

# Regulation of the low density lipoprotein receptor and hydroxymethylglutaryl coenzyme A reductase genes by protein kinase C and a putative negative regulatory protein

(gene expression/Hep G2 cells/phorbol esters/THP-1 cells/transcription factors)

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**ABSTRACT** Transcription of the low density lipoprotein receptor (LDL-R) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase genes was rapidly and transiently induced (8.5- and 2.3-fold, respectively) early during phorbol 12-myristate 13-acetate (PMA)-induced macrophage differentiation of the human monocytic leukemia cell line THP-1. The levels of mRNA coding for LDL-R and HMG-CoA reductase increased soon after induction, reached a maximum (12- and 7-fold increase, respectively) in 2-3 hr, and then rapidly returned to the low constitutive levels observed before induction. The stability of LDL-R mRNA did not change significantly during differentiation, whereas that of HMG-CoA reductase mRNA decreased by about 5-fold 6 hr after the addition of PMA. Transcriptional induction of both LDL-R and HMG-CoA reductase genes (5.6- and 2-fold, respectively) was also observed when undifferentiated cells were treated with cycloheximide (CHX), resulting in a transient increase in steady-state mRNA (7- and 3-fold, respectively). These results suggest that expression of the two genes is maintained at low constitutive levels in uninduced THP-1 cells by a protein with a short half-life. Superinduction of both genes occurred when PMA and CHX were added simultaneously. The induction of LDL-R and HMG-CoA reductase mRNAs during early macrophage differentiation is mediated by protein kinase C. It is hypothesized that protein kinase C acts directly or indirectly to inactivate the labile negative regulatory protein. Induction of LDL-R mRNA was also observed when the human hepatocarcinoma cell line Hep G2 was treated with PMA and CHX, suggesting that this mechanism of regulation may exist in several cell types.

Eukaryotic cells obtain cholesterol either by receptor-mediated endocytosis of lipoprotein cholesterol via the low density lipoprotein receptor (LDL-R) or by *de novo* synthesis, the rate-limiting step of which is catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34) (1, 2). Both of these pathways are regulated by cellular cholesterol content and are therefore under the influence of end-product repression.

The belief that macrophages are the major cellular progenitors of foam cells in the atherosclerotic plaque (3) has led to an intense interest in the regulation of cholesterol homeostasis in this cell type. Differentiation of monocytes to macrophages has been shown to be associated with decreased LDL-R activity, while activity of the scavenger receptor, which recognizes certain modified forms of LDL, has been found to increase during this process (4, 5).

A large number of genes are specifically induced as monocytes differentiate into macrophages. The human

monocytic leukemia cell line THP-1, which readily assumes properties of macrophages upon treatment with phorbol esters (6, 7), is a useful model for the study of macrophage differentiation. The differentiation process in these cells is associated with secretion of lipoprotein lipase (8) and apolipoprotein E (8), enhanced scavenger receptor activity, and decreased LDL-R activity (5). The increase in lipoprotein lipase and apolipoprotein E secretion was shown to be the result of transcriptional activation of the lipoprotein lipase and apolipoprotein E genes (7). This study was undertaken to investigate the mechanism of regulation of expression of the LDL-R and HMG-CoA reductase genes during macrophage differentiation. A transient but substantial induction of LDL-R and HMG-CoA reductase mRNAs was observed upon treatment of THP-1 cells with phorbol esters and cycloheximide (CHX), suggesting the involvement of protein kinase C (PKC) and a negative regulatory protein in modulating transcription of these genes.

## EXPERIMENTAL PROCEDURES

THP-1 cells and Hep G2 human hepatocarcinoma cells were grown, respectively, in RPMI-1640 medium and Eagle's minimal essential medium supplemented with 10% fetal calf serum (7). Phorbol 12-myristate 13-acetate (PMA) at 0.16  $\mu$ M was used to induce differentiation of THP-1 cells into cells with a macrophage-like appearance (7).

RNA isolation and Northern and dot-blot analyses were as described previously (9). LDL-R mRNA was detected by using the 1.9-kilobase (kb) *Bam*HI fragment of the human LDL-R cDNA clone pLDLR-3 (kindly provided by J. L. Goldstein and M. S. Brown and described in ref. 9). HMG-CoA reductase mRNA was detected by using the 2.5-kb *Bgl*II fragment of the pHRed-102 cDNA clone [ref. 10; American Type Culture Collection (ATCC) no. 57042]. A 0.75-kb *Eco*RI-*Hind*III fragment of pA1, a chicken  $\beta$ -actin cDNA clone (11), a 1.1-kb *Pst*I fragment of a *c-fos* cDNA clone (a kind gift of R. Perlmutter, University of Washington), and a 0.45-kb *Taq*I-*Alu*I fragment of the superoxide dismutase cDNA clone pS61-10 (ref. 12; ATCC no. 57231) were used as control probes.

The nuclear isolation and transcription assay was performed essentially as described by Nevins (13). Equivalent amounts of labeled nuclear RNA ( $3 \times 10^6$  cpm) were hybridized for 36 hr at 42°C to 1, 0.5, and 0.25  $\mu$ g of purified cloned cDNAs immobilized on Hybond nylon filters (Amer-

Abbreviations: CHX, cycloheximide; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; HA-1004, *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDL, low density lipoprotein; LDL-R, LDL receptor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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sham). The following cDNA probes were spotted: the 1.36-kb *EcoRI* fragment of pHLPL26 (7), the 1.9-kb *BamHI* fragment of pLDLR-3 (9), the 2.5-kb *Bgl* II fragment of pHRed-102 (10), a full-length apolipoprotein E cDNA (7), and a 0.75-kb *EcoRI-HindIII* fragment of the pA1 actin clone (11). As a control, 5, 2.5, and 1.25  $\mu\text{g}$  of the vector DNA also were applied to the filter.

## RESULTS

**Differentiation of THP-1 Cells Is Associated with Transient Induction of LDL-R and HMG-CoA Reductase mRNAs.** The THP-1 human monocytic leukemia cell line differentiates into mature macrophage-like cells after treatment with phorbol esters such as PMA (6, 7). Soon after addition of PMA, a marked and transient increase in the steady-state levels of LDL-R and HMG-CoA reductase mRNAs was observed (Fig. 1). LDL-R mRNA started accumulating as early as 15 min after the addition of PMA, reached a maximum (12-fold increase) 1–2 hr later, and then decreased to the preinduction level within 3 hr. A similar, but slightly slower, induction was observed for HMG-CoA reductase mRNA, which reached its maximum (7-fold increase) approximately 2 hr after addition of PMA (Fig. 1) and then decreased to preinduction levels by 6 hr. Induction of LDL-R and HMG-CoA reductase was dose dependent (Fig. 1 *Inset*). Blot hybridization of RNA (Fig. 2) from THP-1 cells before and after PMA treatment showed an increase in the mRNA species corresponding to those previously described for LDL-R (14) and HMG-CoA reductase (15) and matching those observed in the human hepatoma cell line Hep G2. The low levels of LDL-R and HMG-CoA reductase mRNA in undifferentiated cells were expected, since they were grown in the presence of fetal bovine serum, the cholesterol content of which represses these two genes.

**Induction of LDL-R and HMG-CoA Reductase mRNA Is Mainly at the Level of Transcription.** To determine the relative contribution of transcriptional and post-transcriptional processes to the increase in steady-state levels of LDL-R and HMG-CoA reductase mRNAs, rates of synthesis and degradation of the mRNA species were determined. Pulse-labeling of nascent mRNA in nuclei of THP-1 cells before and after induction with PMA and hybridization to excess cloned LDL-R and HMG-CoA reductase cDNAs

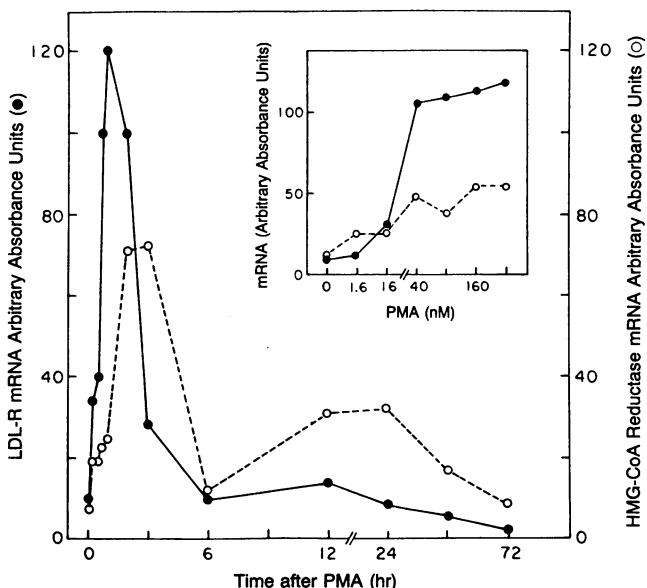


FIG. 1. Time course of accumulation of LDL-R mRNA (●) and HMG-CoA reductase mRNA (○) in THP-1 cells after treatment with PMA. (*Inset*) Dose-response curves generated by treatment with PMA for 2 hr.

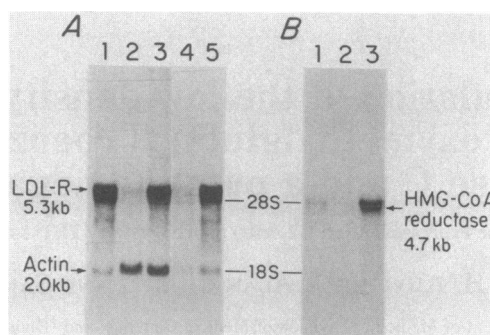


FIG. 2. Electrophoretic analysis of LDL-R and HMG-CoA reductase mRNAs induced in THP-1 and Hep G2 cells. (A) LDL-R mRNA in THP-1 and Hep G2 cells. The cells were grown for 2 hr (THP-1) or 6 hr (Hep G2) in the presence or absence of PMA. Lane 1, total liver RNA (control); lane 2, untreated THP-1 cells; lane 3, THP-1 cells treated with PMA for 2 hr; lane 4, untreated Hep G2 cells; lane 5, Hep G2 cells treated with PMA. Twenty micrograms of RNA was used in each lane. (B) HMG-CoA reductase mRNA (lanes 1, 2, and 3 are the same as in A).

revealed that induction by PMA was largely at the level of transcription (Fig. 3, rows 2 and 3, lanes 1 and 2). Increases of approximately 8.5-fold and 2.3-fold in transcription rate were observed for the LDL-R and HMG-CoA reductase mRNAs, respectively. No change in transcription rate of actin gene was observed. Transcription rates for the LDL-R and HMG-CoA reductase genes dropped back to the low preinduction levels 5 hr after initial stimulation with PMA (Fig. 3, lanes 4). Furthermore, the increase in steady-state levels of LDL-R and HMG-CoA reductase mRNA at 2 hr after addition of PMA and CHX was abolished by prior addition of actinomycin D (5  $\mu\text{g}/\text{ml}$ ) (data not shown).

To assess the role of mRNA stability in the observed changes of the LDL-R and HMG-CoA reductase mRNAs, RNA synthesis was blocked with actinomycin D at 1, 3, 6, and 72 hr after induction with PMA, and the relative rates of disappearance of LDL-R and HMG-CoA reductase mRNA was determined (Fig. 4). Due to the low preinduction levels of both the LDL-R and HMG-CoA reductase mRNAs, no determination of stability was possible before induction with PMA. Whereas the stability of LDL-R mRNA did not change appreciably during the transient induction, that of HMG-CoA reductase decreased substantially, thus contributing significantly to the decrease in HMG-CoA reductase steady-state mRNA. The initial increase in HMG-CoA reductase and LDL-R mRNAs probably is caused by a lag in transcription block by actinomycin D. A similar lag has been observed with

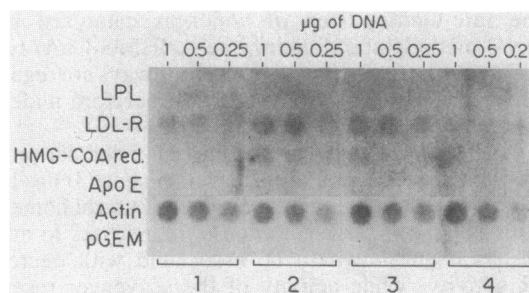


FIG. 3. Analysis of transcription rate of the LDL-R and HMG-CoA reductase genes. Nuclear run-off analysis with labeled RNA obtained from nuclei prepared from THP-1 cells before any treatment (lanes 1), and 1 hr after treatment with PMA (lanes 2), 1 hr after CHX (lanes 3; 10  $\mu\text{g}/\text{ml}$  of media), or 5 hr after PMA (lanes 4). The RNAs were hybridized with 1, 0.5, or 0.25  $\mu\text{g}$  of DNA inserts or pGEM vector (5, 2.5, 1.25  $\mu\text{g}$  of DNA). LPL, lipoprotein lipase; Apo E, apolipoprotein E.

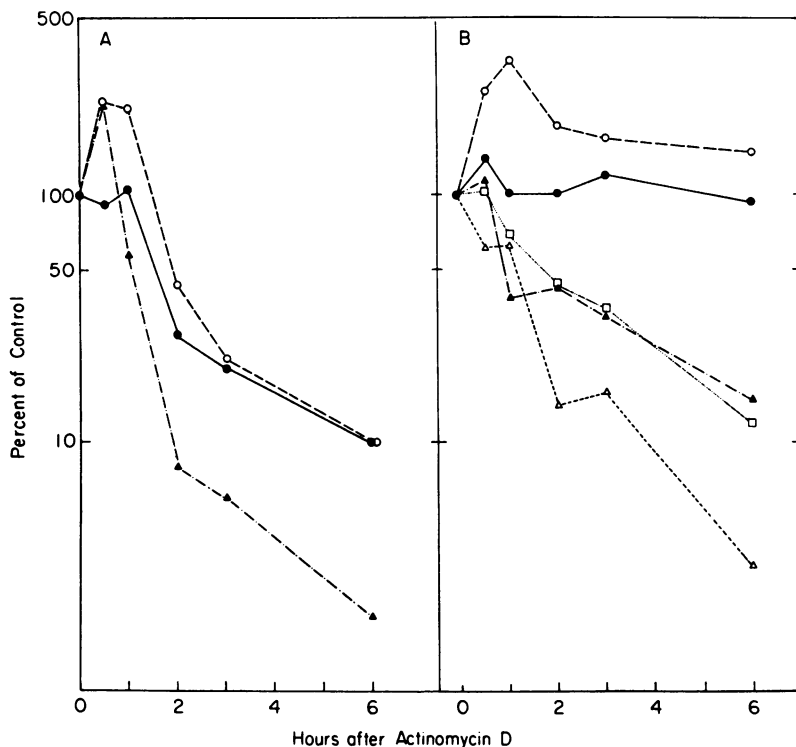


FIG. 4. Stability of LDL-R (A) and HMG-CoA reductase (B) mRNAs. mRNA content was determined at various intervals after the addition of the actinomycin D to THP-1 cells which had been treated with PMA for 1 (●), 3 (○), 6 (▲), 24 (□), or 72 (Δ) hr. Insufficient LDL-R mRNA was present 72 hr after PMA for analysis. The relative concentration of RNA before treatment with actinomycin D was set at 100%.

the *c-fos* protooncogene after treatment of U-937 cells with actinomycin D and PMA (16).

**Induction of the LDL-R and HMG-CoA Reductase Genes by CHX.** Addition of the protein synthesis inhibitor CHX (10 μg/ml) to THP-1 cells resulted in the same magnitude of induction of LDL-R mRNA as that seen with PMA (Fig. 5). HMG-CoA reductase mRNA also was induced by CHX, but to a lesser extent than LDL-R mRNA. The induction of both mRNAs was dose dependent (data not shown). Induction by CHX was shown to be at the level of transcription (Fig. 3, lanes 3). Simultaneous treatment of undifferentiated cells with CHX and PMA resulted in superinduction (approximately 15-fold) of LDL-R and HMG-CoA reductase mRNAs (Fig. 5). PMA and CHX therefore act synergistically to induce RNA transcription. The absence of a change in the capacity of the cells to degrade LDL after treatment of cells with CHX (data not shown) excluded the possibility that induction of these mRNAs was caused by decreased cholesterol delivery to cells by blocking synthesis of LDL-R.

**Induction of LDL-R and HMG-CoA Reductase mRNAs Appears to Be Mediated by PKC.** Activation of PKC is thought to be one of the pleiotropic effects of phorbol esters (17). As with PMA, treatment with *rac*-1,2-dioctanoylglycerol (di-C<sub>8</sub>), a synthetic homolog of diacylglycerol, the naturally occurring PKC activator, resulted in induction (although to a lesser extent than with PMA) of LDL-R and HMG-CoA reductase mRNAs (Fig. 6). Induction of LDL-R mRNA by di-C<sub>8</sub> followed the same time course and was concentration dependent. As a control, induction of the *c-fos* protooncogene by di-C<sub>8</sub> was demonstrated in these cells (data not shown).

The effect of the protein kinase inhibitors 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) and *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride (HA-1004) on the induction of LDL-R and HMG-CoA reductase mRNAs was next tested. H-7 and HA-1004 were shown to have similar *K<sub>i</sub>* values for the inhibition of protein kinase A, protein kinase G, and myosin light chain kinase, whereas H-7 (*K<sub>i</sub>* = 6.0 μM) was shown to be a much

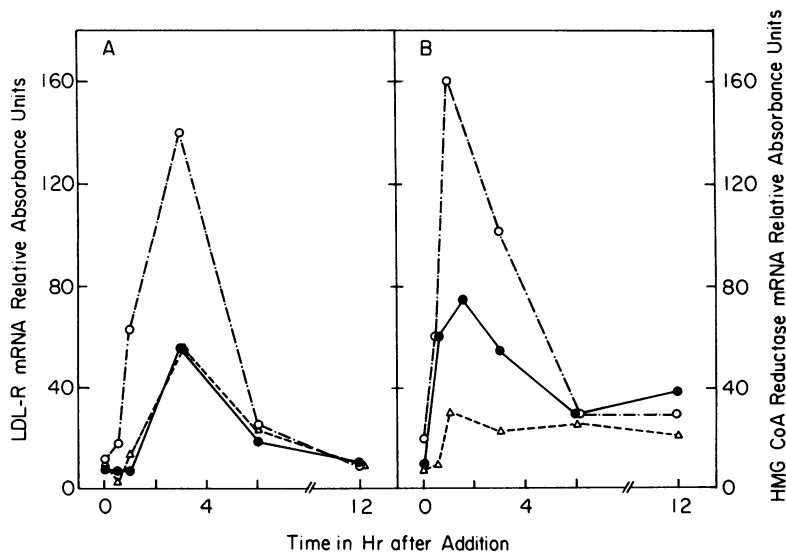


FIG. 5. Induction of LDL-R (A) and HMG-CoA reductase (B) mRNAs by CHX and PMA in THP-1 cells. RNA was isolated at various times after addition of PMA (●), CHX (Δ), or CHX + PMA (○) and was quantitated by dot-blot hybridization.

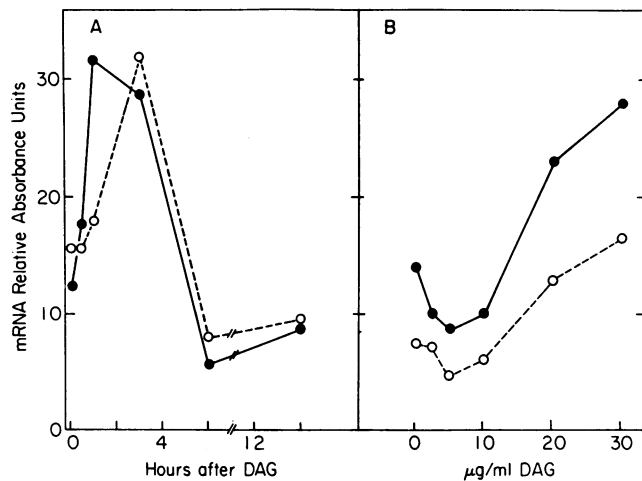


FIG. 6. Time course (A) and dose response (B) of LDL-R (●) and HMG-CoA reductase (○) mRNA accumulation after treatment of THP-1 cells with the synthetic diacylglycerol (DAG) *rac*-1,2-dioctanoylglycerol (20 µg/ml).

more potent inhibitor of PKC than HA-1004 ( $K_i = 40 \mu\text{M}$ ) (18). Whereas H-7 caused a profound dose-dependent inhibition of induction by PMA of both LDL-R and HMG-CoA reductase mRNA, HA-1004 had no effect (Fig. 7). H-7 and HA-1004 did not affect differentiation of THP-1 cells and were not cytotoxic at the concentrations used.

**Induction of LDL-R mRNA by PMA and CHX in Hep G2 Cells.** To assess if induction of LDL-R mRNA by PMA and CHX was limited to THP-1 cells, the effect of these agents on LDL-R mRNA accumulation in the human hepatocarcinoma cell line Hep G2 was studied. An increase in LDL-R mRNA was observed as early as 3 hr after induction by PMA, and by 6 hr the increase was approximately 24-fold (Figs. 2 and 8). CHX resulted in only a 2-fold increase in LDL-R mRNA levels. However, in contrast to THP-1 cells, no superinduction of LDL-R mRNA was observed when CHX and PMA were added simultaneously.

## DISCUSSION

Fully differentiated macrophages and THP-1 cells have decreased LDL-R and increased scavenger receptor and HMG-CoA reductase activities (4, 5, 19). Very early events involving lipid metabolism during macrophage differentiation, however, have not been studied in detail. Treatment of THP-1 cells with PMA (which induces differentiation into

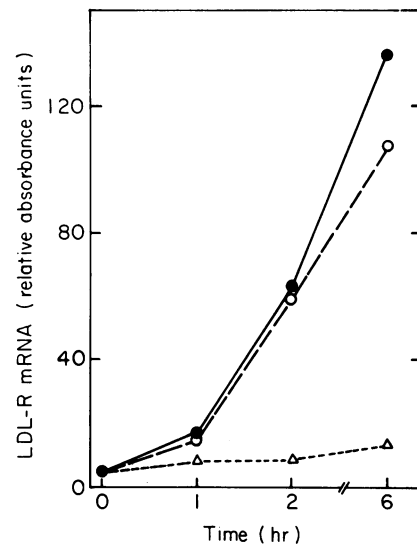


FIG. 8. Induction of LDL-R mRNA in Hep G2 cells. RNA was isolated at various times after addition of PMA (●), CHX (Δ), or CHX + PMA (○) and quantitated by dot-blot hybridization.

macrophage-like cells) resulted early (within 3 hr) in a dramatic and transient increase in LDL-R and HMG-CoA reductase mRNAs. Due to the transient nature of this induction, no change in LDL-R activity was noted in the earlier study by Hara *et al.* (5). Indeed, by the first time point reported in that study (24 hr) LDL-R mRNA had already returned to the low constitutive level. An even greater induction of LDL-R mRNA by PMA was observed in Hep G2 cells.

The increase in the steady-state levels of mRNA in THP-1 cells was shown to be mainly the result of transcriptional activation of both the LDL-R and HMG-CoA reductase genes. The subsequent decline to constitutive mRNA levels was largely due to a decrease in transcription rate of the LDL-R gene, since only a minor change in mRNA stability was observed during this rapid decline phase. In contrast, the stability of HMG-CoA reductase mRNA decreased markedly (approximately 6-fold) after the steady-state level reached its maximum. Thus degradation of this mRNA seems to have contributed significantly to the rapid return to constitutive levels. Similar changes in *c-fos* mRNA stability have been observed during the transient induction by PMA in U-937, another monocytic cell line (16).

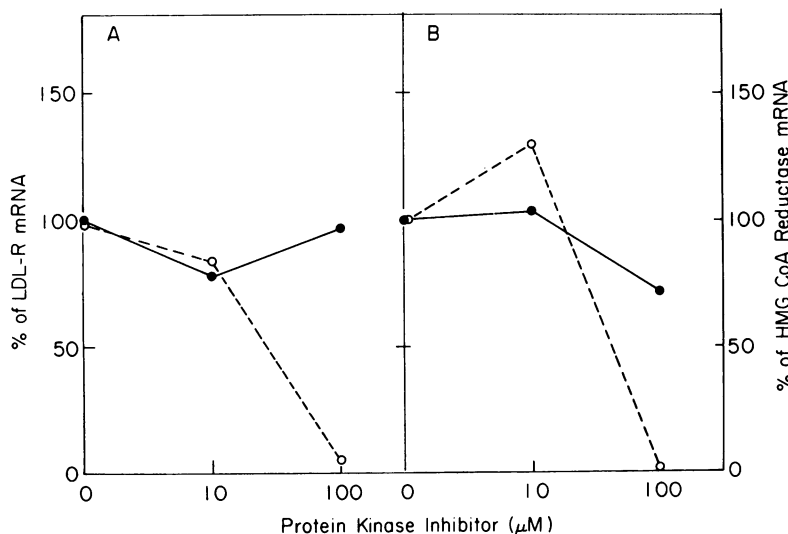


FIG. 7. Accumulation of LDL-R (A) and HMG-CoA reductase (B) mRNAs is inhibited by H-7. THP-1 cells were grown for 2 hr in the presence of either 0.16 µM PMA (used as a reference point) alone or various concentrations of H-7 (○) or HA-1004 (●) added 10 min before PMA (0.16 µM). The amount of mRNA present with a given concentration of protein kinase inhibitor was expressed as a percentage of the amount of mRNA in cells treated with PMA only.

Transcriptional activation of the LDL-R and HMG-CoA reductase genes by PMA was shown to be mediated by PKC. A homolog of diacylglycerol, the natural activator of PKC, also induced LDL-R and HMG-CoA reductase mRNAs. This diacylglycerol, however, did not induce macrophage differentiation when added in a single dose, due to the short-lived stimulation of PKC by these rapidly metabolized homologs (20). Treatment of THP-1 cells with PMA together with H-7, an inhibitor of PKC (18), resulted in a decrease in the extent of induction of both LDL-R and HMG-CoA reductase mRNA, while HA-1004, an inhibitor of other protein kinases, had no effect. The effect of PKC on LDL-R mRNA expression is not limited to macrophages, since LDL-R mRNA also increased after PMA addition to Hep G2 cells. Several other genes such as human metallothionein IIA (21, 22), *c-fos* (16), and collagenase (23) were also shown to be transcriptionally activated by phorbol esters. Activation of these genes involves interaction of transcription factors (such as AP1) with specific 5' upstream phorbol-ester-responsive enhancer sequences with a conserved motif such as TGACTCA (22, 23). In the case of *c-fos*, activation was shown to be mediated by PKC (24). Although the mechanism by which PKC induces gene transcription is not completely elucidated, it has been suggested that induction by PMA involves a post-translational modification such as phosphorylation of one or more trans-acting factors (21–23).

The transient increase in LDL-R and HMG-CoA reductase mRNAs shortly after the addition of PMA could be in response to increased cholesterol needs for the synthesis of internal membranes early in differentiation. The resulting increase in intracellular cholesterol content might in turn be responsible for the subsequent decline in LDL-R and HMG-CoA reductase mRNAs. Classic end-product repression by sterols is mediated largely by changes in transcription of the LDL-R (25, 26) and HMG-CoA reductase (27, 28) genes. Increase in cellular sterol was suggested to repress transcription either by inactivating a positive transcription factor or by inhibiting its binding to specific 5' regulatory sequences (25, 26).

Transcriptional activation of the two genes also was observed upon inhibition of protein synthesis with CHX. It is unlikely that a block in LDL-R synthesis by CHX might limit cellular uptake of cholesterol and therefore cause induction of LDL-R and HMG-CoA reductase. First, Brown and Goldstein (29) have shown that the LDL-R has a half-life on the cell surface of approximately 20 hr, which is much longer than the time required for induction of LDL-R by CHX. Second, exposure of fibroblasts to CHX did not influence binding and internalization of LDL for many hours, showing that protein synthesis is not required for internalization of LDL and recycling of LDL-R (2). Induction of transcription by CHX suggests the participation of a labile protein in repressing transcription. Superinduction of transcription was observed when cells were treated with both PMA and CHX, suggesting that they either affect different pathways or both partially decrease the level of the active form of a labile protein repressor. Precedents for this type of regulation, namely, transcriptional stimulation of gene expression by CHX and hence presumed regulation by an inhibitory protein, have been described for several genes such as the *c-fos* (16, 30), the *c-myc* (30), the actin (30), and the  $\beta$ -interferon (31) genes. These inhibitory proteins are thought to interact either directly or indirectly with regulatory sequences (31). In the latter case, a negative regulatory protein binds to a positive transcription factor to form an inactive complex.

Activation of transcription could then occur either by blocking synthesis of the negative regulatory protein or by converting it (possibly by phosphorylation by PKC) into a form unable to bind to the positive transcription factor. Recently similar mechanisms of regulation have been proposed for the  $\kappa$  enhancer binding protein, NF- $\kappa$ B, which activates transcription of the  $\kappa$  light chain gene (32).

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