation of SRE-1 in the 68 bp promoter fragment (68mut-CAT) significantly reduced the response to stimulation with PMA and CHX (Fig. 3, 1.4 ± 0.1-fold induction, n = 8 transfectants; $P < 0.001$ compared with 68wt-CAT). Indeed, the response of 68mut-CAT to PMA and CHX was comparable in magnitude to that observed with TK-CAT, which served here as a negative control. Thus, within the 142 bp promoter fragment, but 5′ of the 68 bp closest to the transcription start site, there existed a DNA sequence mediating responses to the combination of PMA and CHX that was independent of SRE-1. In addition, a second element within the 68 bp promoter fragment was dependent on SRE-1 for responsiveness induced by PMA and CHX.

The SRE-independent element was defined more precisely with an additional series of reporter constructs containing mutated SRE-1 sequences and extending 5′ from the transcription start site to −88 bp or −115 bp upstream. These constructs terminated on either side of the upstream Sp1 site (repeat 1 in reference 7). Stimulation with PMA and CHX did not induce reporter gene expression linked to the 88 bp promoter fragment with a mutated SRE-1 (see Fig. 3; 1.1 ± 0.1-fold induction, n = 8 transfectants; $P > 0.3$). There was only a minimal response when cells transfected with 115mut-CAT were stimulated with PMA and CHX (1.3 ± 0.1-fold induction, n = 8 transfectants; $P < 0.005$ compared to unstimulated cells) that was comparable in magnitude to the response of TK-CAT ($P > 0.1$). In contrast, the response of 142mut-CAT was 2-fold greater than that of TK-CAT. Thus, a 28 bp sequence, between 142 bp and 115 bp 5′ of the transcription initiation site, is required for the SRE-independent response to PMA and CHX.

The effect of deleting this SRE-independent response element from LDL receptor promoter fragments was therefore examined. Two promoter-reporter constructs with mutated SRE-1 sequences, but of different overall lengths (Δ226mut-CAT and Δ297mut-CAT, see Materials and Methods for details) were generated for these studies. A 30 bp sequence, from −113 bp to −142 bp 5′ of the transcription initiation site, was deleted in these constructs. As seen in **Fig. 4**, when the 30 bp sequence was deleted, there was a significant reduction in the response to stimulation with PMA and CHX (Δ297mut-CAT: 1.5 ± 0.1-fold induction, n = 7 transfectants, compared with 297mut-CAT 3.4 ± 0.4-fold induction, n = 6 transfectants; $P < 0.05$). These findings confirm the requirement for the 30 bp fragment between −113 bp and −142 bp 5′ of the transcription initiation site for induction of reporter gene transcription by PMA and CHX. Similarly, when cells transfected with Δ226mut-CAT were stimulated with PMA and CHX, CAT activity was identical to that observed with TK-CAT (data not shown). Of note, deletion of the 30 bp

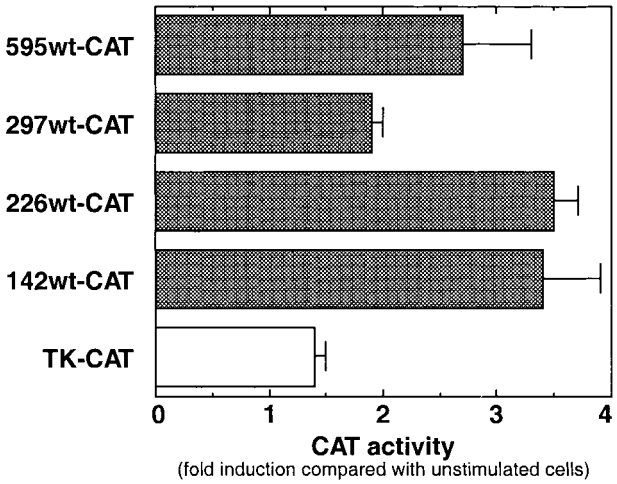


**Fig. 5** An unopposed repressor is revealed by truncation of the LDL receptor promoter. Jurkat T cells permanently transfected with reporter constructs driven by LDL receptor gene promoter fragments of the indicated lengths (142 bp to 595 bp) containing a wild-type SRE-1 element were incubated in complete medium and then stimulated for 2 h with the combination of phorbol 12-myristate 13-acetate (PMA) and cyclohexamide (CHX) before harvesting and assaying for reporter gene expression as detailed in Materials and Methods. Results are mean ± SEM of experiments using 6–10 separate stable transfectants (see text). The results obtained with a reporter construct driven by the minimal Herpes simplex thymidine kinase promoter are included for comparison purposes.

fragment between −113 bp and −142 bp 5′ of the transcription initiation site had no effect on the response to PMA and CHX in the reporter constructs containing a wild-type SRE-1 (data not shown).

Unexpected responses were observed when the effects of PMA and CHX were examined with reporter constructs of intermediate length containing the wild-type SRE-1 (**Fig. 5**). Thus, the presence of the wild-type SRE-1 in the construct containing the 297 bp fragment significantly decreased the response to stimulation with PMA and CHX (297wt-CAT: 1.9 ± 0.1-fold induction, n = 10 transfectants; 297mut-CAT: 3.4 ± 0.4-fold induction, n = 6 transfectants). In contrast, the responses observed with reporter constructs containing the 226 bp fragment or the 595 bp fragment and a wild-type SRE-1 were similar to the responses of longer (1472wt-CAT and 1472mut-CAT) and shorter (142wt-CAT and 142mut-CAT) promoter fragments (Fig. 5). These data are consistent with the possibility that a sequence between 226 bp and 595 bp 5′ of the transcription initiation site represses responses in the setting of a wild-type SRE-1. Either mutation of SRE-1 or increasing the length of the promoter fragment prevented repression.

Transfection analysis indicated that LDL receptor promoter sequences from −142 bp to −115 bp contributed to SRE-independent induction of transcription stimulated by PMA and CHX. Therefore, electrophoretic mobility shift assays (EMSAs) were conducted to determine whether novel DNA binding proteins bound to this regulatory region. Nuclear extracts obtained from unstimulated Jurkat cells were briefly incubated with a $^{32}$P-labeled DNA probe containing sequences from −142 bp to −104 bp (**Fig. 6A and B**). The binding reaction was then fractionated over a

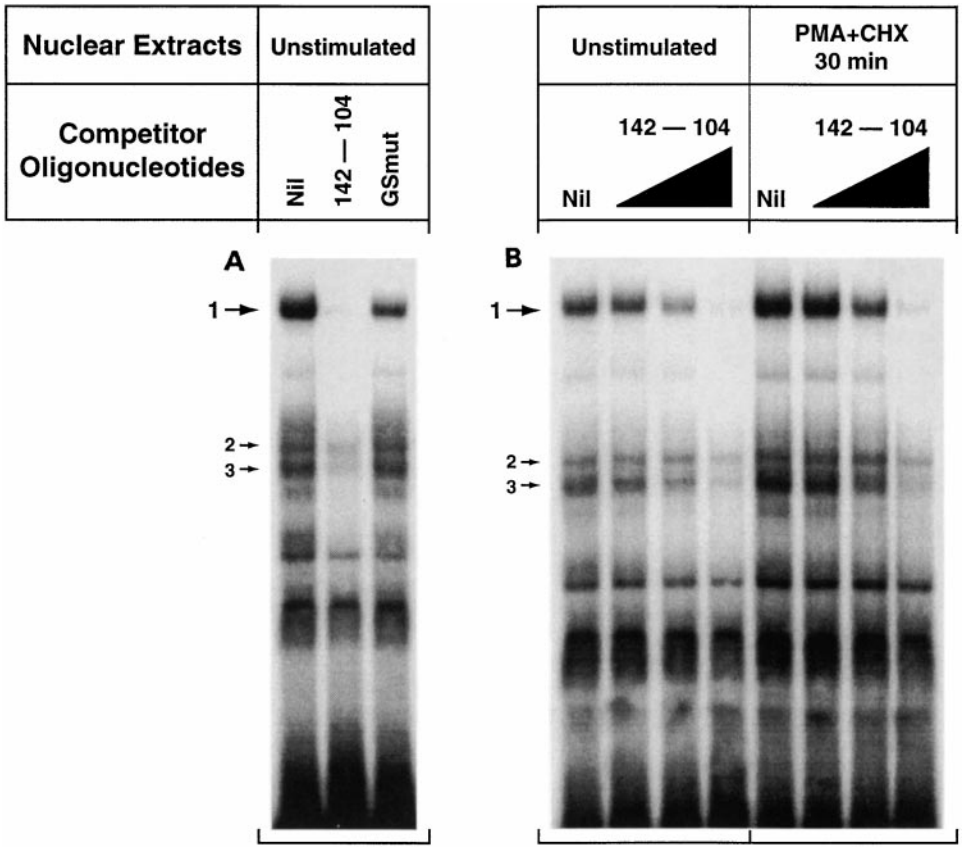


**Fig. 6.** Nuclear proteins bind DNA sequences from −142 to −104 in a sequence-specific, stimulus-independent manner. EMSAs were carried out with the $^{32}$P-labeled 142–04 ds oligo, containing LDL receptor promoter sequences from −142 to −104. Briefly, Jurkat cells cultured in medium containing 10% BCS were stimulated with PMA (10 ng/ml) and CHX (10 μg/ml) for 30 min. Unstimulated cells were treated with vehicle alone. The cells were harvested and nuclear extracts were prepared as described in Materials and Methods. Binding reactions containing aliquots of nuclear extract (5 μg) were incubated at room temperature for 20 min with ~5 fmoles of EMSA probe and then fractionated on a 4% native polyacrylamide gel. Before addition of the probe, the unlabeled competitor oligonucleotides were added to binding reactions where indicated. Binding reactions conducted in the absence of competitor are identified by "Nil." The sequence of the EMSA probe and the competitor oligonucleotides can be found in Table 1. Shifted bands are indicated by arrows and given number designations (1–3). A: The unlabeled competitors were added to the binding reactions at a concentration of ~1000-fold molar excess compared to the EMSA probe. B: Unlabeled 142–104 oligonucleotide was added to the binding reactions at concentrations of ~10, 100, and 1000-fold molar excess. Titration of competitor is represented by wedges.

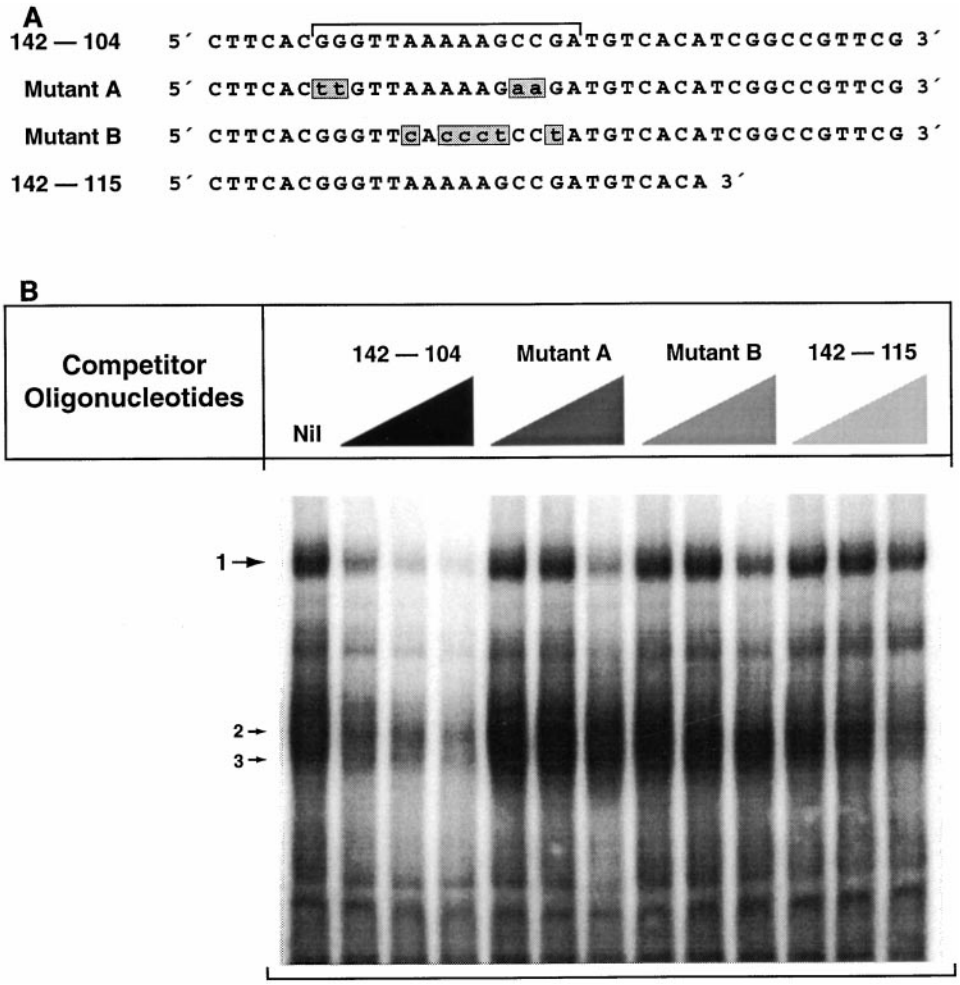


**Fig. 7** Jurkat nuclear proteins recognize specific nucleotides within a purine-rich sequence contained in the PMA and CHX response element. An EMSA was carried out with the $^{32}$P-labeled 142–104 oligonucleotide. Briefly, unstimulated Jurkat cells cultured in medium containing 10% BCS were harvested and nuclear extracts were prepared as described in Materials and Methods. Binding reactions containing aliquots of nuclear extract (5 μg) were incubated at room temperature for 20 min with ~5 fmoles of EMSA probe and then fractionated on a 4% native polyacrylamide gel. Before addition of the probe, the indicated unlabeled competitor oligonucleotides were added to binding reactions at concentrations of ~10, 100, and 1000-fold molar excess compared to the probe. The titration of competitors is represented by wedges. The binding reaction conducted in the absence of competitor is identified by "Nil." The sequence of the EMSA probe and the competitor oligonucleotides can be found in Table 1. Shifted bands 1, 2, and 3 are indicated by arrows. A: The competitor oligonucleotides are shown. The purine-rich sequence is indicated by a bracket. Altered nucleotides in mutant A and B oligonucleotides are in lowercase. B: Autoradiogram of EMSA gel.

native polyacrylamide gel, separating the free probe from the protein-bound. Protein-DNA complexes containing nuclear proteins bound to the probe were resolved on the basis of mobility, resulting in the formation of several distinct bands (Fig. 6A). A low-mobility band (designated 1) was efficiently competed away by addition of molar excess of unlabeled 142–104 DNA, but not by an irrelevant double-stranded oligonucleotide called GSmut. In addition, two higher mobility bands (designated 2 and 3), were competed away by unlabeled 142–104 DNA, although to a lesser degree. Like band 1, bands 2 and 3 were not competed away by excess unlabeled GSmut. These data demonstrated that the Jurkat nuclear proteins bound specifically to LDL receptor promoter sequences between −142 bp and −104 bp, resulting in the formation of three distinct shifted bands.

Stimulation with PMA and CHX did not alter the mobility shift pattern observed with the labeled 142–104 probe (Fig. 6B). Thus, incubation of the probe with nuclear extracts isolated from either unstimulated Jurkat cells or cells stimulated for 30 min with PMA and CHX resulted in formation of the same three sequence-specific, shifted bands. Identical results were obtained with different nuclear extract preparations from Jurkat cells stimulated with PMA and CHX for 30 min or longer. In addition, nuclease protection assay of mRNA isolated from stimulated cells demonstrated that PMA and CHX had induced LDL receptor mRNA levels (data not shown). Therefore, PMA and CHX

did not appear to induce LDL receptor gene transcription by stimulating the binding of novel transcription factors to the promoter sequences from −142 bp to −104 bp.

In order to define more precisely the DNA sequences required for binding by the nuclear factors contained in bands 1–3, an EMSA was carried out using unlabeled, mutated versions of the 142–104 probe as competitors (**Fig. 7A**). The premise for this approach was that mutation of nucleotides required for binding of nuclear proteins would render the competitor unable to inhibit formation of the shifted bands. The mutations were designed to disrupt a purine-rich LDL receptor promoter sequence within −142 bp to −115 bp (Fig. 7A). Whereas excess unlabeled 142–104 oligonucleotide competitively inhibited shifting of all three bands, neither mutant A nor mutant B oligonucleotides was an effective competitor (Fig. 7B). These data demonstrated that LDL receptor promoter sequences from −136 bp to −123 bp were required for binding by the nuclear proteins contained in these complexes. No competition was observed when an unlabeled oligonucleotide containing wild-type promoter sequences from −142 bp to −115 bp was added to binding reactions (Fig. 7B). Therefore, stable binding by the nuclear proteins contained in the shifted bands apparently required DNA sequences 3′ of −115 bp. Taken together, the data from EMSAs were consistent with the hypothesis, suggested by the results of transfection experiments, that LDL receptor promoter sequences from −142 bp to −115 bp contributed to transcriptional regulation of LDL receptor gene expression. Moreover, these data were consistent with the hypothesis that the nuclear proteins contained in the three sequence-specific complexes might contribute to SRE-independent regulation of LDL receptor gene expression.

Recent work has suggested that the DNA sequence between −115 bp and −142 bp 5′ of the transcription start site, implicated here in SRE-independent responses to PMA and CHX, may contribute to induction of LDL receptor gene transcription by sterol deprivation (26, 27). Sterol regulation of transcription was therefore examined with promoter-reporter constructs in which this sequence was deleted. Sterol regulation of CAT activity was modestly diminished (72% of control) in cells transfected with Δ297wt-CAT when compared with 297wt-CAT (297wt-CAT: 2.9 ± 0.3-fold induction by sterol deprivation, n = 4 transfectants; Δ297wt-CAT: 2.1 ± 0.3-fold induction, n = 4 transfectants, $P < 0.05$; data not shown). These findings suggest cooperation between DNA sequences residing in the region −115 bp to −142 bp 5′ from the transcription initiation site and SRE-1 in response not only to mitogenic stimulation but also to sterol deprivation.

A wild-type SRE-1 sequence was required for responsiveness of the 68 bp promoter fragment to PMA and CHX. The role of SREBPs in the response to PMA and CHX was therefore examined. For these experiments, cells were pre-incubated for 48 h in either lipoprotein-depleted medium alone or in the same medium supplemented with regulatory sterols before stimulation with PMA and CHX. Degradation of nuclear SREBPs during stimulation with PMA and CHX was prevented by the addition of a cysteine protease inhibitor (see Materials and Methods). Cells were harvested 30 min to 90 min after the addition of PMA and CHX and proteins were isolated from nuclear extracts. In parallel experiments, total cellular RNA was isolated.

SREBPs were readily detectable by immunoblot in nuclear extracts from Jurkat cells cultured in sterol-depleted medium, but were undetectable in extracts from cells cultured in suppressive sterols (**Fig. 8B and C**). As expected, the increase in nuclear levels of SREBPs observed in cells cultured in sterol-depleted medium was associated with a 5.5-fold increase in LDL receptor mRNA levels (Fig. 8A, compare unstimulated samples in control vs. suppressive ste-

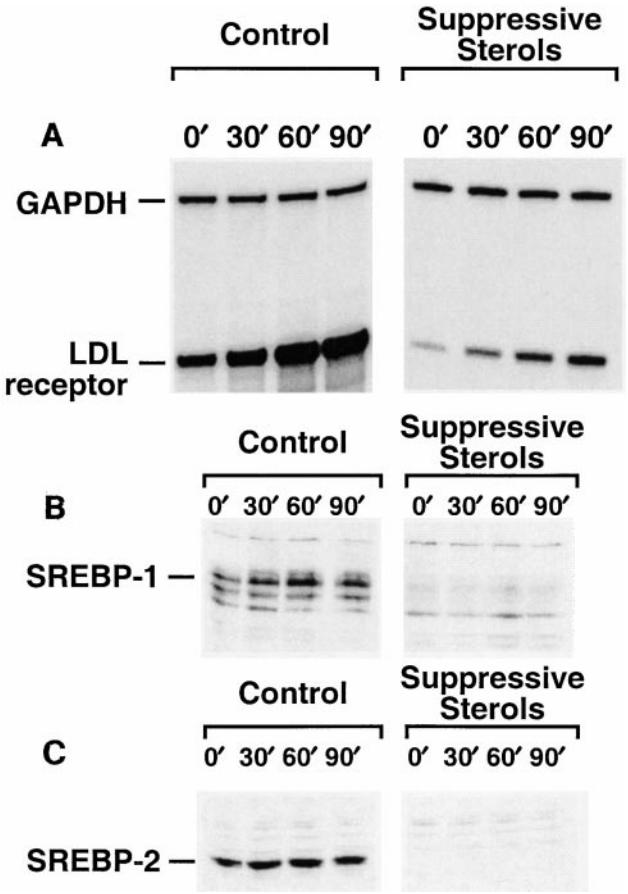


**Fig. 8** SREBPs are not required for LDL receptor gene expression induced by PMA and CHX. Jurkat T cells were preincubated for 48 h either in lipoprotein-depleted medium alone or in the same medium supplemented with suppressive sterols (see Materials and Methods for details) before addition of cysteine protease inhibitors (to prevent degradation of SREBPs) and stimulation with PMA and CHX. Cells were harvested 30 min to 90 min after stimulation and nuclear extracts and total RNA were prepared. CAT mRNA levels were quantified by nuclease protection assay and nuclear levels of SREBPs were detected by immunoblotting as detailed in Materials and Methods. A: Autoradiogram of nuclease protection assay gel; B: immunoblot for SREBP-1. Similar results were obtained when the experiment was repeated in the absence of cysteine protease inhibitors (data not shown); C: immunoblot for SREBP-2. Similar results were obtained when the experiment was repeated in the absence of cysteine protease inhibitors (data not shown).

rols). Stimulation with PMA and CHX further induced LDL receptor mRNA levels in cells cultured in sterol-depleted medium, increasing them 4.9-fold after 90 min (Fig. 8A). Of note, levels of SREBPs in nuclear extracts from cells cultured in sterol-depleted medium modestly increased after stimulation with PMA and CHX (Fig. 8B and C). PMA and CHX stimulation also increased LDL receptor mRNA levels in cells down-regulated by suppressive sterols. Levels of LDL receptor mRNA in sterol-suppressed cells stimulated for 90 min with PMA and CHX were increased 3.3-fold, approximating those in unstimulated cells incubated in sterol-depleted medium (Fig. 8A). Importantly, whereas levels of SREBPs slightly increased in cells cultured in sterol-depleted medium, SREBPs remained undetectable in cells incubated with suppressive sterols, even when stimulated with PMA and CHX (Fig. 8B and C). Thus, PMA and CHX increased LDL receptor gene expression regardless of the presence or absence of regulatory sterols and did so even when nuclear SREBPs were not detectable. Similar results were obtained in a second experiment carried out without the addition of cysteine protease inhibitors (data not shown).

As the response of the 68 bp promoter fragment was dependent on SRE-1, the effect of suppressive sterols on the ability of PMA and CHX to induce reporter gene expression from this construct was determined. CAT activity was induced by stimulation with PMA and CHX regardless of the presence or absence of suppressive sterols (**Fig. 9**). Although the absolute level of CAT activity was uniformly decreased by culturing the transfected cells with suppressive sterols, conditions which had been shown to result in undetectable levels of nuclear SREBPs (Fig. 8B and C), the fold induction after stimulation by PMA and CHX was comparable. Thus, PMA and CHX induced LDL receptor and reporter gene expression by a mechanism that was dependent on SRE-1 but was likely to be independent of the presence of SREBPs.

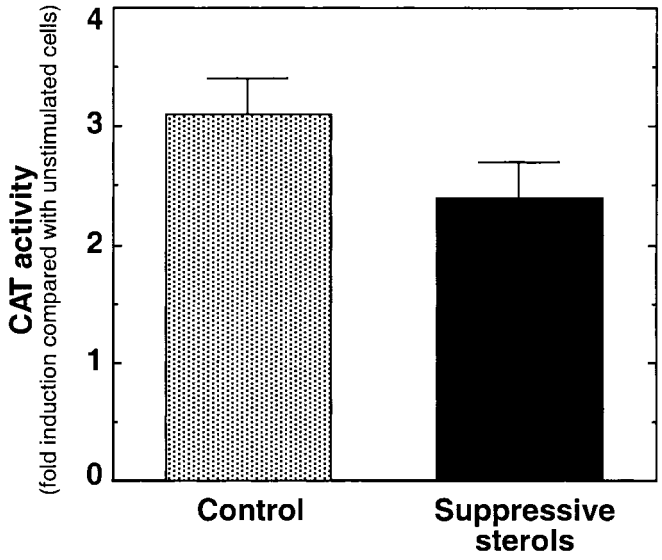


**Fig. 9** Responses dependent on SRE-1 are intact when SREBPs are suppressed. Jurkat T cells permanently transfected with the LDL receptor promoter construct 68wt-CAT were pre-incubated for 48 h in lipoprotein-depleted medium alone or in the same medium supplemented with suppressive sterols (see Materials and Methods for details) and then stimulated with PMA and CHX for 2 h before harvesting and quantifying reporter gene expression. Results are mean ± SEM of 4 experiments using separate stable transfectants.

## DISCUSSION

In the current studies, we have demonstrated that at least two regions within the 5′ promoter of the LDL receptor gene modulate transcription in response to PMA and CHX. One 28 bp region, upstream of the classic regulatory elements (repeats 1 and 3 binding Sp1 and repeat 2 binding SREBPs) conferred transcriptional induction by the combination of PMA and CHX. Jurkat nuclear proteins bound to this novel regulatory region in a sequence-specific, but stimulus-independent manner. The response to stimulation with PMA and CHX was totally independent of sterol regulation. Thus, neither mutation of SRE-1 nor depletion of SREBPs prevented responsiveness. This same region, however, apparently cooperated with SRE-1 in the induction of gene transcription by sterol deprivation. PMA and CHX also induced gene expression from a more 3′ region of the promoter in a manner that was dependent on SRE-1. Nevertheless, SREBPs did not appear to be the transcription factors necessary for the induction of transcription in response to PMA and CHX stimulation. In addition, the LDL receptor promoter contained sequences that repressed responses to PMA and CHX that were revealed by truncation of overall promoter length. Thus, the proximal LDL receptor promoter is implicated in both SRE-1 independent and novel SRE-1-dependent, SREBP-independent responses induced by PMA and CHX.

The regulation of LDL receptor transcription by sterols has been extensively delineated (6–9, 12–14). Mature nuclear forms of SREBPs, that bind to SRE-1 in the LDL receptor promoter, are more abundant when cells are deprived of sterols (12–14). The increase in nuclear levels of SREBPs results from enhanced proteolytic processing, releasing the active transcription factor from the full-length, inactive precursor anchored in the endoplasmic reticulum (14). Nuclear forms of SREBPs were increased in Jurkat T cells in response to sterol deprivation, as predicted by previous experimental data (15, 17), with the expected effect of increasing LDL receptor gene expression. Of interest, there was also a modest but detectable change in the abundance of nuclear SREBPs when Jurkat T cells were stimulated with the combination of PMA and CHX. Similarly, nuclear levels of SREBPs increased when Jurkat T cells were stimulated with the mitogenic combination of PMA and ionomycin (17). These results strongly imply that mitogenic stimulation as well as sterol deprivation can influence the nuclear levels of SREBPs.

However, sterol regulation appears to be the dominant influence regulating levels of nuclear SREBPs. No increase in nuclear levels of SREBPs could be detected when Jurkat T cells cultured in the presence of suppressive sterols were

stimulated with either PMA and CHX, as shown here, or PMA and ionomycin, as shown previously (17). Nevertheless, SRE-1 was required for the induction of LDL receptor gene transcription by the most proximal promoter element in response to either PMA and ionomycin (17) or PMA and CHX. Thus, mutation of SRE-1 in promoter fragments extending 5′ for 115 bp or less from the transcription start site abolished responsiveness to PMA and CHX. Similarly, mutation of SRE-1 in a 142 bp promoter fragment prevented induction by PMA and ionomycin (17). As shown here, despite the requirement for SRE-1, the activity of this element appeared to be independent of SREBPs in that PMA and CHX could induce transcription regulated by this promoter region even in the presence of suppressive sterols when nuclear SREBPs could not be detected. Taken together, these findings implicate a novel transcriptional mechanism that is dependent on SRE-1, yet SREBP-independent. Whether the same mechanism is involved in the induction of transcription by both forms of stimuli (PMA and CHX, PMA and ionomycin) remains unclear. Potential differences between the responses to specific stimuli by the different promoter elements are highlighted by the observation that the SRE-independent element between −142 and −115 bp is not activated by PMA and ionomycin (17) although it can regulate transcription in transfectants activated by PMA and CHX as shown here.

Non-sterol regulation of LDL receptor gene expression is less well-characterized than sterol regulation. Growth factors and mitogenic stimuli, hormones, cytokines, signaling agonists and protein synthesis inhibitors may each increase LDL receptor mRNA levels in different model systems (28–42). Whereas some of these responses are considered related to sterol regulation (40, 43), we have previously demonstrated that some of these responses are independent of sterols, independent of not only SREBPs but also of SRE-1 (17). Thus, PMA and ionomycin increased reporter gene expression associated with the 1472 bp LDL receptor promoter fragment, but not the 142 bp promoter fragment, when SRE-1 was mutated (17). The 28 bp sterol-independent PMA and CHX response element defined in the current studies is contained within the 142 bp promoter. Two separate approaches identified this region. First, truncation of the promoter fragment to 115 bp abolished SRE-1 independent induction of transcription by PMA and CHX. Second, deletion of this region from longer promoter fragments likewise prevented responses.

Within the 28 bp PMA and CHX response element, there are no DNA sequences than coincide with the recognition consensus sequences for known transcriptional activators. Nevertheless, EMSAs demonstrated that Jurkat nuclear proteins bound to the response element in a sequence-specific, but stimulus-independent manner. These data suggest that PMA and CHX may induce LDL receptor gene transcription by activating transcription factors constitutively bound to the 28 bp response element. A similar regulatory mechanism has been demonstrated for the ternary complex factors, which are constitutively bound to the serum response element and are activated by serum stimulation (44–48). Alternatively, PMA and CHX may stimulate transcription through the recruitment of transactivating factors to the response element, but this complex may have been unstable under the conditions used for EMSA and, therefore, not detected.

DNA binding factors in HepG2 cells bind to LDL receptor promoter sequences from −145 bp to −126 bp and participate in sterol-mediated regulation of LDL receptor gene transcription (26, 27). The mutant A oligonucleotide used in the competition EMSAs reported here contains discrete mutations that prevent binding of the HepG2 factors to their target sequence (26, 27). These same mutations also prevented binding of the Jurkat nuclear proteins to the PMA and CHX response element, suggesting that the Jurkat proteins may be similar to the factors previously described in HepG2 cells. This possibility is further supported by the observation that the PMA and CHX response element contributes to sterol regulation of transcription. Thus, deletion of this region from promoter fragments of 297 bp or 226 bp modestly attenuated the induction of reporter gene expression by sterol deprivation (72% of control responses). This suggests that there might be cooperation between this segment of the LDL promoter and SRE-1 in regulating transcription by sterols. However, it should be emphasized that, in both the current studies and in those conducted in HepG2 cells (27), SRE-1 is clearly the dominant element, as it is both necessary and sufficient for sterol-dependent regulation of transcription. In contrast, the region between −142 bp and −115 bp is neither necessary nor sufficient, but apparently is involved in attaining maximal transcription.

The Jurkat nuclear factors contained in the three sequence-specific shifted complexes did not bind the PMA and CHX response element unless additional DNA sequences 3′ of −115 were present. This requirement for flanking DNA sequences distinguishes these proteins from the factors previously described in HepG2 cells (26, 27). Moreover, the sequence-specific nuclear proteins identified in HepG2 cells were resolved as a single, discrete complex in EMSAs, rather than the three complexes described here. It is possible that different cell types may contain distinct DNA binding proteins that recognize similar DNA sequences within the LDL receptor promoter. Alternatively, the capacity of common DNA-binding proteins to recognize the PMA and CHX response element may be modulated by cell-type-specific accessory factors. Finally, the apparent differences between Jurkat cells and HepG2 cells may reflect technical differences in carrying out the EMSA. Further studies will be required to clarify these issues.

In summary, we have demonstrated that the 142 bp of DNA from the LDL receptor promoter just 5′ of the transcription initiation site contains two potential elements for up-regulating LDL receptor gene transcription regardless of sterol-mediated suppression. One of these is dependent on SRE-1, but not nuclear SREBPs and therefore may involve the action of a novel transcriptional activator(s). The second is a 28 bp DNA element that can bind novel nuclear factors and function independently of both

SREBPs and SRE-1. These studies suggest potential targets for pharmacological manipulation aimed at increasing LDL receptor expression even in the presence of sterol-mediated transcriptional repression. By increasing LDL receptor activity a mere 2-fold, major reductions in plasma LDL cholesterol levels can be achieved in patients (1). Atherosclerotic cardiovascular, cerebrovascular, and peripheral vascular diseases continue to be leading causes of death. Because lowering plasma LDL cholesterol levels can decrease the impact of these diseases on morbidity and mortality in developed nations, such a pharmacological approach may be worth considering.

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