Role of mevalonate in regulation of cholesterol synthesis and 3-hydroxy-3-methylglutaryl coenzyme A reductase in cultured cells and their cytoplasts

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Abstract H4-II-E-C3 hepatoma cells in culture respond to lipid-depleted media and to mevinolin with increased sterol synthesis from [^{14}C]acetate and rise of 3-hydroxy-3-methylglutaryl coenzyme A reductase levels. Mevalonate at 4 mM concentration represses sterol synthesis and the reductase, and completely abolishes the effects of mevinolin. Mevalonate has little or no effect on sterol synthesis or reductase in enucleated hepatoma cells (cytoplasts) or on reductase in cytoplasts of cultured Chinese hamster ovary (CHO) cells. The sterol-synthesizing system of hepatoma cell cytoplasts and the reductase in the cytoplasts of CHO cells were completely stable for at least 4 hr. While reductase levels and sterol synthesis from acetate followed parallel courses, the effects on sterol synthesis—both increases and decreases—exceeded those on reductase. In vitro translation of hepatoma cell poly(A)$^+$RNAs under various culture conditions gave an immunoprecipitable polypeptide with a mass of 97,000 daltons. The poly(A)$^+$RNA from cells exposed for 24 hr to lipid-depleted media plus mevinolin (1 μg/ml) contained 2.8 to 3.6 times more reductase-specific mRNA than that of cells kept in full-growth medium, or cells exposed to lipid-depleted media plus mevinolin plus mevalonate. Northern blot hybridization of H4 cell poly(A)$^+$RNAs with [^{32}P]cDNA to the reductase of CHO cells gave two ^{32}P-labeled bands of 4.6 and 4.2 K-bases of relative intensities 1.0, 0.61–1.1, 2.56, and 1.79 from cells kept, respectively, in full-growth medium, lipid-depleted medium plus mevinolin plus mevalonate, lipid-depleted medium plus mevinolin, and lipid-depleted medium. These values approximate the reductase levels of these cells. We conclude that mevalonate suppresses cholesterol biosynthesis in part by being a source of a product that decreases the level of reductase-specific mRNA. —**Popják, G., C. F. Clarke, C. Hadley, and A. Meenan.** Role of mevalonate in regulation of cholesterol synthesis and 3-hydroxy-3-methylglutaryl coenzyme A reductase in cultured cells and their cytoplasts. *J. Lipid Res.* 1985. **26:** 831–841.

Supplementary key words hepatoma cells • Chinese hamster ovary cells • mevinolin • HMG-CoA reductase mRNA

It was first demonstrated in our laboratory that mevalonate powerfully repressed 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase in isolated hepatocytes and in the liver of rats (1). The interesting property of mevalonate is that it is effective only in vivo in whole cells or in animals; it has no effect on the isolated microsomal enzyme. In contrast, cells or the liver of animals deprived of mevalonate by the administration of the two competitive inhibitors of HMG-CoA reductase, compactin (2) or its analogue mevinolin (3), responded with a great rise of reductase when the inhibitors were removed (4–6).

The effects of compactin, mevinolin, and mevalonate could be explained by the hypothesis that metabolism of mevalonate furnishes a repressor of the transcription of the HMG-CoA reductase gene. In the presence of compactin or mevinolin, neither mevalonate nor the putative repressor would be formed and synthesis of reductase-specific mRNA would proceed uninhibited. The work described represents the first stage of our testing the hypothesis. For most experiments we used the cultured H4-II-E-C3 (H4) cells derived from a minimum deviation rat hepatoma (7), although some experiments were made also with Chinese hamster ovary (CHO) cells.

EXPERIMENTAL PROCEDURES

Cell culture

Stocks of the H4-II-E-C3 (H4) cell line, obtained originally from Dr. Van R. Potter in 1971, were kept frozen in liquid N_2. Fresh cultures were started from the frozen stock in a modified Swim's S-77 medium (8) supplemented with 5% fetal bovine serum, 10% horse serum, and 100,000 units of penicillin, 100 mg of streptomycin, and 50,000 units of polymyxin B sulfate per liter. This medium

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; FGM, full-growth medium; LDM, lipid-depleted medium; LDL, low density lipoprotein; HDL, high density lipoprotein; EDTA, ethylenediamine tetraacetic acid disodium salt; CHO, Chinese hamster ovary.

is referred to as full-growth medium (FGM). For subcultivation, the cell layers of confluent cultures grown in 75-cm^2 Lux plastic flasks (Flow Laboratories, Inglewood, CA) were washed twice with a citrate-saline solution (NaCl, sodium citrate, and phenol red; 6.0, 3.0, and 0.01 g per liter, respectively) and then treated with 2 ml of 0.025% trypsin in citrate-saline for 5 min at 37°C. The cell suspension was layered on 10 ml of growth medium and the cells were sedimented by centrifuging and then suspended in 20 ml of FGM. One ml of this suspension (ca. 1.5–2.0 × 10^6 cells) was inoculated either into 20 ml of FGM in 75-cm^2 flasks, or into 4 ml of the same medium in 25-cm^2 Falcon plastic flasks (American Scientific Products, Irvine, CA). The media were changed every second day. The cultures in the large flasks became confluent in 10–12 days and in the small flasks in 5 days. In most experiments confluent cultures in 25-cm^2 flasks were used.

Fresh medium was supplied the day before an experiment. On the day of the experiment, the media were removed by aspiration and the cell layers were washed three times with sterile Ca^{2+}- and Mg^{2+}-free phosphate-saline (NaCl, KCl, $Na_2HPO_4 \cdot 7H_2O$ and KH_2PO_4: 8.0, 0.2, 2.17, and 0.2 g per liter). Cultures in 75-cm^2 flasks were given 10 ml and those in 25-cm^2 flasks were given 3 ml of experimental media.

Experimental media were: *i*) FGM; *ii*) lipid-depleted medium (LDM); *iii*) FGM + mevinolin, 1 μg/ml (2.48 μM); *iv*) LDM + mevinolin (1 μg/ml); *v*) LDM + mevinolin (1 μg/ml) + varying concentrations of potassium (*R*)-mevalonate or normal human low density lipoproteins (LDL). Mevinolin was added as a solution of Me_2SO. The media with mevinolin contained 2 μl of Me_2SO per ml, and controls without mevinolin also contained 2 μl/ml of Me_2SO. Media and other solutions were sterilized by filtration through Nalgene bacterial filters (American Scientific Products, Irvine, CA) or through Millex®-GS filter units (Millipore Corporation, Bedford, MA).

The lipid-depleted medium had the same composition as FGM except that the fetal bovine and the horse sera were extracted by the method of McFarlane (9). This method removed triacylglycerols, free and esterified cholesterol, and approximately one-half of all the phospholipids (10, 11). A further advantage is that there is no protein loss; the pattern of protein bands of the extracted sera after electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (12) was indistinguishable from that of normal sera. In incubations of mixed populations of human leukocytes, such lipid-depleted sera resulted in an increased activity of HMG-CoA reductase (13).

We analyzed the sera for lipoprotein-cholesterol content by the micromethod of Bronzert and Brewer (14), using the Beckman Airfuge® (Beckman Instruments, Palo Alto, CA) and Beckman Cholesterol Analyzer 2 (Beckman Instruments, Fullerton, CA). Fetal bovine serum contained, per dl, 26 ± 2 mg of LDL cholesterol and 5 ± 0.5 mg of HDL cholesterol. After extraction, no cholesterol could be detected. The extraction of the horse serum was incomplete: before extraction it contained 50 ± 5 mg LDL cholesterol and 48 ± 3 mg HDL cholesterol per dl; after extraction it still contained 8 mg LDL cholesterol and 6.5 mg HDL cholesterol per dl. Thus, FGM contained, on the average, 113 μg of total cholesterol per ml, of which 63 μg were in LDL. The LDM contained approximately 15.5 μg of total cholesterol, of which 8 μg were in LDL.

Enucleation of cells

Cells were enucleated by the use of cytochalasin B (Sigma Chemical Company, St. Louis, MO) according to the method of Cavenee, Chen, and Kandutsch (15). Cultures of H4 cells grown to confluence in FGM in 25-cm^2 flasks were switched to LDM medium for 24 hr. The LDM was then removed and the cells were washed with sterile phosphate-saline. The flasks were then filled completely with Swim's S-77 medium (without serum) containing 10 μg of cytochalasin B in Me_2SO solution (0.5 μl/ml medium). The cultures were first incubated for 30 min and then centrifuged in a pre-warmed GSA rotor in a Sorvall centrifuge for 30 min at 6500 *g* at 34–35°C. Control cultures, the cells of which were not to be enucleated, were treated similarly with the cytochalasin B and were kept at 37°C until completion of the centrifugation. The media were then withdrawn from all flasks and the cytoplasts and the intact cells were washed three times with 3 ml of LDM, overlayered with 3 ml of LDM, and incubated for 1 hr at 37°C.

Cytoplasts of H4 cells appear immediately after centrifugation as small round bodies interconnected with fine filaments which, after one hour in LDM, flatten and assume an epithelioid shape. The extent of enucleation was judged after this 1-hr incubation from phase contrast photomicrographs at 200 × magnification. We usually obtained preparations that were 90–95% enucleated. Cells exposed to cytochalasin, but not centrifuged, could not be distinguished from normal cells after 1 hr recovery in fresh LDM.

CHO cells enucleated exactly as described (15) were treated with only 5 μg of cytochalasin per ml of medium and were not incubated before centrifugation. The shape of the cytoplasts of CHO cells after "recovery" in LDM for 1 hr, as described for the cytoplasts of H4 cells, resembled the tip of a lance or a narrow leaf.

Harvesting of cells and cytoplasts for analysis

H4 cells and their cytoplasts were harvested by exposing them for 30 min at 37°C to 2 ml of Ca^{2+}- and Mg^{2+}-free phosphate-saline containing 0.02% sodium EDTA. The loosened layers of cells and cytoplasts were washed into centrifuge tubes with phosphate-saline.

The cytoplasts of CHO cells were similarly treated, but had to be scraped off the substratum with a glass spatula.

Determination of cholesterol synthesis and assay of HMG-CoA reductase

For determination of cholesterol synthesis, media were removed and the cell layers, or cytoplasts, were washed gently three times with sterile phosphate-saline. Their respective media (FGM or LDM), but without either mevinolin or mevalonate that might have been present in the experimental media, were then added. The flasks were returned to the incubator for 1 hr. The purpose of this step was to eliminate the possibility of interference by residual mevinolin or mevalonate. After 1 hr the media were removed and the cell layers or cytoplasts were washed twice with basal Swim's S-77 medium (without serum). Cultures in 75-cm^2 flasks were then supplied with 10 ml of the basal S-77 medium containing 10 μCi of [1-^{14}C]acetate (sp act 50 Ci/mol; Amersham Corp., Arlington Heights, IL). Cultures in 25-cm^2 flasks were given 3 ml of the same medium containing 5 μCi of the [1-^{14}C]acetate. After 2 hr incubation at 37°C, the media were removed and the cells were washed twice with phosphate-saline, harvested with EDTA as described, and collected in tared centrifuge tubes. The cells or cytoplasts were sedimented by centrifugation and the pellets were washed three times by resuspension and sedimentation. Fluid was drained from the pellets, and the sides of centrifuge tubes were wiped with Q-tips. The pellets were then weighed. The mean weight of cell pellets from 75-cm^2 cultures was 73 ± 4 mg (n = 60) and from the 25-cm^2 cultures 22 ± 2 mg (n = 65). The pellets were dissolved in 0.5 ml of 0.5 N NaOH at 50°C for 1 hr. Portions, 100 μl, were set aside for protein determination and the remainder was supplemented with 0.1 ml of 10 N NaOH and 0.5 ml of ethanol containing 1 mg of cholesterol. The mixture was then heated at 70°C under N_2 for 1 hr. Unsaponifiable lipids were extracted and sterols were precipitated with digitonin (16). The pellets of washed digitonides were dried with a stream of N_2 at approx. 40°C and dissolved in 1 ml of absolute methanol. A portion, 0.5 ml, of the methanol solution was counted in 3a70B scintillation fluid (Research Products International, Elk Grove Village, IL) in a Packard Tri-Carb model 3320 scintillation spectrometer.

For assay of HMG-CoA reductase activity, the cell pellets were lysed in the following solution: sucrose, 0.1 M; KCl, 50 mM; KH_2PO_4, 40 mM; Na_2EDTA, 30 mM; dithiothreitol, 5 mM; KYRO EOB detergent (Procter and Gamble Co., Cincinnati, OH), 0.25%. The pH was adjusted to 7.2 with NaOH. The pellets were suspended in 0.5 ml of this solution and incubated at 37°C for 30 min with occasional stirring with a thin glass rod. The suspension of lysed cells was centrifuged at 12,000 *g* for 30 min in an Eppendorf microcentrifuge (Brinkmann Instruments Co., Westbury, NY). The clear supernatants were removed and the reductase present in 10–50 μl of the solutions (40–200 μg of protein) was assayed immediately in a 250-μl final volume for 20 min by the method of Avigan, Bhalthena, and Schreiner (17) as modified by Edwards, Lemongello, and Fogelman (18). Cell lysates were first incubated with the cofactors for 20 min at 37°C and then the reaction was started by the addition of (*R*,*S*)-[3-^{14}C]-HMG-CoA to a concentration of 100 μM. In early experiments we used an old, repurified specimen of (*R*,*S*)-[3-^{14}C]-HMG-CoA (sp act, 11 Ci/mol) (13). In later experiments we used (*R*,*S*)-[3-^{14}C]HMG-CoA (51.5 Ci/mol; New England Nuclear, Boston, MA) diluted with unlabeled HMG-CoA (Sigma, St. Louis, MO) to a specific activity of 4.5 Ci/mol. All assays were carried out in duplicate or triplicate on cells from two identical cultures.

Cultures for preparation of poly(A)$^+$RNA

Seventy-two cultures of H4 cells were initiated in 75-cm^2 flasks in FGM and grown to confluency. On the day of confluence, the cultures were divided into four groups: group *i*), 22 cultures in fresh FGM; group *ii*) 12 cultures in medium changed to LDM; group *iii*) 12 cultures in medium changed to LDM + mevinolin (1 μg/ml); and group *iv*) 22 cultures in medium changed to LDM + mevinolin (1 μg/ml) + 4 mM sodium (*R*)-mevalonate. After 24 hr the cells from each group, in batches of 10 cultures, were harvested with EDTA and collected in tared 50-ml centrifuge tubes.

Preparation of RNA

Total cellular RNA was prepared and poly(A)$^+$RNA was isolated as described by Chirgwin et al. (19). Total RNA was obtained by ultracentrifugation through a cushion of cesium chloride, and the polyadenylated RNA species were prepared from it by chromatography on oligo(dT)-cellulose, type 3 (Collaborative Research, Waltham, MA). Each cell pellet of approx. 0.5 g from the cultures described above was completely dissolved by vortex mixing with 16 ml of 4 M guanidinium thiocyanate, 0.5% sodium N-lauroylsarcosine, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol, and 0.1% Sigma Antifoam A.

Poly(A)$^+$RNA was also prepared as described by Chiappelli et al. (20) from polysomes of two batches of twenty 75-cm^2 cultures each of H4 cells. One batch was exposed for 24 hr to LDM (1.197 g of cells) and the other to LDM + mevinolin, 1 μg/ml (1.33 g of cells).

Translation of poly(A)$^+$RNA

The rabbit reticulocyte translation system as described by Clarke et al. (21) was used. A typical translation system consisted of 50 μl of reticulocyte lysate, 6 μl (approx. 6 μCi) of [^{35}S]methionine (sp act > 1000 Ci/mol; Amersham), and 20 or 40 μg of poly(A)$^+$RNA in 6 μl. This RNA concentration is within the linear range for total

protein synthesis and gave an optimal translation efficiency for the reductase polypeptide. Incorporation of [^{35}S]methionine into total protein was measured as described by Beale, Katzen, and Granner (22).

Immunoprecipitation of radiolabeled reductase polypeptide and its electrophoresis

The antiserum, raised in rabbits to rat liver HMG-CoA reductase, and used for immunoprecipitation of the labeled reductase (21), was the preparation described by Edwards et al. (23). Immunoprecipitates were analyzed by electrophoresis on 8 M urea–7.5% polyacrylamide–sodium dodecylsulfate gels, and the radioactivity associated with the reductase polypeptide was quantitated as previously described (21).

Electrophoresis of RNA and hybridization with cDNAs

Poly(A)$^+$RNAs were denatured with purified glyoxal, separated by electrophoresis on 1% agarose gels (24), and transferred to nitrocellulose paper by the method of Thomas (25). Reference poly(A)$^+$RNA, from the liver of rats in which the HMG-CoA reductase was induced to a high level by cholestyramine feeding and administration of mevinolin (26), was included in the analyses. The RNAs on the nitrocellulose paper were first hybridized with a ^{32}P-labeled cDNA, prepared from the plasmid pRed-10, constructed by Chin et al. (27) from a clone of Chinese hamster ovary cells (UT-1) rich in HMG-CoA reductase. A second hybridization was carried out with a ^{32}P-labeled mouse-actin cDNA, constructed in pUC8 by Dr. Robert LaPolla. Labeling of the cDNAs was carried out by nick-translation with [α-^{32}P]dCTP (Amersham) (cf. 28) to specific activities of about 2×10^8 cpm/μg. Radioautograms were made from the blots on Kodak XAR-5 films. Deoxyribonuclease type 1 (Sigma, St. Louis, MO) and *E. coli* DNA polymerase 1 (Bethesda Research Laboratories, Gaithersburg, MD) were used in the nick-translations. The intensities of the bands of ^{32}P-labeled hybrids were measured on the radioautographic films with a Helena Scanning Densitometer RND (Helena Labs, Beaumont, TX).

Determination of protein

Protein was measured by dye-binding with reagents supplied by Bio-Rad (Richmond, CA) using bovine serum albumin as standard.

RESULTS

Regulation of sterol synthesis in H4 cells

Regulation of sterol synthesis and of HMG-CoA reductase in H4 hepatoma cells has not previously been explored. As this is a malignantly transformed cell-line, it was essential to determine whether cholesterol synthesis and HMG-CoA reductase could be modified in these cells by varying culture conditions.

Fig. 1 summarizes the results of three sets of experiments, carried out on three different batches of cultures. Keeping the cells in lipid-depleted media caused, in 24 hr, approximately a fourfold increase in the incorporation of [^{14}C]acetate into sterols. After 48 hr this effect was further augmented. Addition of mevinolin (1 μg/ml) to full-growth media caused, in 24 hr, a two- to threefold rise in sterol synthesis from acetate but, in combination with lipid-depleted media, mevinolin caused at least a twofold increase in sterol synthesis over and above that seen in cultures kept in lipid-depleted media. These effects of mevinolin appeared only after mevinolin was removed and the cultures were incubated for 1 hr in fresh media without the inhibitor. The third set of experiments, carried out in quadruplicate, are included in Fig. 1 (the last two bars) only to indicate the exceptional reproducibility of observations within any single batch of cultures of the H4 cells.

We next asked whether the "superinduction" of sterol synthesis elicited by mevinolin could be suppressed by mevalonate or by low density lipoproteins (LDL). LDL even at the high concentration of 110 μg/ml only partially suppressed the effect of mevinolin (**Fig. 2**). By contrast,

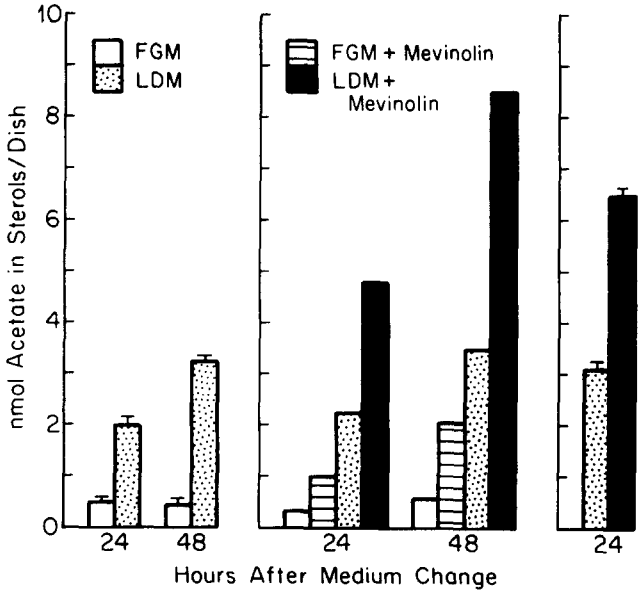

Fig. 1. Regulation of cholesterol synthesis in cultured hepatoma cells. Effects of lipid-depleted media, and of mevinolin (1 μg/ml) in combination with full-growth medium (FGM) or with lipid-depleted medium (LDM) on sterol synthesis from [^{14}C]acetate. Confluent cultures of H4 cells in 75-cm^2 flasks were given a medium change as indicated on day 0 and were analyzed 24 and 48 hr later. Mevinolin causes a "superinduction" over and above that seen with lipid-depleted media alone. Mean protein content of flasks was 6.5 ± 0.5 mg (n = 12).

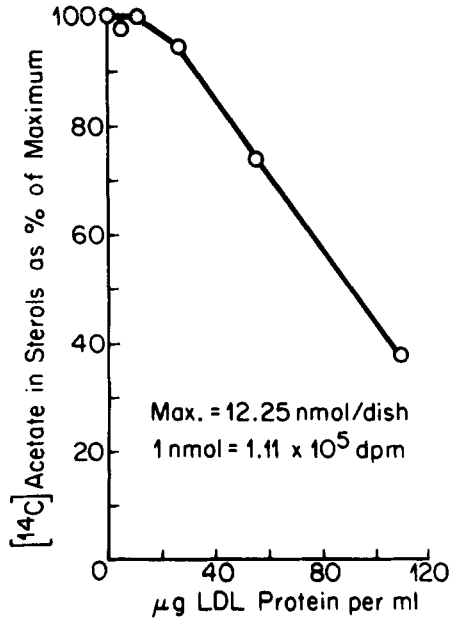

Fig. 2. Suppression of effects of mevinolin on sterol synthesis by low-density lipoprotein. Confluent cultures of H4 cells grown in 75-cm^2 flasks in full-growth medium were given, on day 0, lipid-depleted medium + mevinolin (1 μg/ml) with increasing concentrations of LDL; 24 hr later the cultures were washed with PBS and were given fresh lipid-depleted medium without mevinolin for 1 hr, then analyzed for incorporation of [^{14}C]acetate into sterols. The residual activity observed at the highest concentration of LDL (110 μg/ml), 4.7 nmol acetate incorporated into sterols, is about four times higher than that of a comparable culture kept in full-growth medium.

mevalonate completely suppressed, but only at the highest concentration (4 mM), the inductive effect of mevinolin (**Fig. 3**).

HMG-CoA reductase in the H4 hepatoma cells

We attempted to measure the reductase in microsomes of the H4 cells, but we abandoned this approach because we could not achieve quantitative disruption of the small cell pellets by homogenizing and because it was very difficult to disperse the tiny microsomal pellets in buffers. We turned, therefore, to disruption of the cells by the non-ionic detergent KYRO EOB as described in Experimental Procedures.

KYRO EOB solubilizes only the plasma membranes of the cells, leaving the subcellular particles intact (29). Brown, Dana, and Goldstein (30) have successfully used this detergent to disrupt cultured fibroblasts for assay of reductase. We found that KYRO EOB (0.25%) disrupted all the cells in pellets of H4 cells, and that the lysates could subsequently be used for the preparation of various subcellular elements by differential centrifugation. We assayed the reductase in the postmitochondrial supernatants of such lysates. When cell pellets from 25-cm^2 confluent cultures were disrupted in 0.5 ml of the lysis buffer (cf. Experimental Procedures), the postmitochondrial supernatants contained a total of 2.0–2.5 mg of protein. The assays gave values linearly proportional with protein up to at least 300 μg in 250-μl assay mixtures, and with time up to 20 min (data not shown). We determined the K_m value for the (*R*,*S*)-HMG-CoA in lysates of cells grown in FGM and of cells exposed for 24 hr to LDM. In the standard kinetic assays we used six different concentrations of (*R*,*S*)-HMG-CoA ranging from 2.46 μM to 73.8 μM at a saturating concentration (100 μM) of NADPH. The data were analyzed by Lineweaver-Burk plots and by the direct linear plot method of Eisenthal and Cornish-Bowden (31). Both methods gave, within experimental error, identical K_m values of 11.1 ± 0.2 μM for the (*R*,*S*)-HMG-CoA with extracts of cells kept in FGM and extracts of cells exposed to LDM. The only difference between the two preparations was that the extract of cells exposed to LDM gave a V_{max} 2.4 times higher than the extract of cells kept on FGM. The value of the K_m was almost precisely double that found (18) for the solubilized pure reductase from rat liver.

Table 1 shows analytical data on reductase and a comparison of the relative activities of the enzyme with the relative rates of sterol synthesis from [^{14}C]acetate under comparable conditions. While the trends in changes of reductase activity under the various conditions are similar

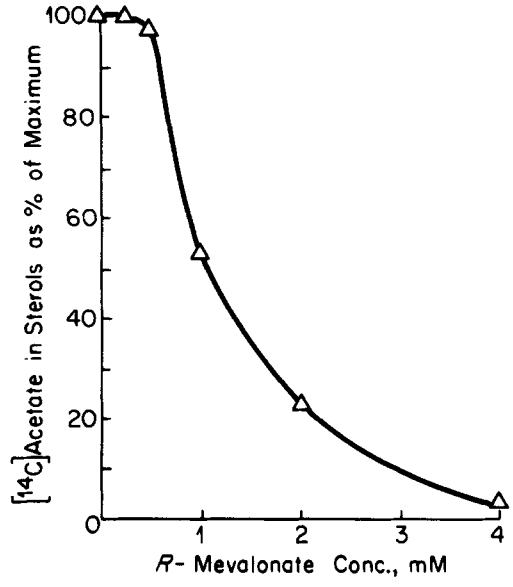

Fig. 3. Suppression of effects of mevinolin on sterol synthesis by mevalonate. Confluent cultures of H4 cells grown in full-growth medium in 75-cm^2 flasks were given, on day 0, lipid-depleted medium + mevinolin (1 μg/ml) with increasing concentrations of mevalonate; 24 hr later the cultures were washed with PBS and were given fresh lipid-depleted medium for 1 hr. They were then analyzed for incorporation of [^{14}C]acetate into sterols. The maximum incorporation was 14.49 ± 0.13 nmol per dish, or 2.54 ± 0.02 nmol/mg protein. The residual activity (0.086 nmol/mg protein) after exposure to the highest concentration of mevalonate was 38.7% of that observed in control culture kept in full-growth medium alone (1.27 nmol per dish, or 0.222 nmol/mg protein).

TABLE 1. Effects on HMG-CoA reductase in H4 cells exposed for 24 hr to various media. Comparison of the relative activities of the reductase with the relative incorporation of [^{14}C]acetate into sterols

Medium	Reductase pmol · min^{-1} · mg^{-1}	Relative Activities of the Reductase	Relative Synthesis of Sterols from [^{14}C]Acetate
Full growth	52 ± 12 (n = 12)	1.0	1.0
Full growth + mevinolin (1 μg/ml)	133.0 ± 16.0 (n = 12)	2.56 (range 2.2–2.8)	4.2 ± 1.6 (range 2.5–5.5; n = 18)
Full growth + (*R*)-mevalonate (4 mM)	28.0 ± 5.0 (n = 12)	0.54 (range 0.45–0.72)	0.31 ± 0.01 (n = 8)
Lipid-depleted	86.0 ± 7.8 (n = 12)	1.65 (range 1.4–1.9)	3.2 ± 1.5 (range 2.4–6.7; n = 18)
Lipid-depleted + mevinolin (1 μg/ml)	291 ± 29 (n = 12)	5.6 (range 5.3–6.30)	11.1 ± 1.7 (range 9.5–14.1; n = 18)
Lipid-depleted + mevinolin + (*R*)-mevalonate (4 mM)	40 ± 4.2 (n = 4)	0.77 (range 0.60–0.85)	0.045 ± 0.005 (n = 4)
Lipid-depleted + (*R*)-mevalonate (4 mM)	31.0 ± 2.1 (n = 12)	0.60 (range 0.50–0.70)	0.38 ± 0.01 (n = 4)

to those seen in changes of acetate incorporation into sterols, the magnitude of the changes seen by the two types of analysis differ significantly. Almost invariably the incorporation of [^{14}C]acetate into sterols gave a more exaggerated change on changing conditions than the reductase.

Sterol synthesis and HMG-CoA reductase in cytoplasts (enucleated cells)

If our hypothesis has any validity, mevalonate should not repress sterol synthesis from acetate or HMG-CoA reductase in enucleated cells.

We tried several methods for enucleation of H4 cells, but found the method of Cavenee et al. (15), with minor modifications (cf. Experimental Procedures), to be the most suitable. All the enucleations were carried out on confluent cultures exposed to LDM for 24 hr because we expected, on the basis of the data of Cavenee et al. (15), that the cytoplasts of H4 cells might have much lower sterol-synthesizing capacities per mg protein than the whole cells.

Before beginning the experiments, the extent of enucleation was judged by microscopic examination of each preparation after 1 hr incubation of the cells and cytoplasts in LDM to allow recovery from the effects of cytochalasin B. This was essential because, for reasons that we could not account for, various batches of cultures of H4 cells—although grown under identical conditions—responded with different degrees of enucleation. Hence we could not rely on DNA synthesis from [^{3}H]thymidine in representative preparations to assess the success of enucleation. Only preparations judged by the microscopic examination to be 90–95% enucleated were used for experiments. In successful enucleations of confluent H4 cells, one-half to two-thirds of cellular material was usually lost as judged by the weight of the cytoplast pellets and their protein content.

We first examined whether the cytoplasts of H4 cells retained the sterol-synthesizing system. Not only did the cytoplasts retain their sterol-synthesizing ability, but the values of incorporation of [^{14}C]acetate into sterols expressed per mg of protein were slightly higher in the cytoplasts than in the control nucleated cells (**Table 2**). This was not an invariable finding, although the sterol-synthesizing capacity of the H4 cytoplasts was usually within 10% of the nucleated cells.

Fig. 4 illustrates that in nucleated cells the HMG-CoA reductase was depressed by more than 75% in 4 hr by 4 mM (*R*)-mevalonate. However, in cytoplasts, after an initial suppression during the first 2 hr by about 40%, there was no further loss of activity up to 4 hr of exposure to mevalonate.

The sterol-synthesizing capacity of cytoplasts was similarly very little affected by mevalonate. In the experiment illustrated in **Fig. 5**, we included control cytoplasts incubated in LDM without mevalonate and show that the sterol-synthesizing ability of the cytoplasts does not decay spontaneously. The mean incorporation of [^{14}C]acetate into sterols over the 4-hr experiment in the cytoplasts exposed to mevalonate was 129.7 ± 12.8 pmol · mg^{-1} · hr^{-1} and in the control cytoplasts, 123.5 ± 11.5 pmol · mg^{-1} · hr^{-1}. In the nucleated cells, sterol synthesis was depressed from an initial 118 to 48 pmol · mg^{-1} · hr^{-1} by exposure to mevalonate.

TABLE 2. Sterol synthesis from [1-^{14}C]acetate in cytoplasts of H4 cells and their nucleated controls at 37°C in 1 hr

Preparation		Weight of Cell Pellet	Protein per Dish	Acetate in Sterols[a] per Dish	Acetate in Sterols[a] per mg Protein
		mg	*mg*	*pmol*	*pmol*
#86	Enucleated	8.8	0.83	237	285
#87	Enucleated	11.2	1.07	297	278
#88	Nucleated[b]	23.0	1.60	392	244
#89	Nucleated[b]	22.9	1.71	382	223

[a] One pmol = 111 dpm.
[b] Exposed to cytochalasin B for 1 hr (but not centrifuged) and then to lipid-depleted medium for 1 hr before addition of [^{14}C]acetate in Swim's S-77 medium containing no serum. The enucleated preparations (cytoplasts) were similarly treated after centrifugation.

To examine whether the diminished suppression of reductase and sterol synthesis by mevalonate in cytoplasts was applicable to cytoplasts of cells other than those of H4 cells, we carried out experiments with Chinese hamster ovary (CHO) cells. Cavenee et al. (15) demonstrated that oxygenated sterols had little effect on reductase or sterol synthesis in the cytoplasts of CHO cells, but depressed these activities in the nucleated cells.

The enucleation of CHO cells differs from the enucleation of H4 cells in that complete enucleation is obtained, but at the expense of very large losses of cellular material. For this reason we had to combine cytoplasts from two flasks in order to have sufficient material for duplicate or

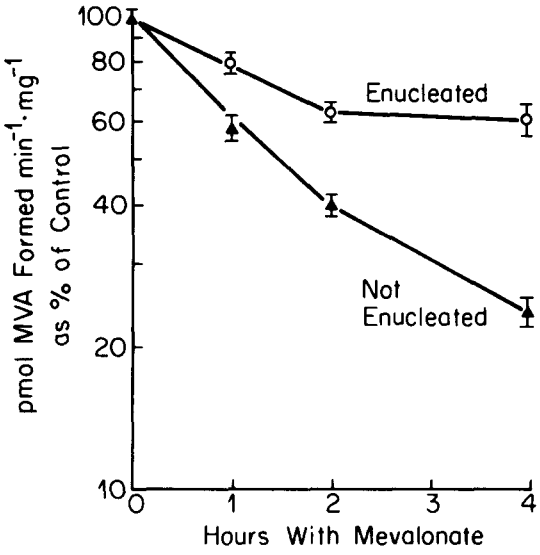

Fig. 4. Effects of mevalonate on HMG-CoA reductase in cytoplasts and nucleated H4 cells. Confluent H4 cells were given LDM for 24 hr, then were treated with cytochalasin B (10 μg/ml). One-half of the flasks were centrifuged for enucleation of the cells. After enucleation and removal of cytochalasin-containing medium, the enucleated cells (cytoplasts) and the nucleated cells (treated with cytochalasin but not centrifuged) were given fresh lipid-depleted medium for 1 hr. Control cells were harvested for assay of reductase; others were given fresh lipid-depleted medium + 4 mM (*R*)-mevalonate, harvested 1, 2, and 4 hr later, and assayed for the reductase.

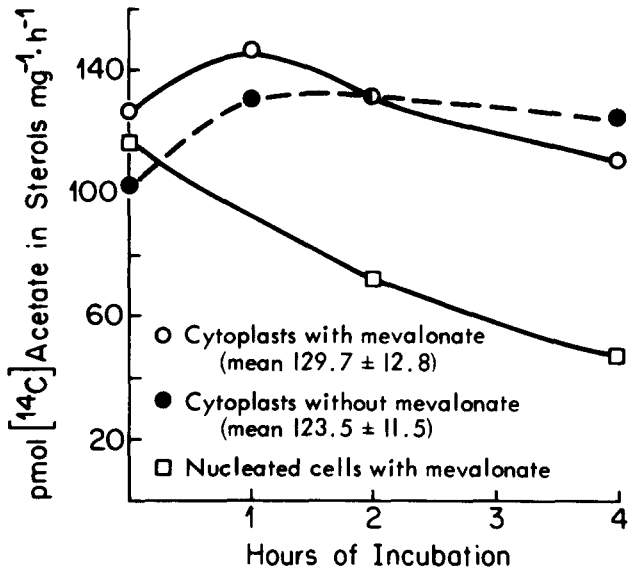

Fig. 5. Lack of suppression of sterol synthesis by mevalonate in cytoplasts. The experiment is identical to that shown in Fig. 4 except that, after the 1-hr "recovery" period in lipid-depleted medium, the cytoplasts and nucleated cells were tested for sterol synthesis from [^{14}C]acetate. Control cytoplasts were also incubated in lipid-depleted media but without mevalonate.

triplicate assays of reductase and protein. The cytoplasts of CHO cells also differ from the cytoplasts of H4 cells in that they are much smaller and cannot be dislodged from the substratum by EDTA alone; they have to be scraped off.

Because we used culture media for the CHO cells different from those used by other workers, we examined how HMG-CoA reductase reacted to a change of medium from FGM to LDM. We found invariably (n = 12) that after a 24-hr exposure to LDM the level of reductase rose more than fourfold (× 4.34 ± 0.08) above that seen in cells kept in FGM. The mean basal level of the reductase (from cells in FGM) in eight cultures was 157 ± 13 pmol · min^{-1} · mg^{-1}; only four cultures had values as low as 102 ± 12 pmol · min^{-1} · mg^{-1}.

Fig. 6 represents three identical experiments. We found, in confirmation of the observations of Cavenee et al. (15), that—in contrast to the behavior of the H4 cells—the cytoplasts of CHO cells had much lower levels of reductase than the intact nucleated cells. Mevalonate (4 mM) had no effect on the reductase in the cytoplasts of CHO cells, whereas in the nucleated cells (treated with cytochalasin B but not centrifuged) it reduced the activity of the enzyme from an initial value of 600 pmol · min^{-1} · mg^{-1} to 120 pmol · min^{-1} · mg^{-1} over a period of 4 hr.

Poly(A)$^+$RNA in H4 cells

We next examined whether the various treatments of H4 cells that affected HMG-CoA reductase and sterol

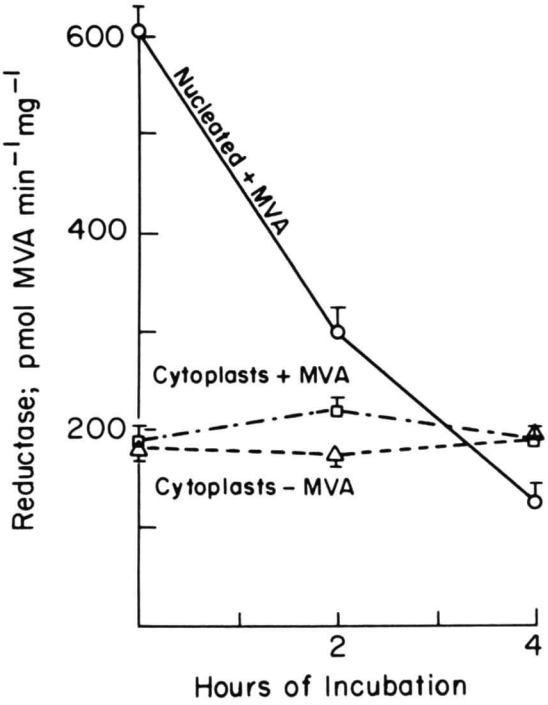

Fig. 6. Lack of effect of mevalonate on HMG-CoA reductase in cytoplasts of CHO cells. The experimental conditions were the same as with the H4 cells and their cytoplasts shown in Fig. 5. Control cytoplasts were incubated in lipid-depleted media without mevalonate. Note the much lower initial activity of the reductase in the cytoplasts than in the nucleated cells.

synthesis also affected the mRNA specific for the reductase. Poly(A)$^+$RNAs were isolated from the large cultures described under Experimental Procedures and were translated to protein in a rabbit reticulocyte protein-synthesizing system with [^{35}S]methionine as marker. The total translation products were immunoprecipitated and analyzed by 8 M urea/NaDodSO$_4$ polyacrylamide gel electrophoresis and fluorography of the gels. Lanes 1 and 2 of **Fig. 7** show that the antiserum specifically precipitated a 97,000-dalton polypeptide, now known to be that of the reductase (27, 21), apart from some minor components precipitated also by the preimmune serum (lane 3). By quantitation of the radioactivity of the reductase polypeptide as described (21), we calculated that the reductase-specific mRNA represented 0.0017 ± 0.00004% of the total translatable mRNA in H4 cells grown in LDM, and increased approximately threefold to 0.0047 ± 0.00025% of the total mRNA in cells exposed to LDM and mevinolin (1 μg/ml) for 24 hr. Similar results were obtained with the two poly(A)$^+$RNA preparations made from polysomes of H4 cells exposed for 24 hr to LDM, and to LDM and mevinolin (cf. Experimental Procedures). mRNA isolated from H4 cells exposed to lipid-depleted media and mevinolin (1 μg/ml) and 4 mM (*R*)-mevalonate, or from cells kept in full-growth media were similarly analyzed. Under the latter conditions 0.0013 ± 0.00048% and 0.0013 ± 0.00095% of the total translation products, respectively, were found in the 97,000 Da reductase polypeptide. Thus the addition of mevalonate to media containing mevinolin abolished the threefold increase in reductase mRNA.

The poly(A)$^+$RNAs (5 μg each) prepared from six batches of differently treated H4 cells (described in Experimental Procedures) and coded as FGM$_1$, FGM$_2$, MVA$_1$, MVA$_2$, LDM, and Mev, were also hybridized first with a ^{32}P-labeled cDNA of the reductase mRNA and then with a ^{32}P-labeled cDNA specific for actin. The hybridization with the reductase-specific cDNA gave only two bands corresponding to 4.6 and 4.2 K-bases and the hybridization with the mouse actin cDNA gave a single band of 2.1 K-bases. We assumed that the actin mRNAs represented transcriptions of a structural gene not influenced by the changes of culture media of the H4 cells. Hence we expressed the densities of the hybridized reductase-mRNAs as a percentage of the respective densities of the hybridized actin-mRNA and calculated the intensities of the hybridized reductase-mRNAs relative to the mean of the FGM$_1$ + FGM$_2$ bands (**Table 3**). The data show that the reductase-specific mRNA is increased both in LDM-treated cells, and also particularly in cells exposed to LDM + mevinolin, and that mevalonate suppresses completely the inductive effect of mevinolin.

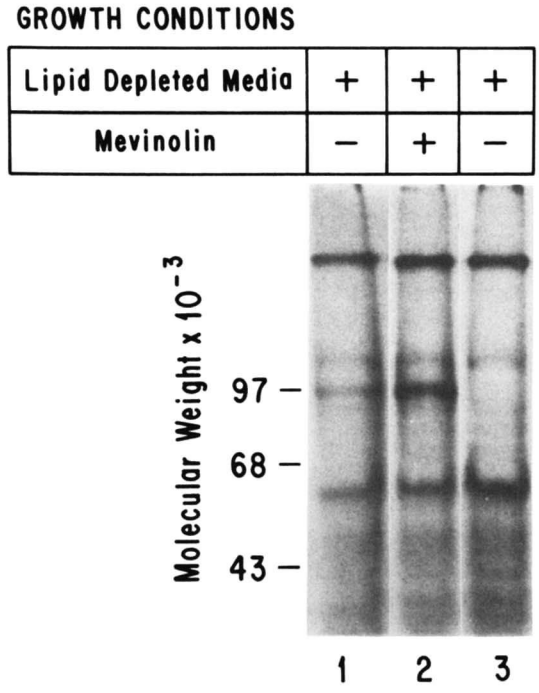

Fig. 7. Photographs of fluorograms made from polyacrylamide gels after electrophoresis of translation products of poly(A)$^+$RNAs from H4 cells. Lanes 1 and 2 are from immunoprecipitation with antiserum to HMG-CoA reductase and lane 3 is from precipitation of total translation products with preimmune serum. Identical numbers of radioactive counts of total translation products were used in the precipitations.

TABLE 3. Peak areas of HMG-CoA reductase-mRNA and actin-mRNA hybridized with their respective [^{32}P]cDNAs measured by densitometry on radioautographic films

	Peak Areas in Arbitrary Units					
mRNA preparation[a]	LDM	Mev	MVA_1	MVA_2	FGM_1	FGM_2
Reductase 4.6 + 4.2 K-base bands (from film exposed for 8 hr)	53.09	81.34	23.33	14.49	34.30	29.43
Actin bands 2.1 K-base	90.30	96.92	64.43	72.56	94.31	100.50
Reductase mRNA bands as % of respective actin band	58.79	83.92	36.21	19.97	36.26	29.28
Peak areas of reductase mRNAs relative to mean (32.77) of FGM_1 + FGM_2	1.79	2.56	1.11	0.61	1.00	

[a]The notation for the RNA preparation is the same as that given in Experimental Procedures where the cultures for the preparation of the RNAs are described.

DISCUSSION

The data demonstrate that sterol synthesis and HMG-CoA reductase are regulated in the transformed H4 hepatoma cell-line just as they are in a variety of non-transformed cultured cells. Contrary to previous thoughts that cholesterol synthesis and HMG-CoA reductase are resistant to regulation in transformed tumor-producing cells (32), Watson (33) and Kirsten and Watson (34) have shown that these processes respond in cultured HTC cells—also a transformed cell-line—to lipoproteins, or to their absence, just as they do in the more commonly used fibroblasts. Ours is another demonstration that absence of regulation of HMG-CoA reductase is not an inherent property of cultured malignantly transformed cells.

We conclude that our hypothesis that mevalonate provides a repressor of the transcription of the HMG-CoA reductase gene has been proved by the following observations. *A*) Cells largely deprived of mevalonate, by the inhibitory action of mevinolin on HMG-CoA reductase, respond with an induction of the reductase. *B*) Mevalonate completely counteracted the inductive effect of mevinolin. *C*) Mevalonate was ineffective in repressing sterol synthesis or HMG-CoA reductase in cytoplasts, i.e., in the absence of the cell nucleus. The small effects of mevalonate seen in the cytoplasts of the H4 cells are very similar to those observed by Cavenee et al. (15) in cytoplasts of CHO cells treated with 25-hydroxycholesterol, and can be attributed to the incomplete enucleation of the cells. We have never achieved complete enucleation of the H4 cells. On the other hand, we never saw nucleated cells among the cytoplasts of CHO cells, and, in these, mevalonate had no effect whatever on HMG-CoA reductase. *D*) Treatment of H4 cells with mevinolin in LDM resulted in an increase of the functional reductase-specific mRNA in the H4 cells and this effect was completely abolished by mevalonate.

It is noteworthy that high concentrations (>1 mM) of mevalonate are needed for the suppression of the effects of mevinolin. Faust, Brown, and Goldstein (35) observed that human fibroblasts treated with compactin converted [^{3}H]mevalonate at low concentrations (<100 μM) and in the presence of LDL into isopentenyl tRNA and ubiquinone, but not into cholesterol. We have also noted that confluent H4 cells (25-cm^2 culture) in FGM (in the absence of compactin or mevinolin) converted (*R*)-[2-^{14}C]-mevalonate at low concentration (8.5 μM) only into minute amounts of cholesterol (43.4 ng/5 hr); but at high concentration of mevalonate (2.3 mM), an identical culture synthesized as much as 13.5 μg cholesterol in 5 hr.

Quantitative comparison of changes in HMG-CoA reductase levels in H4 cells and their ability to convert [^{14}C]acetate into sterols (cf. Table 1) reveals substantial discrepancies. The most notable differences were in the much higher relative rates of sterol synthesis from acetate in mevinolin-treated H4 cells as compared to the changes in reductase levels. Also, incorporation of acetate into sterols was more depressed than the reductase in cultures treated for 24 hr with mevalonate: a difference particularly striking in the instance of cultures exposed to LDM + mevinolin + mevalonate. HMG-CoA reductase is generally regarded as the rate-limiting enzyme of sterol synthesis and it is widely held that acetate incorporation into sterols parallels the reductase activities (30, 36–38). These differences may be attributed to technical inadequacies in estimating sterol synthesis from [^{14}C]acetate and assaying the reductase, particularly if the increases in the levels of the enzyme had been underestimated. However, there may be a physiologic explanation for the differences noted. Bergstrom et al. (39) have found recently that two enzymes preceding the reductase, acetoacetyl-CoA synthetase and HMG-CoA synthetase, are coordinately regulated in rat liver by mevalonate and mevinolin in a manner similar to the regulation of the reductase. Thus, induction of these enzymes by mevinolin would facilitate the availability of [^{14}C]HMG-CoA from [^{14}C]-acetate, but their suppression by mevalonate would have the opposite effect and reduce the synthesis of sterols from

[^{14}C]acetate. It is also known that cultured cells transferred from lipoprotein-containing media to media free of lipids or depleted in lipids respond not only with an increase in HMG-CoA reductase, but also with an increased activity of acetyl-CoA synthetase, acetoacetyl-CoA thiolase, HMG-CoA synthetase, and mevalonate kinase (for review cf. 40).

In the experiments of the translation of poly(A)$^+$RNAs isolated from H4 cells treated in various ways, we found a 2.8- to 3.6-fold increase in the translation of the mRNA from H4 cells exposed to LDM + mevinolin to a 97,000-dalton polypeptide precipitated by an antiserum specific against rat-liver HMG-CoA reductase. In these translation experiments, the mRNAs specific for the reductase, derived from cells other than those exposed to LDM + mevinolin, could not be distinguished from one another. However, under all these conditions, the reductase-specific mRNA is a very small proportion of the total translatable poly(A)$^+$RNA (approximately 1 in 10^5 molecules) and this may preclude an accurate measurement of changes in functional mRNA levels. On the other hand, the hybridization experiments with the ^{32}P-labeled cDNAs gave relative values for the reductase-specific RNAs not too different from the relative activities of the reductase in the H4 cells under the various conditions (cf. Tables 1 and 3). It is worth noting that the hybridization of the mRNAs from the H4 cells under all conditions gave the same two bands of 4.6 and 4.2 K-bases as were found by Chin et al. (27) in the poly(A)$^+$RNAs of UT-1 cells and by Clarke, Fogelman, and Edwards (41) in rat liver poly(A)$^+$RNA.

HMG-CoA reductase is regulated in a variety of ways, e.g., by phosphorylation and dephosphorylation (cf. 42), by specific uptake of LDL by cells (cf. 43), and by oxygenated sterols (cf. 44). To this list we need to add mevalonate which, from our evidence, provides a repressor of the transcription of the HMG-CoA gene, and for whose action the cell nucleus is essential. Luskey et al. (45) have shown also that mevalonate repressed the HMG-CoA reductase mRNA even in the UT-1 cells which contain a much amplified reductase gene. Mevalonate seems also to have another effect as Edwards, Lan, and Fogelman (46) have shown that mevalonate decreases the stability of the reductase and suggest that some product derived from mevalonate is responsible for the decreased stability. Since the reductase is stable in cytoplasts, one might speculate that even the factor increasing the degradation of the enzyme in response to mevalonate is produced by the interaction of some mevalonate derivative with the cell nucleus.

The nature of the repressor of the transcription of the reductase gene derived from mevalonate is as yet uncertain and is the subject of our current investigations.

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