Inhibition of protein synthesis in baby-hamster kidney cells blocks oxysterol-mediated suppression of 3-hydroxy-3-methylglutaryl-CoA reductase mRNA at a post-transcriptional level

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The effects of the protein-synthesis inhibitor cycloheximide on 25-hydroxycholesterol-mediated suppression of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase mRNA levels were evaluated in the baby-hamster kidney cell line C100. Cells cultured in medium supplemented with delipidized fetal bovine serum and 25 μ M lovastatin for 12–24 h had a 5-fold higher level of HMG-CoA reductase mRNA than cells grown in medium supplemented with non-delipidized fetal bovine serum (FBS). The higher level was due to increased transcription, as determined by run-on assays with isolated nuclei. Addition of 25-hydroxycholesterol to lovastatin-treated cells lowered HMG-CoA reductase mRNA levels within 4 h of treatment to those of cells grown in FBS-supplemented medium. This decrease was due in part to a decrease in gene transcription. Cycloheximide

added in conjunction with 25-hydroxycholesterol to lovastatin-treated cells blocked the suppression of mRNA levels, but did not block oxysterol-mediated suppression of transcription. In addition, cycloheximide added to cells grown in FBS-supplemented medium rapidly increased mRNA levels by 10-fold relative to untreated cells, with no comparable increase in transcription. No comparable increase in either the mRNA level or rate of transcription for β -actin was observed under such conditions. These results indicate that cycloheximide specifically stabilizes HMG-CoA reductase mRNA in the presence of oxysterols and suggests that continuous synthesis of a short lived protein regulator is required for oxysterol-mediated suppression of HMG-CoA reductase mRNA at a post-transcriptional level.

INTRODUCTION

In animal tissues the rate-limiting enzyme for synthesis *de novo* of cholesterol from acetyl-CoA is 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase [1]. Specifically, HMG-CoA reductase synthesizes mevalonate from HMG-CoA; subsequently, mevalonate is either converted into cholesterol through multiple enzymic steps or used in the production of isoprenoids such as ubiquinone, dolichol, isopentenyl-tRNA and prenylated proteins [2]. HMG-CoA reductase levels are regulated by feedback control of both synthesis and stability of the enzyme. Feedback regulation of reductase synthesis by both sterol and non-sterol intermediates of the cholesterol-biosynthetic pathway [1] occurs at both transcriptional [3–9] and translational [10–14] levels. When cells are deprived of cholesterol or mevalonate, there is an increase in reductase mRNA and a corresponding increase in the synthesis of reductase from this mRNA. The increase in mRNA is primarily due to enhanced transcription of the gene, an effect mediated through an octameric nucleotide sequence in the 5' promoter region known as the sterol regulatory element [15] or SRE. The translational efficiency, or rate of synthesis of reductase from its mRNA, is mediated either by mevalonate or by a non-sterol intermediate of cholesterol biosynthesis derived from mevalonate [10–14]. Levels of HMG-CoA reductase are also modulated through a post-translational degradative process; in the presence of sterols and mevalonate, the 97 kDa form of reductase associated with the endoplasmic reticulum is rapidly degraded [13,16].

Although the steady-state level of reductase mRNA is thought to be controlled generally by oxysterol-regulated transcription, post-transcriptional control mechanisms modulating these levels

have been largely ignored. At present, the only characterized example of post-transcriptional control is thyroid-hormone-mediated stabilization of rat liver mRNA [17]. When transcription of HMG-CoA reductase mRNA is inhibited by oxysterols, an as yet uncharacterized post-transcriptional degradative process lowers its mRNA level to a new steady state. Because many degradative processes that act on other mammalian mRNA species appear to be mediated by labile proteins [18,19], possibly RNAases [19], we investigated the role of protein synthesis in oxysterol-mediated suppression of reductase mRNA in baby-hamster kidney C100 cells treated with lovastatin, a competitive inhibitor of HMG-CoA reductase [20]. In this study we have demonstrated that protein-synthesis inhibition by cycloheximide in lovastatin-treated cells prevents 25-hydroxycholesterol-mediated suppression of reductase mRNA, and that this blockage occurs at a post-transcriptional level.

MATERIALS AND METHODS

Cell culture and media

C100 cells were provided by Dr. Robert Simoni, Stanford School of Medicine. Cells were maintained in Minimal Essential Medium (MEM) containing 5% delipidized (lipid-deficient) fetal bovine serum (FBS), prepared from whole FBS as described previously [21], and 2 μ g/ml lovastatin. For experimental purposes, cells were cultured in MEM supplemented with either 5% whole FBS (FBS-MEM) or 5% delipidized FBS (DFBS-MEM). Lovastatin was kindly given by Dr. A. Alberts, Merck, Sharp and Dohme Laboratories, and was prepared as described by Endo et al. [22]. Cycloheximide and actinomycin D were from Sigma Chemical

Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; MEM, minimum essential medium; FBS, fetal bovine serum; 1 $\times$ SSC, 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; 1 $\times$ Denhardt's solution, 0.02% BSA, 0.02% Ficoll and 0.02% polyvinylpyrrolidone.

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Co., and 25-hydroxycholesterol was from Steraloids. Cycloheximide was dissolved in water to a final concentration of 10 mg/ml, actinomycin D in ethanol to a final concentration of 20 mg/ml, and 25-hydroxycholesterol in 100 % ethanol to a final concentration of 1 mg/ml. Concentrations of ethanol in media did not exceed 0.01 % (v/v).

RNA isolation

The single-step method of RNA isolation by acid guanidinium thiocyanate/phenol/chloroform extraction as described by Chomczynski and Sacchi [23] was used throughout this study. After precipitation with propan-2-ol, pelleted RNA was washed with 80 % ethanol, vacuum dried, and resuspended in 0.05 M Tris/HCl, pH 7.5, 0.01 M $MgCl_2$ and 0.05 mg/ml acetylated BSA containing 6 μ g of RNAase-free DNAase and 10 units of RNasin (both from United States Biochemicals). Samples were incubated at 37 °C for 45 min, and the RNA was extracted by using acid phenol as described previously [13]. RNA was precipitated with ethanol, dried under vacuum, and resuspended in diethyl pyrocarbonate-treated water.

Analysis of RNA

Slot-blots

Samples of total RNA ranging from 1 to 16 μ g were denatured with 6 M formaldehyde in 10 $\times$ SSC (1 $\times$ SSC is defined in title-page footnote) for 15 min at 65 °C. Denatured samples were transferred to nitrocellulose membranes by a slot-blotting vacuum manifold (Schleicher and Schuell). After baking the membrane at 80 °C in a vacuum for 2 h, membranes were pre-hybridized for 16 h at 42 °C in hybridization buffer, consisting of 6 $\times$ SSC, 50 % formamide, 5 $\times$ Denhardt's solution (1 $\times$ Denhardt's is defined in title page footnote), 0.5 % SDS, 50 mM sodium phosphate (pH 7.0) and 100 μ g/ml sheared salmon sperm DNA. Cloned cDNA probes were labelled with [α - 32 P]dCTP to a specific radioactivity of at least 10^9 d.p.m./ μ g [13]. For estimates of relative changes in HMG-CoA reductase mRNA levels, membranes were hybridized to a 32 P-labelled 4.2 kb *Eco*R1 cDNA insert from pDGS2 [5] containing sequences for Syrian hamster reductase mRNA. Hybridization and washing conditions were done as described previously [14]. As a control, blots hybridized to the reductase-specific probe were stripped of cDNA and subsequently hybridized to 32 P-labelled pHF β A-1, a recombinant plasmid containing a full-length cDNA insert for human β -actin [24]. Levels of reductase and β -actin mRNA were estimated by scanning densitometry of autoradiographs prepared from such blots.

Northern blots

Samples of total RNA were denatured at 65 °C for 15 min in gel buffer (0.1 M Mops, 40 mM sodium acetate and 5 mM EDTA, pH 8.0) containing 2.2 M formaldehyde and 50 % formamide. Samples were electrophoresed on 1 % agarose gels containing 2.2 M formaldehyde and gel buffer. RNA was then transferred to a charged nylon membrane (MSI) in 20 $\times$ SSC by passive capillary diffusion, and covalently linked to the membrane either by u.v. cross-linking (Stratagene) or by baking at 80 °C. Membranes were pre-hybridized at 42 °C for 16 h in hybridization buffer described previously for slot-blots analysis. Northern blots were hybridized as described above, first to 32 P-labelled reductase-specific cDNA probe, then to β -actin probe, and subjected to autoradiography.

Transcriptional run-on assays

The relative transcriptional rates of HMG-CoA reductase, HMG-CoA synthase and β -actin genes were determined on isolated nuclei by modification of a method described by Celano et al. [25]. For each experimental treatment, 5×10^7 C100 cells were lysed in 2 ml of lysis buffer (0.5 % Nonidet P-40, 20 mM Tris/HCl, pH 7.4, 10 mM NaCl and 3 mM $MgCl_2$). Lysates were disrupted in a glass homogenizer and nuclei were pelleted at 100 g for 5 min at 4 °C. Nuclei were washed once with lysis buffer lacking Nonidet P-40, and pelleted. Nuclear pellets were then resuspended in 40 % glycerol/50 mM Tris/HCl (pH 8.0)/5 mM $MgCl_2$ /0.1 mM EDTA, frozen, and stored at -70 °C.

For transcription reactions *in vitro*, nuclei were thawed on ice and incubated at 26 °C for 30 min in vol. of 2 $\times$ reaction buffer, containing 10 mM Tris/HCl, pH 8.0, 0.3 M KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM each of rATP, rCTP and rGTP, and 100 μ Ci of [α - 32 P]UTP (sp. radioactivity, 800 μ Ci/mmol). The reaction was terminated by the addition of a solution containing 10 mM Tris/HCl, pH 7.4, 0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$ and 40 μ g of RNAase-free DNAase I, followed by incubation for 5 min at 26 °C. Proteinase K buffer (200 μ l) containing 0.5 M Tris/HCl, pH 7.4, 0.125 M EDTA, pH 8.0, 5 % SDS and 200 μ g of proteinase K was then added, and samples were incubated at 42 °C for 30 min. RNA was extracted once with phenol/chloroform (1:1, v/v), and the aqueous phase was ethanol-precipitated. Approx. 2×10^6 d.p.m. of 32 P-labelled RNA was added to 1 ml of slot-blot hybridization buffer. RNA was then hybridized to slot-blots containing 5 μ g each of hamster HMG-CoA reductase, HMG-CoA synthase, human β -actin, or pSP6 plasmid DNA. Hybridizations were done at 42 °C for 2 days. The blots were washed sequentially in 2 $\times$ SSC/0.1 % SDS and 0.1 $\times$ SSC/0.1 % SDS, dried and exposed to X-ray film for 3 days.

RESULTS

Lovastatin increases HMG-CoA reductase mRNA levels in C100 cells

Previous studies on both hepatocytes [8,9] and fibroblasts [3,6,13,14,26] have demonstrated that the HMG-CoA reductase inhibitor lovastatin increases reductase mRNA levels. Similarly, in this study, incubating cells with lovastatin in DFBS-MEM for periods ranging from 12 to 24 h increased reductase mRNA levels 5-fold compared with cells grown in FBS-MEM (Figure 1). There was no further increase in mRNA when cells were incubated with lovastatin for 36 and 48 h (results not shown). As a control, β -actin levels were also measured by hybridization assay with RNA extracted from lovastatin-treated cells (Figure 1). β -Actin is a constitutively expressed protein, and its mRNA level has been shown to remain unchanged under similar conditions [14]. Indeed, during the time course studied, there was no comparable change in β -actin mRNA.

Cycloheximide blocks oxysterol-mediated suppression of HMG-CoA reductase mRNA

In lovastatin-treated cells, it was previously demonstrated that 25-hydroxycholesterol, a potent suppressor of reductase mRNA [27], decreased mRNA levels of approx. 4-fold [14]. Presumably, 25-hydroxycholesterol mimics the effect of endogenous oxysterol regulators of reductase mRNA by interacting with an oxysterol-binding complex, which in turn acts as a negative regulator of transcription of binding to the SRE-1 element in the reductase promoter [15]. To determine the role of protein synthesis in the

Figure 1 Time course for induction of HMG-CoA reductase mRNA levels in C100 cells

Cells were grown in FBS-MEM; the medium was then changed to DFBS-MEM plus 25 μ M lovastatin to induce reductase mRNA. Cells were incubated for various time intervals, and total RNA was extracted. Reductase and β -actin mRNA levels were measured by the hybridization protocol described in the Materials and methods section. Relative levels of reductase mRNA in the main Figure were determined by scanning densitometry of autoradiographs shown in the inset, which were prepared from blots hybridized to 32 P-labelled reductase cDNA. For each time point, the area of the autoradiograph representing an individual RNA concentration was scanned in three separate regions, and the slope (mean $\pm$ S.D.) determined by regression analysis of absorbance values for at least three RNA concentrations. Slot-blot representing β -actin mRNA are not shown. Reductase (Red.; $\square$) and β -actin ($\blacktriangle$) mRNA levels in uninduced cells were set at 100%, and all other slopes are reported as a percentage of these values (mean $\pm$ S.D.).

regulation of oxysterol-mediated mRNA suppression, the protein-synthesis inhibitor cycloheximide was added together with 25-hydroxycholesterol to lovastatin-treated cells, and mRNA levels were measured at various time intervals. In the absence of cycloheximide, 25-hydroxycholesterol decreased mRNA levels 4-fold (Figures 2a and 2b) compared with control cells treated only with lovastatin; this degree of suppression was observed within 4 h. By contrast, in the presence of both 25-hydroxycholesterol and cycloheximide, there was a 2-fold decrease in reductase mRNA within 1 h, but, after 2 h of incubation, reductase mRNA levels returned to those measured in control (lovastatin-treated) cells. These results indicate that continuous protein synthesis is required for the oxysterol-mediated suppression of mRNA. In addition, cycloheximide increased reductase mRNA within 2–4 h when added to lovastatin-treated cells. However, during the 4 h period, reductase mRNA in cells treated with lovastatin only did not increase (results not shown). There was no comparable change in β -actin mRNA levels in lovastatin-treated cells subsequently treated with cycloheximide alone, 25-hydroxycholesterol alone, or both 25-hydroxycholesterol and cycloheximide at any time point studied (Table 1). This suggests that the effects of cycloheximide under the above conditions are specific to reductase mRNA.

When RNA from these lovastatin-treated cells was analysed by Northern blots, the increase in mRNA was most apparent in the 4.7 kb reductase transcript (Figure 3, lanes 1 and 2). Lovastatin treatment also co-ordinately increased a series of heterogeneous reductase transcripts smaller than the 4.7 kb mRNA species. Such transcripts have been attributed to the

Figure 2 Cycloheximide blocks 25-hydroxycholesterol-mediated suppression of HMG-CoA reductase mRNA

Cells were incubated for 16 h in DFBS-MEM and 25 μ M lovastatin (L). The medium was then supplemented with 1.2 μ M 25-hydroxycholesterol (25-OH C) alone, or with both 1.2 μ M 25-OH C and 10 μ g/ml cycloheximide (CHX). Incubations were done for the indicated times and total RNA was extracted as described in the Materials and methods section. Levels of both reductase and β -actin mRNA were estimated by slot-blot hybridizations. Relative changes in mRNA were based on laser-densitometric scanning of autoradiographs prepared from slot blots, and relative amounts of RNA were determined by regression analysis as described in Figure 1. (a) Autoradiographs of slot-blot hybridized with 32 P-labelled reductase probe. Control refers to HMG-CoA reductase levels in cells treated only with lovastatin for 16 h; this value was set at 100%. (b) Time course for changes in reductase (Red.) mRNA that were estimated from laser densitometry of autoradiographs shown in (a). The level of reductase mRNA in cells grown in DFBS-MEM and 25 μ M lovastatin was set at 100%; all other values are reported as a percentage of this value (mean $\pm$ S.D.).

alternate use of polyadenylation sites found in the 1.9 kb 3' untranslated region of the hamster HMG-CoA reductase transcript [4,28,29]. The cycloheximide-induced block to oxysterol-mediated suppression of reductase mRNA was likewise observed when Northern blots of total cellular RNA were hybridized to the same reductase-specific cDNA probe used for slot-blot analysis (Figure 3, lanes 4 and 5). Compared with lovastatin-treated cells, reductase mRNA levels were greatly decreased by

Table 1 Effects of lovastatin, 25-hydroxycholesterol and cycloheximide on β -actin mRNA levels in C100 cells

Slot-blots shown in Figure 2(a) were stripped of reductase probe, hybridized to pHF β A-1, and quantified by laser densitometry as described in Figure 1. The level of β -actin mRNA in cells grown in DFBS-MEM and 25 μ M lovastatin was set at 100%, and all other values are reported as a percentage of this RNA level (mean $\pm$ S.D.).

Time	Treatment		
	Lovastatin	Lovastatin + 25-hydroxy-cholesterol	Lovastatin + 25-hydroxy-cholesterol + cycloheximide
1 h	100 $\pm$ 9	100 $\pm$ 9	89 $\pm$ 9
2 h	118 $\pm$ 9	117 $\pm$ 7	95 $\pm$ 7
4 h	91 $\pm$ 19	113 $\pm$ 6	100 $\pm$ 22

Figure 3 Northern-blot analysis of RNA from C100 cells

Cells were incubated in the presence of 25 μ M lovastatin for 16 h; treatment with 1.2 μ M 25-hydroxycholesterol and 10 μ g/ml cycloheximide was for 2 h. Cellular RNA was electrophoresed on a 1%-agarose gel (40 μ g/lane), transferred to a nylon membrane, and hybridized to a 32 P-labelled reductase probe. Lanes: 1, no treatment (cells grown in FBS-MEM); 2, lovastatin only; 3, lovastatin and cycloheximide; 4, lovastatin and 25-hydroxycholesterol; 5, lovastatin, 25-hydroxycholesterol and cycloheximide. Markers include 28 S (5.1 kb), 23 S (3.0 kb), 18 S (1.9 kb) and 16 S (1.5 kb) rRNA.

25-hydroxycholesterol alone. This decrease was co-ordinate for all reductase mRNA species, indicating that there was no different stability for any of the transcripts. Cycloheximide, when added simultaneously with 25-hydroxycholesterol, co-ordinately blocked the suppression of all transcripts, and mRNA levels remained the same as those observed in cells treated only with lovastatin. Furthermore, compared with the lovastatin-only treatment, cycloheximide did not change the size or relative amounts of transcripts, indicating that inhibition of protein synthesis did not alter normal processing or differentially stabilize any of the mRNA species hybridizing to the reductase-specific probe. The same Northern blots were subsequently hybridized to the β -actin probe, and no comparable change in the level of this mRNA was apparent under any condition (Figure 3).

Cycloheximide increases HMG-CoA reductase mRNA levels in cells cultured in FBS-MEM

Cells grown continuously in FBS-MEM have reductase mRNA levels equivalent to those in cells treated with both lovastatin and 25-hydroxycholesterol [14]. Cholesterol in the serum enters the cells and suppresses reductase mRNA synthesis in a manner analogous to that mediated by 25-hydroxycholesterol added to

Figure 4 Cycloheximide increases HMG-CoA reductase mRNA in cells grown in FBS-MEM

Cells were grown in FBS-MEM for 48 h. Cycloheximide (10 μ g/ml) was added and cells incubated for the indicated times. RNA was extracted and levels of both reductase and β -actin mRNAs were determined by the appropriate hybridization assay. (a) Autoradiographs of slot-blots hybridized with either 32 P-labelled reductase or β -actin probes. (b) Relative levels of reductase (Red.) and β -actin mRNA estimated by laser-densitometric scanning of the autoradiograph in (a) as described in Figure 1. The amount of reductase and β -actin mRNA in cells grown in FBS-MEM was set at 100%; all other values are reported as a percentage of this value (mean $\pm$ S.D.).

lovastatin-treated cells [1]. To determine whether inhibition of protein synthesis altered reductase mRNA levels in cells grown in FBS-MEM, cycloheximide was added and mRNA levels were measured at various times. As shown in Figures 4(a) and 4(b), within 6 h cycloheximide increased reductase mRNA 10-fold relative to untreated cells. When the blots were subsequently hybridized to 32 P-labelled pHF β A1, there was no comparable increase in β -actin mRNA. These results were confirmed by Northern blots of total RNA from cycloheximide-treated cells. The level of reductase transcripts increased with cycloheximide treatment and corresponded in both sizes and distribution to mRNA transcripts induced by cellular lovastatin treatment (results not shown).

Cycloheximide enhances the lovastatin-induced increase of HMG-CoA reductase mRNA

To address the role of protein synthesis in the lovastatin-mediated increase of reductase mRNA, cycloheximide was added to cells together with lovastatin. Typically, cells grown in FBS-MEM must be treated with lovastatin for 8 h to see increased reductase mRNA. However, due to possible irreversible cell damage during an 8 h exposure to cycloheximide, cells were pretreated with DFBS-MEM for 16 h to decrease endogenous metabolic pools of sterols that suppress reductase transcription. This pretreatment,

Figure 5 Cycloheximide enhances lovastatin-mediated increase of HMG-CoA reductase mRNA

Cells were grown in DFBS-MEM for 16 h. The medium was then supplemented with either 25 μ M lovastatin alone or both 25 μ M lovastatin and 10 μ g/ml cycloheximide. Cells were incubated for the times indicated and total RNA extracted. Reductase and β -actin mRNA levels were estimated by densitometric scanning of autoradiographs prepared from slot blots as described in Figure 1. The lack of treatment refers to cells grown in DFBS-MEM alone. The amount of HMG-CoA reductase (Red.) and β -actin mRNA in cells grown in DFBS-MEM was set at 100%, and all other values are reported as a percentage of this value (mean $\pm$ S.D.).

used in previous studies [10,13,14], accelerates the effects of lovastatin on reductase mRNA.

Results from these experiments are shown in Figures 5(a) and 5(b). When lovastatin was added to cells cultured initially in DFBS-MEM, within 2 h reductase mRNA increased 2-fold relative to control cells. When cycloheximide and lovastatin were added to the same medium, reductase mRNA levels increased 4-fold. A 4-fold enhancement was also seen in cells treated for up to 10 h with cycloheximide and lovastatin. No change in β -actin mRNA was observed under the same conditions. Thus, rather than blocking lovastatin-induced mRNA increases, inhibition of protein synthesis enhanced reductase mRNA levels.

Actinomycin D blocks 25-hydroxycholesterol-mediated suppression of reductase mRNA

To determine whether 25-hydroxycholesterol mediated any of the effects shown in Figure 2 post-transcriptionally, cells were treated with actinomycin D to inhibit RNA transcription. If there were post-transcriptional regulation of reductase in RNA in the presence of actinomycin D, mRNA levels would be lower in cells treated with lovastatin plus 25-hydroxycholesterol than in cells treated only with lovastatin. If 25-hydroxycholesterol exerted no post-transcriptional effect, mRNA levels would be approximately equal. Results from these experiments are shown in Figure 6 and expressed quantitatively in Table 2. The effects of actinomycin D on reductase mRNA were concentration-

Figure 6 Actinomycin D attenuates the oxysterol-mediated suppression of HMG-CoA reductase mRNA

Cells were incubated in DFBS-MEM and 25 μ M lovastatin for 16 h. The medium was then supplemented with 0.1 μ g/ml, 1.0 μ g/ml or 5 μ g/ml actinomycin D (Act D) in the presence or absence of 1.2 μ M 25-hydroxycholesterol (25-OH C). Cells were incubated for an additional 4 h, and total RNA was extracted. Relative changes in both reductase and β -actin mRNA were estimated by slot-blot hybridization assays. Shown is a representative slot-blot autoradiograph of 8 μ g of total RNA hybridized to 32 P-labelled reductase or β -actin probes. Lanes 1 and 2 (controls) represent reductase mRNA levels in cells incubated for 16 and 20 h respectively.

dependent. The addition of 0.1 and 1 μ g/ml actinomycin D to lovastatin-treated cells increased reductase mRNA by approx. 2-fold relative to cells treated with lovastatin only (compare lanes 1, 4 and 6). In contrast, cells treated with lovastatin and 5 μ g/ml actinomycin D had mRNA levels equal to those of cells treated only with lovastatin and either 0.1 or 1 μ g/ml actinomycin D, reductase mRNA levels were suppressed approx. 2-fold (lanes 5 and 7), but only to a level equal to that found in lovastatin-treated cells (lane 1). In contrast, in the absence of actinomycin D, 25-hydroxycholesterol decreased reductase mRNA levels in lovastatin-treated cells 4-fold (lane 3) compared with cells treated with lovastatin only. When actinomycin D concentrations were increased to 5 μ g/ml in cells treated with both lovastatin and 25-hydroxycholesterol (lane 9), mRNA levels remained 4-fold higher than in cells treated with lovastatin and 25-hydroxycholesterol in the absence of actinomycin D. β -Actin mRNA remained unchanged under any of these conditions (Table 2). The effects of actinomycin D on reductase mRNA are analogous to those for cycloheximide and support the hypothesis that a labile cellular regulator mediates steady-state levels of reductase mRNA.

Cycloheximide does not block oxysterol-mediated suppression of HMG-CoA reductase mRNA transcription

Results from experiments described in Figure 2 suggested that a short-lived protein mediated oxysterol control of HMG-CoA reductase mRNA at a transcriptional or post-transcriptional level. To address this issue, run-on transcriptional assays were conducted on isolated nuclei from cells treated with lovastatin and 25-hydroxycholesterol, in either the presence or the absence of cycloheximide. If this putative regulator acted transcriptionally, in cells treated with both lovastatin and 25-hydroxycholesterol, cycloheximide would block oxysterol-mediated suppression of transcription. Alternatively, if this regulator acted at a post-transcriptional level, 25-hydroxycholesterol would suppress transcription in cells treated with both cycloheximide and lovastatin, whereas reductase mRNA levels would remain unchanged.

Representative results from one of two run-on transcriptional assays are shown in Figure 7(a). Lovastatin increased HMG-CoA reductase transcription approx. 4-fold compared with cells

Table 2 Effects of actinomycin D on HMG-CoA reductase and β -actin mRNA levels

Relative changes in mRNA levels were estimated by laser-densitometric scanning of autoradiographs represented in Figure 6. For each treatment, the amount of 32 P-labelled cDNA probe hybridizing to at least four RNA concentrations from three separate RNA preparations fixed to nitrocellulose membranes indicated relative changes in mRNA levels. Values for β -actin mRNA are shown in parentheses. HMG-CoA reductase and β -actin mRNA in cells treated only with lovastatin in DFBS-MEM were set at 100%, and all other values are reported as a percentage (mean $\pm$ S.D.) of this value.

Treatment	[Actinomycin D] (μ g/ml) ...	mRNA level			
		0	0.1	1.0	5.0
Lovastatin		100 $\pm$ 4 (100 $\pm$ 1)	175 $\pm$ 8 (126 $\pm$ 8)	177 $\pm$ 6 (96 $\pm$ 11)	103 $\pm$ 9 (89 $\pm$ 6)
Lovastatin + 25-hydroxycholesterol		17 $\pm$ 10 (96 $\pm$ 6)	108 $\pm$ 3 (131 $\pm$ 7)	104 $\pm$ 3 (96 $\pm$ 12)	134 $\pm$ 5 (111 $\pm$ 1)

Figure 7 Cycloheximide blocks 25-hydroxycholesterol-mediated suppression of HMG-CoA reductase mRNA at a post-transcriptional level

Cells were grown in FBS-MEM for 48 h. In (a) the medium was then changed to DFBS-MEM plus 25 μ M lovastatin and cells were incubated for 16 h. Subsequently, 25-hydroxycholesterol (25-OH C) was added to a final concentration of 1.2 μ M in the presence or absence of 10 μ g/ml cycloheximide (CHX). Cells were incubated for an additional 4 h. In (b), cycloheximide was added to a final concentration of 10 μ g/ml and cells were incubated for 1, 2 or 6 h. The relative level of transcription was estimated by using run-on transcription assays on isolated nuclei as described in the Materials and methods section. 32 P-labelled nuclear RNA from cells was hybridized to slot-blots containing 5 μ g each of cDNAs for HMG-CoA reductase, HMG-CoA synthase and β -actin mRNAs. Plasmid DNA pSP6 was used to estimate background hybridization. Shown is an autoradiograph of representative slot blots. The 0 h control represents the transcription rate in nuclei from cells grown in FBS-MEM (see a). Relative transcription rates were estimated by laser-densitometric scanning of autoradiographs; these values are reported in the text.

grown in FBS-MEM. Conversely, 25-hydroxycholesterol decreased reductase transcription in lovastatin-treated cells 5-fold, comparable with the level in cells grown in FBS-MEM. When cycloheximide was added in conjunction with 25-hydroxycholesterol to lovastatin-treated cells, transcription was similarly decreased 5-fold. β -Actin transcription did not change concordantly. The slight decrease in β -actin transcription after lovastatin and 25-hydroxycholesterol treatment in the presence of cycloheximide was not significant ($t = 1.571$; $P = 0.26$) when compared with that after treatment with FBS-MEM, lovastatin

alone, or a combination of lovastatin and 25-hydroxycholesterol, which together had a mean absorbance of 0.28 ± 0.04 . These results indicate that inhibition of protein synthesis by cycloheximide did not result in the loss of a protein that was involved in oxysterol-mediated suppression of reductase mRNA at the level of transcription. As a positive control, transcription of another sterol-responsive gene, HMG-CoA synthase, which is involved in the initial steps of cholesterol biosynthesis [1], was similarly increased by lovastatin. In addition, 25-hydroxycholesterol decreased transcription of HMG-CoA synthase, and cycloheximide did not prevent this oxysterol-mediated suppression (Figure 7a).

As shown in Figure 4, cycloheximide treatment of cells cultured in FBS-MEM increased reductase mRNA 10-fold. If this increase were due to the loss of a repressor protein mediating oxysterol suppression of reductase transcription, it was expected that cycloheximide treatment would likewise increase reductase transcription. Therefore, run-on transcription assays were conducted on nuclei isolated from cells cultured in FBS-MEM plus cycloheximide for various time intervals (Figure 7b). However, we found no increase in reductase transcription after cycloheximide treatment, as determined by laser-densitometric scanning. The mean value for the cycloheximide-treated groups (0.23 ± 0.08) did not differ significantly ($t = 1.154$; $P = 0.33$) from the value (0.26) for untreated cells. Compared with cells treated with only FBS-MEM, actin transcription increased approx. 2-fold after 2 h exposure to cycloheximide. By 6 h, however, actin mRNA returned to basal levels. A similar transient increase in actin transcription mediated by cycloheximide has been observed by Greenberg et al. [30], who attributed the increase to a release of transcriptional repression. Thus these results support our hypothesis that cycloheximide mediates an effect on reductase mRNA at the post-transcriptional level.

DISCUSSION

This study had demonstrated that inhibition of protein synthesis by cycloheximide blocked 25-hydroxycholesterol-mediated suppression of HMG-CoA reductase mRNA in lovastatin-treated hamster cells at a post-transcriptional level. In the presence of cycloheximide, 25-hydroxycholesterol suppressed transcription of reductase mRNA in lovastatin-treated cells, but reductase mRNA levels were no lower than in cells treated only with lovastatin. These results indicate that proteins modulating oxysterol regulation of transcriptional control of reductase mRNA synthesis are relatively stable compared with protein regulators of mRNA degradation in the presence of 25-hydroxycholesterol. Although Trzaskos et al. [31] observed cycloheximide-induced

blockage of oxysterol-mediated suppression of reductase mRNA in fibroblasts, they did not determine whether the effects were mediated at the post-transcriptional or transcriptional level.

Cycloheximide blockage of 25-hydroxycholesterol-induced mRNA degradation suggests that protein-synthesis inhibition stabilizes reductase mRNA. This hypothesis is supported by two observations in our study. First, when cycloheximide and 25-hydroxycholesterol were added together to lovastatin-treated cells, reductase mRNA levels decreased for only 1 h, then returned to those observed in lovastatin-treated cells. This result was not unexpected, because 25-hydroxycholesterol suppresses reductase transcription by only 70% [12,32]. Therefore, if reductase transcripts are stabilized by cycloheximide, reductase mRNA levels could increase, due to the residual 30% transcription. Secondly, when cycloheximide was added to cells cultured in FBS-MEM, reductase mRNA levels increased rapidly without any apparent increase in transcription. This suggests that mRNA levels increased because of increased transcript stability. Thus steady-state levels of rodent cell reductase mRNA appear to be closely regulated by two independent oxysterol-regulated processes. One of these is the well-characterized transcriptional control mediated by the SRE in the 5' promoter region of the gene [15], and the second is an uncharacterized translation-dependent degradation of HMG-CoA reductase transcripts.

In human leukaemia cells, cycloheximide increased levels of reductase mRNA as well as the mRNAs for low-density-lipoprotein receptor, farnesyl pyrophosphate synthase and HMG-CoA synthase [33,34]. However, in contrast with the results of our study, these earlier reports concluded that changes in reductase mRNA levels were due to the loss of a labile transcription factor. In these same studies [33,34], phorbol ester treatment increased low-density-lipoprotein receptor, farnesyl pyrophosphate synthase, HMG-CoA reductase and HMG-CoA synthase mRNA 2–7-fold. Treatment with both phorbol esters and cycloheximide superinduced all four mRNAs. In the study by Wilkin et al. [34], addition of phorbol esters plus cycloheximide to cells treated with both 25-hydroxycholesterol plus mevalonic acid further increased mRNA levels for farnesyl pyrophosphate synthetase, HMG-CoA reductase and HMG-CoA synthase. The authors of both reports concluded that protein kinase C acted directly or indirectly to inactivate a labile negative regulator of transcription. However, in a more recent work, cycloheximide decreased HMG-CoA reductase mRNA levels when added to human lymphocytes in medium supplemented with lipoprotein-deficient serum and phytohaemagglutinin [35]. Results from these previous reports as well as our study indicate that regulation of reductase steady-state levels is a translation-dependent process. The diversity of responses to cycloheximide treatment in various cell lines may reflect differences in cell-specific factors involved with either transcriptional or post-transcriptional control of reductase gene expression.

Stabilization of mRNA, a common effect of protein-synthesis inhibitors, is a secondary result of translational arrest. Stabilization could be due to either ribosome-associated nucleases or a labile protein that requires continuous synthesis [36]. The best-characterized examples are the proto-oncogenes *c-myc* and *c-fos*, both of which are stabilized by protein-synthesis inhibitors after transient transcriptional activation by mitogen stimulation [18,19,37–45]. For *c-myc* mRNA, the increase in transcriptional activation by mitogens or growth factors cannot account for the 10–40-fold increase in *c-myc* mRNA [43]. In addition, cycloheximide treatment increases *c-myc* mRNA, and can superinduce this mRNA in the presence of mitogens or growth factors [19,44], a result analogous to that observed in our study. Together, these

observations indicate that stabilization of the extremely labile *c-myc* transcript in the presence of cycloheximide accounts for the majority of its induction. Similarly, *c-fos* transcription is transiently induced by growth factors which increase *c-fos* mRNA [40–43]. The addition of cycloheximide not only prolongs the duration of the transcriptional response but also stabilizes *c-fos* mRNA [36]. In contrast, there was no evidence in our study that HMG-CoA reductase mRNA transcription was enhanced by cycloheximide treatment. This suggests that transcription factors regulating oxysterol suppression of reductase transcription are relatively stable and their functions are unaffected by protein-synthesis inhibition.

RNA sequences that mediate mRNA degradation include both 5' and 3' untranslated [18,19,37,38,42,44,46–51] as well as coding regions [37,38]. For both *c-myc* and *c-fos*, the nucleotide sequence AUUUA found in the 3' untranslated end plays a key role in specifying rapid transcript turnover [18,19,37,41,44,51]. These proto-oncogenes also have sequences within the coding region that mediate rapid mRNA turnover and induction by protein synthesis inhibitors [37,38]. In addition, the 5' untranslated region of *c-fos* mRNA contributes to transcript stability [51]. For HMG-CoA reductase mRNA, AUUUA motifs characteristic of unstable mRNAs are found in the 3' untranslated region of the 4.7 kb transcript, but their role in post-transcriptional stabilization of reductase mRNA by cycloheximide has not yet been established [28]. Although the 5' untranslated regions of hamster reductase mRNA are highly heterogeneous in length [52], the role of these sequences in translation-dependent stabilization likewise remains unexplored.

The actinomycin D-mediated block of suppression of reductase mRNA levels by 25-hydroxycholesterol in lovastatin-treated cells in the first report of its kind regarding the regulation of HMG-CoA reductase. Because cycloheximide treatment likewise blocks oxysterol-mediated suppression of reductase mRNA at the post-translational level, these results suggest that reductase mRNA is stabilized when RNA synthesis is inhibited. This phenomenon would also explain the 2-fold increase in mRNA when 0.1 or 0.5 $\mu\text{g/ml}$ actinomycin D was added to lovastatin-treated cells, if there were either incomplete inhibition of transcription or a lag in the transcriptional block. Similarly, Auwerx et al. [33] observed a transient 2-fold increase in HMG-CoA reductase mRNA in phorbol 12-myristate 13-acetate-treated cells after the addition of actinomycin D, which they attributed to a lag in the transcriptional block induced by actinomycin D.

In our study, the 2-fold reductase mRNA suppression by 25-hydroxycholesterol in the presence of 0.1 or 0.5 $\mu\text{g/ml}$ actinomycin D may reflect oxysterol-mediated suppression of transcription, a result expected because of the stable nature of transcription factors involved in oxysterol regulation. However, in the presence of 25-hydroxycholesterol and all three actinomycin D concentrations, the level of reductase mRNA was 4-fold greater than in cells treated with 25-hydroxycholesterol in the absence of actinomycin D. This indicates that 25-hydroxycholesterol did not suppress reductase mRNA completely when RNA synthesis was inhibited, most likely because mRNA is stabilized under these conditions. Overall, the block to oxysterol-mediated suppression and concomitant increase in reductase mRNA in the presence of actinomycin D can be explained by the existence of short-lived regulators that mediate reductase mRNA degradation. When synthesis of such regulators is blocked, by inhibitors of either RNA or protein synthesis, reductase transcripts become more stable.

Several other studies have attributed mRNA stabilization in the presence of actinomycin D to decreased synthesis of labile regulators of mRNA turnover [53–56]. Pontecorvi et al. [53]

observed in differentiating rat myoblasts that induction and de-induction of creatine activity, as well as its corresponding mRNA, were due to selective stabilization and destabilization of creatine kinase mRNA by insulin. Furthermore, either cycloheximide or actinomycin D prevented degradation of creatine kinase mRNA in a reversible manner when insulin was removed; this mRNA was superinduced if either inhibitor was added during induction by insulin. Similarly, Nemoto and Sakurai [54] reported that actinomycin D increased the half-life of CYP1A1 gene transcripts in mouse hepatocytes and delayed the de-induction of these transcripts when an inducer of CYP1A1, benz[a]anthracene, was removed. In *Dictyostelium discoideum*, inhibition of either RNA or protein synthesis prevented disaggregation-mediated destabilization of prepore mRNAs [55]. Although stabilization was attributed initially to the loss of labile proteins that mediate degradation of pre-spore transcripts [55], a later study demonstrated that mRNA destabilization of a pre-spore gene, EB4-PSV, was linked to an antisense transcript originating from the same gene locus [56]. It is not known whether an antisense transcript is involved in degradation of HMG-CoA reductase mRNA.

Our nuclear run-on assays have demonstrated that proteins involved in oxysterol-mediated control of reductase mRNA transcription are relatively stable. A 4 h treatment with cycloheximide and 25-hydroxycholesterol suppressed transcription of both HMG-CoA reductase and synthase, indicating that sufficient levels of transcription repressors were available to mediate the oxysterol effects. Therefore, the cycloheximide-induced block of oxysterol-mediated suppression of reductase mRNA must occur at a post-transcriptional level, most likely through stabilization of the transcripts when protein synthesis is inhibited.

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