Determination of mRNA levels of cholesterol biosynthesis enzymes and LDL receptor using ribonuclease protection assay

Teruhiko Shimokawa,[1],* Yoshiki Kawabe,*, Makoto Honda,*,† Yoshio Yazaki,* Akiyo Matsumoto,** Hiroshige Itakura,** and Tatsuhiko Kodama***

The Third Department of Internal Medicine* and Department of Psychiatry,† University of Tokyo, Tokyo, Japan, and National Institute of Health and Nutrition,** Tokyo, Japan

Abstract We designed a rapid method for determining mRNA content of cholesterol biosynthesis enzymes and LDL receptor (LDLR) using a ribonuclease protection assay (RPA). ^{32}P-labeled cRNA fragments for genes of human LDLR and the enzymes HMG-CoA synthase (HMGS), HMG-CoA reductase (HMGR), mevalonate kinase (MK), farnesyl pyrophosphate synthase (FPPS), and squalene synthase (SQS) were prepared by in vitro transcription. Total RNA prepared from HepG2 cells was hybridized with the cRNA probe and the hybridized mRNA was determined under protection from RNase digestion. Probe content used in this assay was excess in determining the desired mRNA in total RNA, and surplus probes were completely digested using RNase under standard conditions. When cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), mRNA levels of FPPS, SQS, and LDLR were about 4- to 7-fold higher than those of HMGS, HMGR, and MK. On incubation with DMEM supplemented with 10% lipoprotein-deficient serum (LPDS) for 8 h, all messenger RNA levels increased 1.5- to 3.5-fold. In addition, when the HMG-CoA reductase inhibitor compactin was added to 10% LPDS-DMEM, these levels increased even further and the change in mRNA level seemed to differ between the enzymes and LDLR. From these results, we conclude that RPA is a useful method for determining the very small amount of mRNA level of cholesterol biosynthesis enzymes and LDLR in the cell.—**Shimokawa, T., Y. Kawabe, M. Honda, Y. Yazaki, A. Matsumoto, H. Itakura, and T. Kodama.** Determination of mRNA levels of cholesterol biosynthesis enzymes and LDL receptor using ribonuclease protection assay. *J. Lipid Res.* 1995. **36:** 1919–1924

Supplementary key words HMG-CoA synthase • HMG-CoA reductase • mevalonate kinase • farnesyl pyrophosphate synthase • squalene synthase • compactin • HepG2 cells

Cholesterol homeostasis in mammalian cells is maintained by complex biological regulation, primarily in the liver. Cellular cholesterol content in hepatocytes is controlled by strict regulation of the uptake of exogenous cholesterol through the LDL receptor (LDLR), the synthesis of endogenous cholesterol, and the conversion of cholesterol to bile acids or cholesteryl esters (1, 2). When cells need new cholesterol, they increase both their uptake of exogenous cholesterol through the LDLR and increase endogenous cholesterol biosynthesis (3–5). Both LDLR and cholesterol biosynthesis enzymes are known to be transcriptionally and post-transcriptionally regulated by sterols and nonsterols (5–7).

cDNAs for LDLR (8) and cholesterol biosynthesis enzymes, including HMG-CoA synthase (HMGS) (9), HMG-CoA reductase (HMGR) (10), mevalonate kinase (MK) (11), farnesyl pyrophosphate synthase (FPPS) (12), and squalene synthase (SQS) (13) from human cells have been cloned and sequenced. Information so gained enabled us to examine the mRNA levels of these genes using a ribonuclease protection assay (RPA), a quick and extremely sensitive technique for quantitative analysis of mRNA (14).

DNA transfection studies on the promoter region of LDLR and HMGS genes have shown that sterol regulatory element-1 (SRE-1) may be involved in transcriptional regulation in the presence or absence of cholesterol (7, 15, 16). In recent studies, SRE-1-binding proteins (SREBPs) were also isolated and their cDNA was sequenced (16, 17). We have previously reported

Abbreviations: LDL, low density lipoprotein; FCS, fetal calf serum; LPDS, lipoprotein-deficient serum; RPA, ribonuclease protection assay; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; HMGS, HMG-CoA synthase; MK, mevalonate kinase; FPPS, farnesyl pyrophosphate synthase; SQS, squalene synthase; GAPHD, glyceraldehyde-3-phosphate dehydrogenase; SREBP, sterol regulatory element binding protein.

[1]To whom correspondence should be addressed at present address: Cardiovascular and Atherosclerosis Research Laboratories, Tsukuba Research Center, Yamanouchi Pharmaceutical Co. Ltd., 21 Miyukigaoka, Tsukuba City, Ibaraki 305, Japan.

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TABLE 1. Summary of primer pairs used to make cDNA as a template of riboprobe preparation

Probes	Primer (Forward)	Primer (Backward)	Size[a] (nt)
HMGS	5′-^{576}TATGCCCTGGTAGTTGCAGGAG^{597}-3′	5′-^{867}TTGCCTCTTTCTGCCACTGG^{848}-3′	292
HMGR	5′-^{546}TACCATGTCAGGGGTACGTC^{565}-3′	5′-^{792}CAAGCCTAGAGACATAATCATC^{771}-3′	247
MK	5′-^{139}TCATGGAGAACATGCCGTGG^{158}-3′	5′-^{383}GCAAGCCTGCAACCTCCTTTAG^{362}-3′	245
FPPS	5′-^{1081}AAAATTGGCACTGACATCCAGG^{1103}-3′	5′-^{1317}GGGTGCTGCGTACTGTTCAATG^{1296}-3′	237
SQS	5′-^{2}TGGAGTTCGTGAAATGCCTTC^{22}-3′	5′-^{331}ACCGCCAGTCTGGTTGGTAAAG^{310}-3′	330
LDLR	5′-^{1218}GCACGAGGTCAGGAAGATG^{1236}-3′	5′-^{1515}GCCCTTGGTATCCGCAACAG^{1496}-3′	298
GAPDH	5′-^{963}TGCCCTCAACGACCACTTTG^{982}-3′	5′-^{1245}GATGGTACATGACAAGGTGCGG^{1224}-3′	283

Superscripts indicate the nucleotide number of the cDNA for human enzymes and receptor. Nucleotide #1 is the A of the ATG codon that encodes the initiator methionine in each cDNA. Each primer pair and total RNA prepared from HepG2 cells was mixed and the cDNA[a] fragment was amplified by RT-PCR. The desired fragment was inserted into pCR™II vector and the linearized plasmid was used for [^{32}P]cRNA riboprobe preparation as described under Methods. HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; MK, mevalonate kinase; FPPS, farnesyl pyrophosphate synthase; SQS, squalene synthase; LDLR, low density lipoprotein receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
[a]Size protected from RNase.

that mRNA levels for these SREBPs (-1a, -1b, -1c, -2) are under sterol-mediated regulation by using RPA (18, 19).

In this paper, we describe a quick and sensitive method RPA for the determination of mRNA levels for cholesterol biosynthesis enzymes and LDLR and their cellular response to cholesterol depletion or supplementation in HepG2 cells.

MATERIALS AND METHODS

Materials

The human hepatoma cell line HepG2 was purchased from American Type Culture Collection (Rockville, MD). The TA cloning kit was purchased from Invitrogen (San Diego, CA) and the GeneAmp RNA PCR kit from Perkin-Elmer (Norwalk, CT). [α-^{32}P]UTP was from Amersham (Tokyo, Japan), and the Ribonuclease Protection Assay kit (RPA II) was from Ambion (Austin, TX). The HMG-CoA reductase inhibitor compactin was a generous gift from Dr. Akira Endo (Tokyo Noko University, Tokyo, Japan). All other reagents were commercially obtained from standard sources.

Cell culture

HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine calf serum (FCS). They were plated at a density of 5×10^5 cells per well in 6-well plates and incubated for 72 h in the same medium. At this time, mRNA content in the cells (0 h) was determined, or the medium was replaced with fresh medium supplemented with 10% lipoprotein-deficient serum (10% LPDS-DMEM) with or without compactin and the incubation was continued for the indicated times. For control cells, 10% FCS-DMEM was replaced with new 10% FCS-DMEM at 0 h and the cells were incubated for the indicated time. At each time point, the medium was aspirated, mixed with 1 ml ISOGEN (Nippon Gene, Toyama, Japan), and total RNA was isolated according to the manufacturer's protocol.

Riboprobe preparation

Plasmid constructs used to make ^{32}P-labeled probes were prepared by cloning a cDNA fragment into a pCR™II vector (Invitrogen) (18). **Table 1** shows the list of primer pairs used to make the cDNA fragment for each enzyme. The desired cDNA fragments were obtained by reverse transcription and polymerase chain reaction (RT-PCR) using a GeneAmp RNA PCR kit (Perkin-Elmer). cDNA fragments ligated into pCR™II were sequenced using the dideoxy method. The plasmid constructs were then amplified and linearized with HindIII or XbaI to make cRNA by in vitro transcription with [α-^{32}P]UTP and T7 or SP6 RNA polymerase using a Riboprobe Gemini II kit (Promega, Madison, WI). The labeled transcripts were isolated by phenol–chloroform–isoamyl alcohol extraction and used for RNA hybridization.

Ribonuclease protection assay (RPA)

RPA was performed according to the manufacturer's protocol (RPA™II kit, Ambion) (14). In Fig. 1, we used increasing amounts of total RNA (10, 20, 40 μg) in hybridization buffer to check whether the amounts of probes were in excess. In another experiment, 5–10 μg of total RNA sample was mixed with ^{32}P-labeled riboprobe in the hybridization buffer. For efficient detection of mRNA content, a mixed probe of two kinds of riboprobe of different sizes (e.g., LDLR + HMGR,

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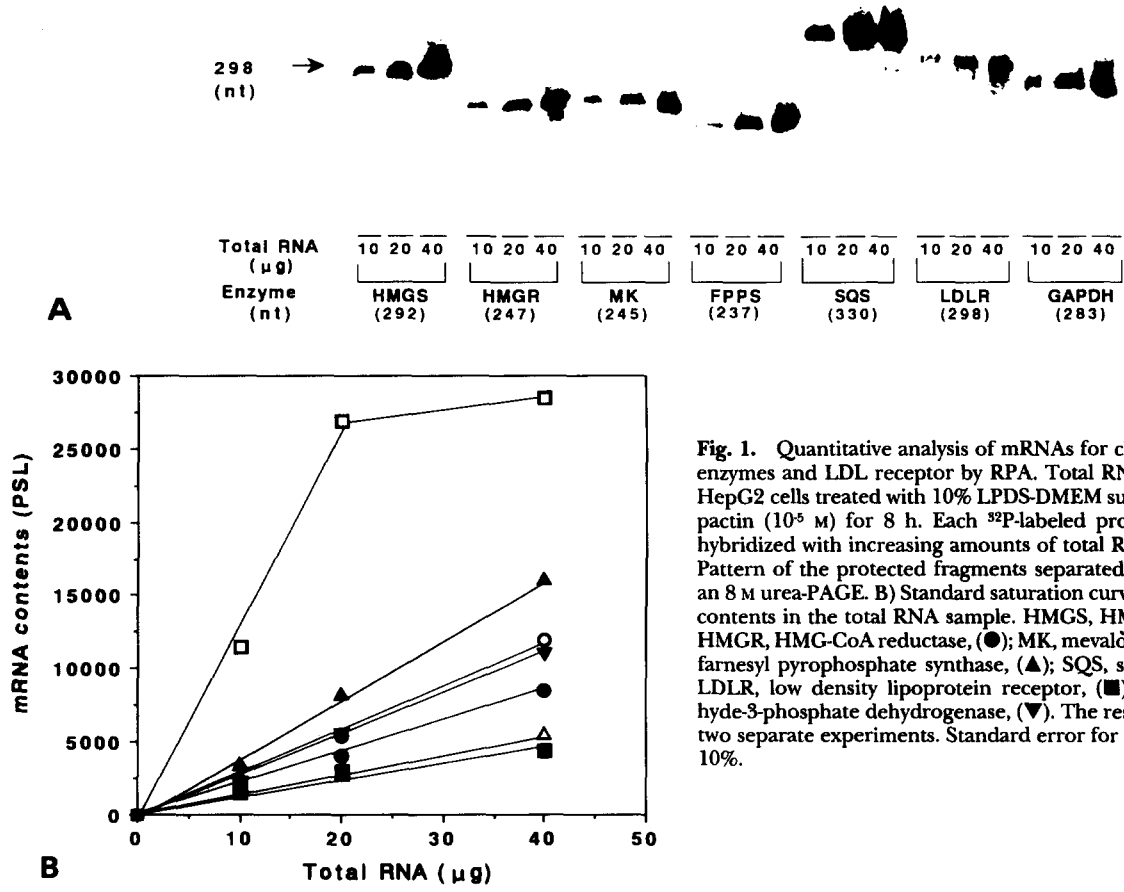

Fig. 1. Quantitative analysis of mRNAs for cholesterol biosynthesis enzymes and LDL receptor by RPA. Total RNA was prepared from HepG2 cells treated with 10% LPDS-DMEM supplemented with compactin (10^{-5} M) for 8 h. Each ^{32}P-labeled probe was prepared and hybridized with increasing amounts of total RNA (10, 20, 40 μg). A) Pattern of the protected fragments separated by electrophoresis on an 8 M urea-PAGE. B) Standard saturation curve of protecting mRNA contents in the total RNA sample. HMGS, HMG-CoA synthase, (○); HMGR, HMG-CoA reductase, (●); MK, mevalonate kinase, (△); FPPS, farnesyl pyrophosphate synthase, (▲); SQS, squalene synthase, (□); LDLR, low density lipoprotein receptor, (■); GAPDH, glyceraldehyde-3-phosphate dehydrogenase, (▼). The results are the average of two separate experiments. Standard error for each datum was within 10%.

HMGS + MK, SQS + or FPPS + GAPDH) was used. The mixture was denatured at 95°C for 5 min and then hybridized at 55°C overnight in the hybridization buffer. The samples were then incubated with RNase solution (1/100 dilution mixture of an equal volume of RNase A (250 units/ml) and RNase T1 (15,000 units/ml)) at 37°C for 30 min and the protected fragment was precipitated, dissolved in 7 μl of gel loading buffer, and analyzed by electrophoresis on a 3.5% polyacrylamide gel containing 8 M urea (urea-PAGE). The gels were dried at 80°C and exposed to an imaging plate (Fuji Film, Tokyo, Japan) for 3 h. mRNA levels were determined by estimation of the photostimulated luminescence (PSL) of the corresponding band with an imaging analyzer (BAS2000, Fuji Film). The data obtained were normalized for GAPDH content and uridine content in each probe. The results were calculated as a percentage of control value and are shown in Fig. 3 and Fig. 4.

RESULTS AND DISCUSSION

The ribonuclease protection assay (RPA) used here is quicker and more sensitive than Northern blot analysis (14). Each primer pair (listed in Table 1) was prepared and the desired cDNA fragment was amplified by PCR with HepG2 total RNA as a template. Plasmids containing the cDNA fragments were then constructed and the [^{32}P]cRNA probes were synthesized using the linearized plasmid by in vitro transcription. The radiolabeled cRNA probe was hybridized with total RNA prepared from HepG2 cells. The intensity of photostimulated luminescence (PSL) of the band proportional to the absorbed radiation energy on the imaging plate was normalized with uridine content in the probe.

To examine whether the hybridization of the cRNA probe and the target mRNA in total RNA had excess probe, we used increasing amounts of total RNA prepared from HepG2 cells under cholesterol depletion. **Figure 1A** shows the pattern of each band corresponding to the desired mRNA. We used a 1/100 dilution solution of RNase mixture (RNase A plus RNase T1) as recommended in the manufacturer's protocol and obtained good digestion of remaining excess probe after hybridization. Figure 1B shows the saturation curve plotting the intensity of PSL of each band for an increasing amount of total RNA. SQS mRNA level showed a linear increase up to 20 μg of total RNA under this condition. In contrast, other mRNAs increased propor-

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tionally to total RNA up to 40 μg. The results show that the probe content used in this assay was sufficient to quantitate each target mRNA for cholesterol biosynthesis enzymes and LDLR at least up to 20 μg of total RNA prepared under cholesterol depletion. When total RNA prepared from HepG2 cells cultured in 10% FCS-DMEM was used, all of these mRNA contents including that of SQS mRNA showed a proportional increase against total RNA content used in this assay up to 40 μg (data not shown). From these results, 5–10 μg of total RNA prepared from HepG2 cells to hybridize with each constructed cRNA probe was enough and this quantity was used for quantification for further experiments.

Using this assay method, we examined the accumulation of mRNAs for each cholesterol biosynthesis enzyme and LDLR in HepG2 cells under exogenous cholesterol supplementation. **Figure 2** shows the relative mRNA levels of cholesterol biosynthesis enzymes and LDLR in HepG2 cells cultured in 10% FCS-DMEM. The cells were seeded at the density of 5×10^5 cells per well in 6-well plates and incubated for 72 h in 10% FCS-DMEM. Under these conditions, the mRNA contents of HMG-CoA synthase (HMGS), HMG-CoA reductase (HMGR), and mevalonate kinase (MK) were relatively lower than those of farnesyl pyrophosphate synthase (FPPS), squalene synthase (SQS), and LDLR. These findings raise the possibility that enzymes upstream of the cholesterol biosynthesis pathway such as HMGS, HMGR, and MK are relatively suppressed at the mRNA level in the presence of cholesterol in the medium, although other explanations such as differences in basic promoter strength or mRNA stability are also possible.

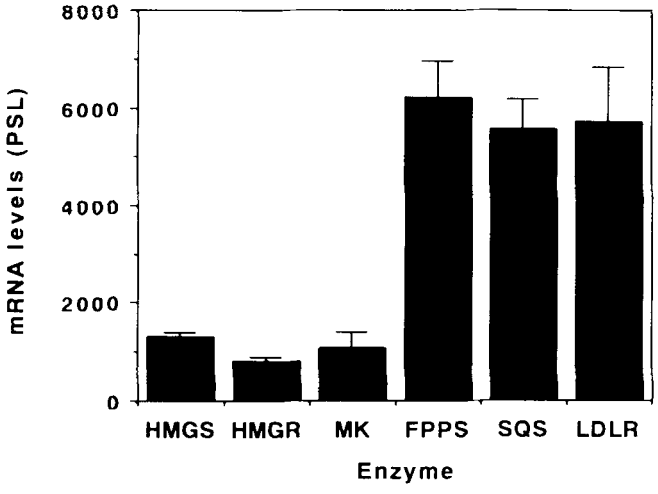

Fig. 2. mRNA levels of cholesterol biosynthesis enzymes and LDL receptor expressed in HepG2 cells cultured in DMEM supplemented with 10% FCS. HepG2 cells were plated at a density of 5×10^5 cells per well in 6-well plates and incubated for 72 h. Total RNA was isolated and individual mRNA levels were measured using RPA. PSL values were normalized by uridine content in the probe. The results are the average of three separate experiments. Enzyme abbreviations as in Fig. 1.

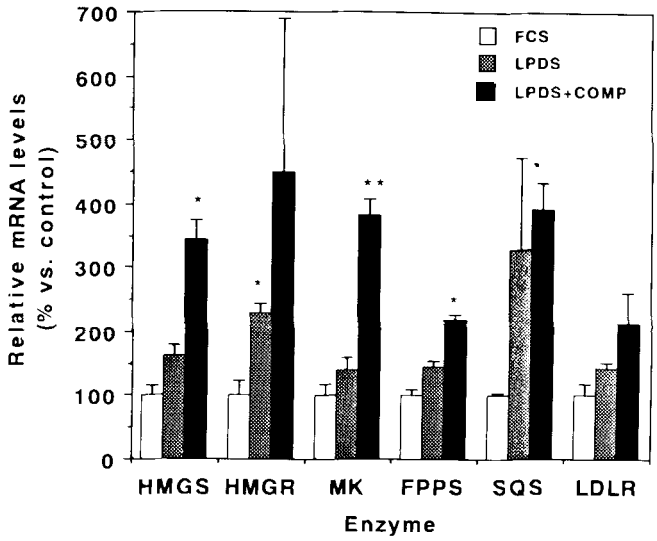

Fig. 3. Effect of DMEM supplemented with 10% LPDS or 10% LPDS plus the HMG-CoA reductase inhibitor compactin on mRNA levels of cholesterol biosynthesis enzymes and LDL receptor. HepG2 cells were incubated in 10% FCS-DMEM (FCS) or 10% LPDS-DMEM (LPDS) with or without compactin for 8 h. The total RNA was then isolated and the content of each mRNA was determined using RPA as described in Materials and Methods. PSL values were normalized by correcting GAPDH content with uridine content in the probe. Data are expressed as percentage versus the control value (10% FCS-DMEM treatment). Compactin (COMP) was added to the medium at the concentration of 10^{-6} M. Enzyme abbreviations as in Fig. 1. Statistical significance between control and treatment: *, $P < 0.02$; **, $P < 0.01$.

To examine the effect of exogenous cholesterol depletion on mRNA levels of cholesterol biosynthesis enzymes and LDLR, the medium was replaced with 10% LPDS-DMEM and incubation was continued for 8 h. As shown in **Fig. 3,** all mRNA levels increased about 1.5- to 3.5-fold under exogenous cholesterol depletion. Moreover, addition of the HMG-CoA reductase inhibitor compactin at a concentration of 10^{-6} M to the 10% LPDS-DMEM increased mRNA levels of all enzymes even further, this increase being much greater for HMGS, HMGR, and MK than FPPS, SQS, and LDLR at 8 h. Similar results have been reported previously (20, 21). These results may indicate that all of these enzymes and LDLR are under strict control at the mRNA level according to the decrease not only in sterol but also nonsterol products.

Figure 4 shows the time course of these changes in mRNA levels of cholesterol biosynthesis enzymes and LDLR due to cholesterol depletion. In 10% LPDS-DMEM supplemented with compactin (10^{-6} M), mRNA content of HMGS and MK reached a maximum at 6 h and then decreased gradually to about 200% of basal level (0 h) at 48 h. Likewise, HMGR, FPPS, and SQS

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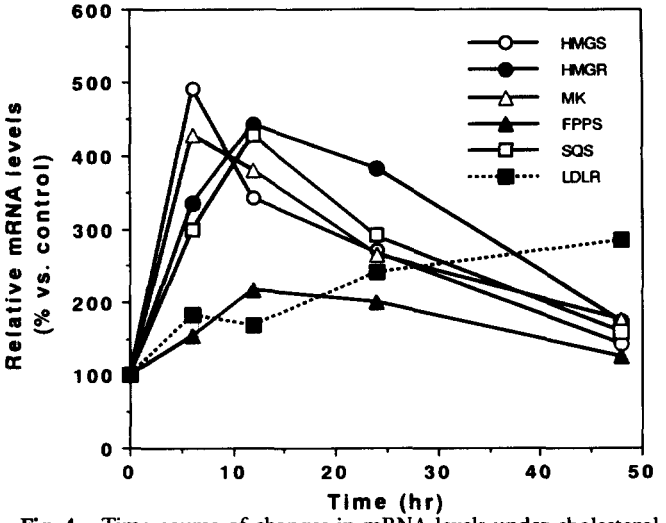

Fig. 4. Time course of changes in mRNA levels under cholesterol depletion. On day 3 after seeding in 10% FCS-DMEM, medium was replaced with 10% LPDS-DMEM supplemented with compactin (10^{-6} M). Total RNA was isolated at each time point and individual mRNA levels were measured. Enzyme abbreviations as in Fig. 1. The results are the average of three separate experiments.

showed the same pattern with a maximum level at 12 h, although the change in FPPS mRNA level was not large. At present, the cause of this transient increase and subsequent decay of these mRNAs is unknown. This point should be clarified by further study, which should include investigation of the stability of mRNA and transcriptional activity under the same conditions. In contrast, LDLR mRNA level increased slowly to 300% of the initial level at 48 h. Our data showed that the induction of accumulation of mRNA for cholesterol biosynthesis enzymes and LDLR was coordinate, but the time course pattern was different between enzymes and LDLR mRNA accumulation. Molowa and Cimis (22) showed similar results for HMGS, HMGR, and LDLR mRNAs. These results indicate that the regulation of mRNA level of these enzymes and LDLR is different under these conditions.

In summary, we studied cellular response at the mRNA levels of cholesterol biosynthesis enzymes and LDLR to depletion of cholesterol or decrease of cholesterol and nonsterol products using RPA. We conclude that this method is a useful technique for determining mRNA contents of several cholesterol biosynthesis enzymes and LDLR in the cell.

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