Exogenous VLDL stimulates apolipoprotein B secretion from HepG2 cells by both pre- and post-translational mechanisms

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Abstract Previous studies have suggested that oleic acid (OA), by increasing availability of triglyceride (TG) and/or cholesteryl ester (CE), increases the secretion of apolipoprotein B (apoB) from HepG2 cells. The present studies were conducted to determine the effect of exogenous very low density lipoproteins (VLDL), which can provide TG and cholesterol to cells and be returned to the liver after secretion, in the regulation of hepatic apoB secretion. Addition of exogenous VLDL (50 μg protein/ml) to culture media was found to significantly stimulate apoB secretion from HepG2 cells. This effect was observed consistently with VLDL isolated from either normal or hyperlipidemic subjects. The effects of addition of VLDL were compared to that of OA, which was previously shown to stimulate apoB secretion by a posttranslational mechanism. VLDL appeared to increase apoB secretion by two mechanisms; the dominant one being posttranslational. Thus, VLDL protected newly synthesized apoB from rapid intracellular degradation in a manner similar to OA. Although treatment with VLDL increased the mass of both TG and CE, 3-fold and 2.6-fold, respectively, it appeared that the increase in TG was the critical factor associated with increased apoB secretion. Triacsin D, which is a potent inhibitor of TG synthesis, significantly inhibited the VLDL-induced stimulation of apoB secretion. Inhibition of apoB secretion by Triacsin D was associated with the loss of the protective effect of VLDL on newly synthesized apoB. In addition to its posttranslational effects, exogenous VLDL also regulated apoB secretion at the pretranslational level. Thus, we also observed that VLDL treatment consistently increased synthesis of apoB protein by 20–30%, an effect that is not observed after treatment of HepG2 cells with OA. A sensitive solution hybridization/RNase protection assay indicated that the increased apoB synthesis was associated with a 20–30% increase in apoB mRNA in VLDL-treated HepG2 cells. OA treatment had no effect on apoB mRNA levels. We conclude that VLDL treatment stimulates apoB secretion in HepG2 cells primarily by supplying fatty acids for TG synthesis. However, 20–30% of the stimulatory effect was due to a second mechanism that appeared to be pretranslational. Based on studies with OA, it appears that some component of VLDL other than TG-derived fatty acid was responsible for this effect.—**Wu, X., N. Sakata, J. Dixon, and H. N. Ginsberg.** Exogenous VLDL stimulates apolipoprotein B secretion from HepG2 cells by both pre- and posttranslational mechanisms. *J. Lipid Res.* 1994. **35:** 1200–1210.

Supplementary key words apolipoprotein B • triglyceride • cholesteryl ester

Overproduction of apolipoprotein B (apoB)-containing lipoproteins appears to be a common cause of hyperlipoproteinemia in humans (1–4). Although progress has been made recently in understanding the regulation of the secretion of apoB-containing lipoproteins from hepatocytes, the exact mechanisms involved remain incompletely understood (5). Most studies using primary cultures of rat hepatocytes (6), whole livers (7–10), and cultured HepG2 cells (11–15) suggest that apoB secretion is regulated posttranslationally. Thus, apoB mRNA levels and the incorporation of labeled amino acids into apoB were not significantly changed in those studies under conditions where apoB output was significantly increased or decreased. As a result, recent studies have focused on posttranslational degradation of apoB as an important mechanism for regulating the assembly and secretion of apoB-containing lipoproteins (13, 16–21).

Results from our laboratory have shown that treatment of HepG2 cells with oleic acid (OA) increased apoB secretion several-fold by facilitating the translocation of newly synthesized apoB away from one or more proteases (22) associated with the endoplasmic reticulum (ER) (13, 16, 17, 21), thereby protecting newly synthesized apoB from early degradation (13, 21). Demonstration that OA increased triglyceride (TG) synthesis without significantly altering cholesteryl ester (CE) or phospholipid synthesis (21) suggested that TG availability played an important role in regulating apoB degradation. This belief was consistent with previous studies indicating that TG delivered to hepatocytes by lipoproteins such as chylomicron remnants (23, 24) could stimulate the assembly and secretion

Abbreviations: VLDL, very low density lipoproteins. apoB, apolipoprotein B; TG, triglyceride; CE, esterified cholesterol; OA, oleic acid; BSA, bovine serum albumin; ER, endoplasmic reticulum; ACAT, acyl-CoA:cholesterol acyltransferase.

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of apoB-containing lipoproteins. These earlier studies (23, 24), however, did not differentiate between lipoprotein-delivered TG or cholesterol as the stimulus for apoB secretion. Nor did they determine whether the effects of circulating lipoproteins on apoB secretion involved pre- or posttranslational changes.

In the present studies we report that incubation of HepG2 cells with exogenous VLDL, isolated from either normal or hyperlipidemic individuals, can significantly stimulate the secretion of apoB-containing lipoproteins from HepG2 cells. Our data also indicate that VLDL stimulates apoB secretion both by providing TG-derived fatty acids and thereby protecting newly synthesized apoB from early intracellular degradation, and by increasing apoB synthesis.

EXPERIMENTAL PROCEDURES

Materials

L-[4, 5-^{3}H]leucine (135 Ci/mmol, catalog No. TRK.683), [2-^{3}H]glycerol (1.0 Ci/mmol, catalog No. TRA.118), and [2-^{14}C]acetic acid, sodium salt (57 mCi/mmol, catalog No. CFA. 14) were purchased from Amersham Corp. (Arlington Heights, IL). Monospecific antihuman apoB antiserum was raised in a goat (25). Protein A-Sepharose CL 4B was obtained from Pharmacia LKB Biotechnology, Inc. (Uppsala, Sweden). Minimum essential medium (MEM), nonessential amino acids, sodium pyruvate, and penicillin/streptomycin were from GIBCO laboratories (Grand Island, NY). Fetal bovine serum was from Integen (Purchase, NY). Leucine-free medium was generated from a minimum essential selectamine kit (CIBCO, catalog No. 300 9050AV). Leupeptin and pepstatin A were from Peninsula Laboratories, Inc. (Belmont, CA). Bovine serum albumin (BSA) (essentially fatty acid-free) and oleic acid (sodium salt) (catalog No.0 7501) were from Sigma Chemical Co. (St. Louis, MO). Triacsin D was a gift from Fujisawa (Osaka, Japan). Sandoz 58-035 was provided by Sandoz Pharmaceutical Corp. (Hanover, NJ). All other chemicals were of the highest purity available.

Growth of cells

HepG2 cells, obtained from ATCC, were grown in 35-mm dishes which had been coated with collagen. The cells were maintained in MEM containing 0.1 mM nonesential amino acids, 1 mM sodium pyruvate, penicillin (100 units/ml)/streptomycin (100 μg/ml each), and 10% FBS for 4 days with medium replenishment at day 3. The medium was then changed to serum-free MEM experimental medium containing 1.5% BSA as described below.

Isolation of VLDL from plasma

Human fasting plasma containing 0.1 mM EDTA was centrifuged in a Beckman TL-100 ultracentrifuge (100,000 rpm, 210 min, 4°C). The $d < 1.006$ g/ml fraction (VLDL) was collected and dialyzed against phosphate-buffered saline (PBS) containing 0.1 mM EDTA overnight. VLDL protein content was determined by the BCA (bicinchoninic acid) method (26) and added to the experimental medium at 50 μg protein per ml.

Pulse-chase experiments

Cells were preincubated in serum-free MEM containing: *1)* 1.5% BSA for 20 h; or *2)* 1.5% BSA plus VLDL (50 μg/ml protein) for 20 h; or *3)* 1.5% BSA for 19 h, followed by 1.5% BSA plus VLDL (50 μg/ml protein) for 1 h; or *4)* 1.5% BSA for 19 h, followed by 1.5% BSA plus OA (0.4 mM) for 1 h. Cells were washed twice with warm PBS (37°C), labeled for 25 min with 1 ml of leucine-free MEM containing 100 μCi of [^{3}H]leucine, and chased for 60 min in serum-free medium. Medium was collected in tubes containing a mixture of protease inhibitors (1 mM benzamidine, 5 mM EDTA, 0.86 mM phenylmethylsulfonyl fluoride, 100 kallikrein-inactivating units/ml of aprotinin, and 10 mM HEPES, pH 8.0), and the cells were harvested in lysis buffer (62.5 mM sucrose, 0.05% sodium deoxycholate, 0.05% Triton X-100, 50 μg/ml leupeptin, 50 μg/ml pepstatin A, 150 μg/ml PMSF, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl). To determine the intracellular decay of nascent apoB, cells were preincubated as above, labeled for 10 min, and chased for various periods (10–60 min). At every time point, medium was collected and cells were harvested as above. In some experiments, VLDL was present in the chase medium. To determine the early incorporation of [^{3}H]leucine into apoB, cells were preincubated as above, labeled for 5 min, and chased for short periods (0–10 min).

Immunoprecipitation

Immunoprecipitation of apoB in medium and cell homogenates was carried out exactly according to the method of Dixon, Furukawa, and Ginsberg (13). Briefly, samples were mixed with NET buffer (150 mM NaCl, 5 mM EDTA, and 50 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, and 0.1% SDS) and an excess amount of anti-apoB antiserum, and the mixture was incubated on a shaker for 10 h at 4°C. Protein A-Sepharose CL 4B was added to the mixture, the incubation was continued for an additional 3 h, and the beads were washed extensively. ApoB was extracted from the protein A pellet with sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% mercaptoethanol) by boiling. An aliquot of each sample was used for scintillation counting and another aliquot was used for SDS-PAGE (3–15% gradient gel). The gel was

treated with autofluor and, after drying, was exposed to a film (Kodak, X OMAT AR) at −80°C.

Uptake of newly secreted apoB-containing lipoproteins

HepG2 cells, pretreated with serum-free medium for 20 h in the presence of VLDL, were labeled for 30 min and chased with serum-free medium in the absence of VLDL for 60 min. The medium, containing [^{3}H]apoB-containing lipoproteins was collected and placed onto other cells that had been pretreated with serum-free medium in the absence or presence of VLDL for 20 h (the VLDL-containing medium had been washed out immediately before the addition of the medium containing [^{3}H]apoB-containing lipoproteins). After the cells were incubated in the labeled medium for 60 min, the medium was collected, and apoB was immunoprecipitated. A second protocol used labeled lipoproteins. Thus, after a pulse-chase, the labeled medium was collected and its density was adjusted with KBr to 1.063 g/ml. The d < 1.063 g/ml fraction was isolated by ultracentrifugation, and the endogenously labeled lipoproteins were added to serum-free medium. This mixture was incubated for 30 or 60 min with cells that had been pretreated as described above.

Northern blotting and solution hybridization/RNase protection for apoB mRNA measurement

HepG2 cells were incubated in serum-free medium in the absence or presence of VLDL for 20 h. Total RNA was prepared by a one-step extraction method described in the manufacturer's instructions (Biotex, CA). The total RNA was demonstrated to be intact by agarose electrophoresis which showed two bands corresponding to 18s and 28s RNA, respectively. ApoB cDNA (1.6 kb) (27) and a full length actin cDNA were labeled with a random primer method (28). For Northern blotting, 10 μg total RNA was run on 0.8% agarose denaturing gels containing formadehyde, and then transferred to nitrocellular membranes by capillary blotting. Prehybridization and hybridization were both performed at 42°C for 16 h. The membranes were placed into cassettes and exposed to film for 24 h.

To achieve greater sensivity, apoB mRNA levels were also measured by solution hybridization/RNase protection (29). An apoB riboprobe (168 bp) was transcriptionally labeled and mixed with 10 μg of the total RNA sample. The mixture was incubated at 63°C for 4 h. RNase A and RNase T1 were added to the mixture and the contents were incubated at 34°C for 40 min. Cold tricholoracetic acid was added to the mixture and the entire contents were filtered through a glass-fiber filter and washed with 7% cold trichloroacetic acid. The filter, after being air-dried, was put into a scintillation vial containing 5 ml of hydrofluor, and radioactivity was determined. To verify the specificity of the riboprobe for apoB mRNA, after treatment with RNase A and T1, the protected fragment was examined by gel electrophoresis and autoradiography. The riboprobe resulted in a RNase-resistant band (125 bp) after hybridization with HepG2 cell total RNA, but did not result in a detectable RNase-resistant band after hybridization with yeast tRNA (data not shown). In our assay system, hybridization was linear upto 150 pg of apoB cRNA. Every assay was accompanied with a standard curve; R values for these curves were between 0.94 and 0.99, and the slopes were between 20 and 30 cpm/pg of apoB cRNA. Multiple measurements of total RNA samples were shown to be reproducible with standard deviations within 10% of the means.

Lipid synthesis and mass determination

HepG2 cells, grown in 35-mm dishes, were pretreated in serum-free medium with BSA for 20 h in the absence or presence of VLDL. After the incubation period, the medium was removed, and the cell monolayer was washed twice with cold PBS. Lipids were extracted from cells with hexane–isopropanol 3:2 (v/v). The organic solvent was removed under nitrogen, and the lipids were resuspended in hexane. TG mass was determined with a kit from Sigma (UV334), and cholesterol mass was determined by gas–liquid chromatography.

RESULTS

Effects of VLDL on cell lipid mass

To determine the effects of VLDL on cell lipid composition, HepG2 cells were incubated with 1.5% BSA (control) or 1.5% BSA plus VLDL (50 μg/ml) for 20 h. Cellular TG content was increased 3-fold by VLDL plus BSA compared to BSA alone ($P < 0.05$). Unesterified cholesterol increased slightly (NS), while CE content was increased 2.6-fold by VLDL ($P < 0.05$) (**Table 1**). When HepG2 cells were incubated with VLDL for 1 h, no significant changes in cellular lipids were seen.

TABLE 1. Effect of VLDL treatment on lipid composition of HepG2 cells

Treatment	TG	UC	CE
	μg/mg protein		
BSA (1.5%)	45.5 ± 12.5	18.8 ± 4.6	3.3 ± 1.2
BSA/VLDL	135.4 ± 35.6[a]	23.0 ± 3.5	8.6 ± 2.3[a]

HepG2 cells were incubated with or without VLDL for 20 h. Lipids were extracted from cells by hexane–isopropanol 3:2 (v/v). TG was determined with a TG kit from Sigma (UV 334); unesterified cholesterol (UC) and esterified cholesterol (CE) were determined by gas–liquid chromatography. Results are from three experiments and reported as mean ± SD.

[a] $P < 0.05$ (BSA/VLDL vs. BSA).

Effects of VLDL and OA on apoB secretion

In these experiments, cells that had been incubated with BSA alone, BSA plus VLDL, or BSA plus OA were pulse-labeled with [^{3}H]leucine for 25 min and chased for 60 min, and apoB content in the medium was determined by immunoprecipitation. As shown in **Fig. 1.**, VLDL preincubation for either 20 h or 1 h resulted in a significant stimulation of apoB secretion into the medium. VLDL preincubation for 1 h resulted in less stimulation than VLDL preincubation for 20 h. OA at a concentration of 0.4 mM stimulated apoB accumulation in the medium to a much greater extent than either short-term or long-term incubation with VLDL. The effects of exogenous VLDL on apoB secretion were observed with VLDL isolated from many individuals, including normal and dyslipidemic subjects.

The second band present in all lanes, which migrated more rapidly than apoB, is an unidentified protein that is nonspecifically precipitated from the medium (but not cell extract) by our procedures. This protein is also nonspecifically precipitated from the medium by antisera against human apoA-I, apoA-II, apoC-II, apoE, albumin, antitrypsin, and α-fetoprotein, etc. (data not shown). Our preliminary data have shown that this nonspecific protein is also immunoprecipitated by apoB antibody from the medium of CHO cells, a cell line that does not synthesize and secrete apoB. It is not recognized by polyclonal apoB antibody in an immunoblot. It is unaffected by perturbations that alter apoB secretion. Furthermore, we do not see this band when we immunoprecipitate apoB from cell homogenates (see Fig. 3B). This protein was present in

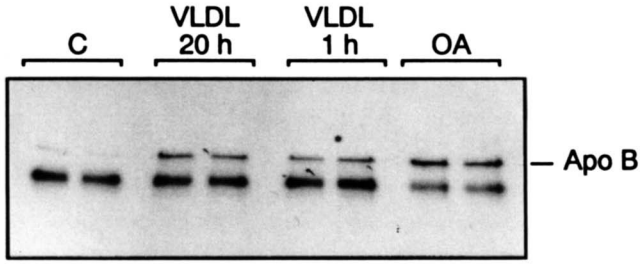

Fig. 1. VLDL increases apoB secretion from HepG2 cells. HepG2 cells were preincubated with control medium, VLDL or OA as described in Experimental Procedures, pulse-labeled with [^{3}H]leucine for 25 min, and chased for 60 min. OA was present in preincubation, pulse, and chase medium at 0.4 mM. VLDL was not included in chase medium because it would interfere with [^{3}H]apoB immunoprecipitation. Medium apoB content was immunoprecipitated using a polyclonal apoB antibody. An aliquot of the sample was electrophoresed on a 3–15% gradient polyacrylamide gel containing SDS. The labeled protein which migrates more rapidly than apoB is nonspecifically precipitated from the medium. It is particularly prominent in the controls because the long-term incubation (19–20 h) of HepG2 cells in BSA-containing serum-free medium inhibits apoB secretion (11). This is representative of numerous experiments in which a 1-h VLDL incubation modestly and a 20-h incubation more significantly stimulated apoB secretion. OA stimulated apoB secretion to a greater degree than VLDL.

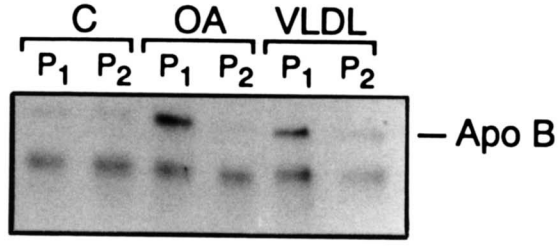

Fig. 2. The stimulation of apoB secretion by VLDL and OA disappears shortly after the removal of OA or VLDL from medium. After preincubation with control medium or medium containing VLDL (20 h) or OA (1 h), HepG2 cells were labeled for 25 min and chased for 60 min. The medium was collected, the cells were washed twice with warm PBS, and the pulse-chase protocol was repeated. The new medium was collected, and apoB secreted into both the initial (P1) and the second media (P2) was determined by immunoprecipitation, electrophoresis, and fluorography. The experiment was repeated with different preparations of VLDL from different individuals.

our earlier studies (13, 21, 22) but is more pronounced in the current experiments because the HepG2 cells were incubated for 20 h in serum-free medium containing 1.5% BSA, which inhibits apoB secretion (11).

Effects of VLDL and OA on apoB secretion are rapidly reversed by removal of VLDL or OA from the medium

We had previously demonstrated that the stimulation of apoB secretion by OA disappeared completely by 40 min after removal of OA from the medium (13). In the present studies, VLDL was compared with OA in a double pulse-chase protocol. After preincubation with VLDL or OA, cells were labeled for 25 min and chased for 60 min in the presence of either substance. The media were collected, the cells were washed twice with warm PBS, and the pulse-chase protocols were repeated. VLDL and OA were omitted from the respective media during this second pulse-chase period. The new media were collected, and apoB secretion into both the initial and the second media was determined. It was found that both VLDL and OA stimulated apoB secretion during the first pulse-chase period (note the ratio of apoB/nonspecific protein), and that the effects of both were lost during the second pulse-chase period (**Fig. 2**). These results suggested similar, evanescent mechanisms for VLDL- and OA-induced apoB secretion.

Both OA and VLDL protect apoB from degradation

In order to better define the mechanism responsible for the stimulation of apoB accumulation in the VLDL-treated cell medium, HepG2 cells were preincubated as described above, then labeled for 10 min and chased for various periods of time. ApoB radioactivity was determined in both the cells and in the medium at each time by immunoprecipitation. Because the media immunoprecipitates contained both labeled apoB and a nonspecific band, apoB radioactivity in the medium was obtained by

scanning the autoradiogram and those data was used to correct the immunoprecipitation data. Based on previous studies, we used the apoB radioactivity in the cells at 10 min as maximal incorporation of labeled amino acid into apoB and normalized all the other points to the 10 min apoB counts. The percent of apoB degraded in the cells at any time was obtained by subtracting the sum of the apoB secreted into the medium plus that remaining in the cells from the initial counts at 10 min. In the control cells, after a 60-min chase, about 75% of nascent apoB was degraded, whereas in VLDL-treated or OA-treated cells, about 65% and 60% of initially synthesized apoB was degraded, respectively (**Fig.3A**). The percent of apoB remaining was significantly greater for VLDL- and OA-treated cells, compared to control cells at all time points after 10 min of chase ($P < 0.01$). In addition, protection by OA was greater than by VLDL ($P < 0.05$). Fig. 3B shows the protective effect of VLDL on intracellular apoB. After chase for 20, 30, and 60 min, more apoB radioactivity was immunoprecipitated from VLDL-treated and OA-treated cells than from control cells. Overall, these studies indicated that VLDL, like OA, could protect apoB from rapid intracellular degradation. It appeared that, at the doses used, VLDL was not as potent as OA, and this probably was reflected in the greater stimulation of apoB secretion seen with OA (Fig. 1).

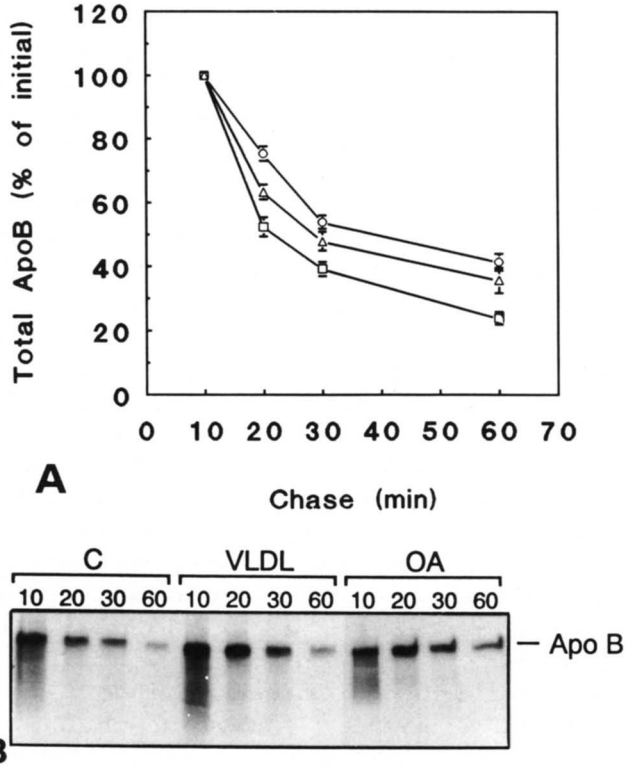

Fig. 3. VLDL protects nascent apoB from intracellular degradation. After preincubation with 1.5% BSA for 20 h (□), or VLDL for 20 h (△), or OA for 1 h (○), HepG2 cells were labeled for 10 min and chased for various periods (10, 20, 30, and 60 min). Labeled apoB content was determined in extracts of cells and medium by immunoprecipitation. Samples were quantitated by scintillation counting and visualized by electrophoresis. After 10 min of chase, apoB radioactivities recovered from control cells, OA-treated cells, and VLDL-treated cells were 396,234 ± 25,708 cpm/mg protein, 404,046 ± 35,249 cpm/mg protein, and 482,006 ± 38,165 cpm/mg protein, (mean ± SE), respectively. ApoB radioactivity in the medium was obtained by scanning the autoradiogram and using those data to correct for the nonspecific protein present in the immunoprecipitates. A) Total apoB radioactivity (cellular and medium) at each chase point is presented as percentage of initially synthesized apoB ± SE. B) ApoB radioactivity remaining in the cells during chase. At each point after 10 min of chase, significantly more apoB radioactivity was immunoprecipitated from VLDL-treated ($P < 0.01$) and OA-treated cells ($P < 0.01$).

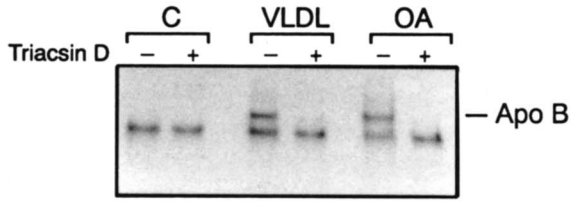

Fig. 4. Triacsin D blocks OA-stimulated or VLDL-stimulated apoB secretion from HepG2 cells. After preincubation with VLDL for 20 h or OA for 1 h, HepG2 cells were labeled for 25 min and chased for 60 min. When cells were incubated with Triacsin D (12.5 μM), it was added concurrently (VLDL-treated cells), or 5 min before (OA treatment) preincubation. Medium apoB content was determined by immunoprecipitation. Samples were electrophoresed on a 3–15% gradient polyacrylamide gel containing SDS. The minimal apoB secretion from control (–) cells is the result of 20 h incubation with BSA. Triacsin D further reduced apoB secretion from control cells, and inhibited the stimulation of apoB secretion usually observed with VLDL or OA. This gel is representative of three different experiments.

Triacsin D, an inhibitor of acyl-CoA synthetase, blocks both VLDL-stimulated and OA-stimulated apoB secretion

As OA stimulation of apoB secretion appears to be linked to stimulation of TG synthesis (13), we investigated the link between TG synthesis and VLDL secretion. Triacsin D is a competitive inhibitor of fatty acyl-CoA synthetase (30, 31), and a potent inhibitor of OA-induced TG synthesis in HepG2 cells. When Triacsin D (12.5 μM) and VLDL were co-incubated with cells for 20 h, a significant decrease in apoB accumulation in the medium was observed (**Fig. 4**). Triacsin D inhibited the VLDL-associated increase in TG mass by 60% without affecting the VLDL-associated increase in CE mass (data not shown). Triacsin D also blocked the stimulation of apoB secretion by OA. The inhibition of both OA-stimulated and VLDL-stimulated apoB secretion by Triacsin D was associated with a loss of the protective effects of OA and VLDL on newly synthesized apoB (**Fig. 5**). The curves representing the percent apoB remaining in the cells treated with VLDL alone and OA alone are significantly different from those respective Triacsin D treatment cells ($P < 0.01$). These results indicate that VLDL, similar to OA, protects apoB from rapid intracellular degradation

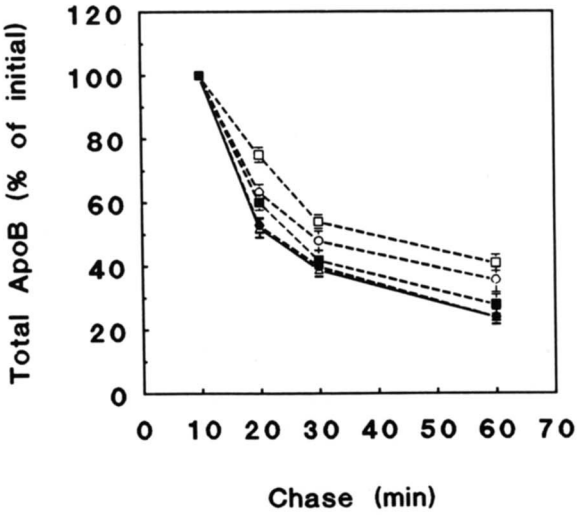

Fig. 5. Triacsin D blocks the protective effect of both OA and VLDL on intracellular nascent apoB in HepG2 cells. After preincubation with 1.5% BSA alone for 20 h (△), BSA and VLDL (50 μg/ml) (○) for 20 h, or BSA and OA (0.4 mM) for 1 h (□), HepG2 cells were labeled for 25 min and chased for various periods (10, 20, 30, and 60 min). When cells were incubated with Triacsin D (12.5 μM), it was added concurrently (VLDL-treated cells) (●), or 5 min before (OA-treated cell) preincubation (■). After 10 min of chase, apoB radioactivities recovered from control cells, OA-treated cells, VLDL-treated cells, OA/Triacsin D-treated cells, and VLDL/Triacsin D-treated cells were 396,234 ± 25,708 cpm/mg protein, 404,046 ± 35,249 cpm/mg protein, 482,006 ± 38,165 cpm/mg protein, 395,769 ± 36,492 cpm/mg protein, and 468,154 ± 29,767 cpm/mg protein, respectively. After each chase time point, cellular apoB and apoB secreted into the medium were determined by immunoprecipitation. Medium data were corrected for the nonspecifically precipitated protein as described in Fig. 3a. The recovered apoB activity from cell and medium was combined to provide an estimate of the proportion of apoB that was protected under each experimental condition. Data represent the % of initial apoB recovered in cells after 10 min of chase ± SE. The data for control cells and VLDL plus Triacsin D are superimposable. After 60 min of chase, a significantly greater portion of apoB was precipitated from VLDL- ($P < 0.01$) or OA-treated cells ($P < 0.01$), compared to control cells. There were no significant differences in apoB radioactivity between control cells and VLDL/Triacsin D- or OA/Triacsin D-treated cells.

by providing newly synthesized TG. As VLDL delivers both cholesterol and TG to cells, we used the ACAT inhibitor, Sandoz 58-035, to inhibit VLDL-stimulated CE formation. The VLDL-induced rise in CE mass was inhibited 70%, while concomitant increase in TG mass was unaffected. However, although Sandoz 58-035 inhibited the VLDL-stimulated rise in HepG2 CE content, stimulation of apoB secretion by VLDL was not affected.

Uptake of nascent apoB is not affected by VLDL treatment

Some apoB-containing lipoproteins, once secreted into the medium, may be taken up rapidly by the same cells. If this occurred to a significant degree, the accumulation of apoB in the medium that we observed would be the sum of both secretion and reuptake. To determine to what extent apoB accumulation was affected by reuptake of nascent lipoproteins, and to see whether preincubation of cells with VLDL could affect rapid uptake, the following experiments were carried out. HepG2 cells were preincubated with VLDL for 20 h, labeled for 30 min, and chased for 60 min. The media were collected and then placed onto another set of HepG2 cells that had been preincubated in serum-free MEM with or without VLDL for 20 h, and the VLDL-containing medium had been washed out. The cells were incubated for 60 min, and the amount of apoB remaining in the medium was determined by immunoprecipitation. There were no differences in medium apoB radioactivity between the control cells and VLDL-treated cells (**Fig. 6**). Identical results were obtained with medium from cells treated with 1.5% BSA and added to cells incubated with or without VLDL (data not shown).

Because the nonspecific band was the predominant band in the gel shown in Fig. 6, we wanted to eliminate the possibility that this nonspecific protein might confound our uptake results. Endogenously labeled apoB-containing lipoproteins were first isolated by ultracentrifugation at d < 1.063 g/ml and then placed onto HepG2 cells pretreated as described above (the nonspecific protein has a density greater than 1.063 g/ml). Incubation was continued for 30 min and 60 min, and the [^{3}H]apoB-containing lipoproteins remaining in the medium were determined by directly counting the radioactivity of the medium and by apoB immunoprecipitation. We found that 6.9% of the radioactivity disappeared from the medium of the control cells, and 3.7% of the radioactivity disappeared from the medium of the VLDL-treated cells after 60 min of incubation (**Table 2**). There were also no differences between control cells and VLDL-treated cells in the amount of apoB remaining in the medium (**Fig. 7**). These results suggested that the reuptake of newly secreted apoB-containing lipoproteins was minimal, and did not significantly affect our estimation of the accumulation of apoB in the medium. Furthermore, the presence of the nonspecific protein did not confound the interpretation of our results.

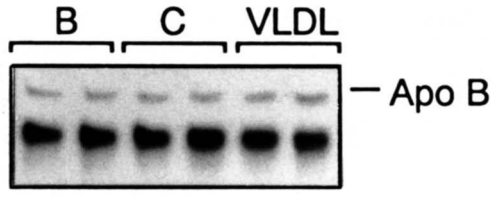

Fig. 6. ApoB uptake from HepG2 cell medium was not affected by VLDL treatment. HepG2 cells were preincubated with VLDL for 20 h, labeled for 30 min, and chased for 60 min. The media containing [^{3}H]apoB were collected and then placed onto another set of HepG2 cells that had been preincubated with BSA (C) or with VLDL (VLDL) for 20 h. The cells were incubated for 60 min and the amount of apoB in the medium before (B) and after incubation was determined by immunoprecipitation. The results from duplicate wells are presented.

TABLE 2. Effect of VLDL on apoB uptake from HepG2 cell medium

Treatment	Added	Recovered	Disappeared
	cpm	*cpm*	%
BSA (1.5%)			
30 min	314,470	295,536	6.0
60 min	314,470	292,722	6.9
BSA/VLDL			
30 min	314,470	305,036	3.0
60 min	314,470	302,834	3.7

HepG2 cells were preincubated with VLDL for 20 h, labeled for 30 min, and chased for 60 min. The medium was collected and the d < 1.063 g/ml fraction was prepared. Aliquots of the d < 1.063 g/ml fraction were placed onto other sets of HepG2 cells that had been preincubated with or without VLDL for 20 h. The incubation was continued for 30 and 60 min. Aliquots of medium containing d < 1.063 g/ml fraction were taken to count before and after incubation.

VLDL treatment increases apoB synthesis

At the 10-min point in the pulse-chase experiment described earlier (Fig. 3B), there was approximately 20% more apoB radioactivity found in VLDL-treated cells than in BSA-treated cells, suggesting that VLDL treatment increased incorporation of [^{3}H]leucine into apoB molecules (i.e., treatment with VLDL might have increased apoB synthesis). This effect was observed consistently in several experiments. In contrast, as we had previously observed, OA treatment did not affect initial (10 min) apoB radioactivity compared to control cells. To better define the effects of VLDL incubation on apoB synthesis, HepG2 cells were preincubated in serum-free MEM, in the absence or presence of VLDL for 20 h, pulse-labeled for 5 min, and chased for 0, 5, and 10 min. These early time points were used to focus on the early incorporation of [^{3}H]leucine into apoB, before any significant intracellular degradation could occur. Approximately 20% more leucine was incorporated into apoB in VLDL-treated cells compared to control cells ($P < 0.05$, **Fig. 8**). These results contrasted with the lack of effect of OA at these time-points in earlier studies (13) and in parallel experiments (data not shown). As neither apoB degradation nor secretion are detectable during the 0 and 5 min chase periods, the intracellular radioactivity data may be used as a reflection of apoB synthesis. The results indicated that VLDL, in contrast to OA, stimulated apoB synthesis.

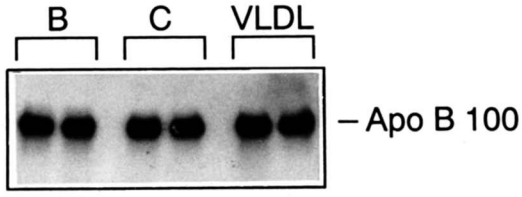

Fig. 7. ApoB uptake from HepG2 cells was not affected by VLDL treatment. HepG2 cells were preincubated with VLDL for 20 h labeled for 30 min, and chased for 60 min. Endogenously labeled apoB-containing lipoproteins were isolated by ultracentrifugation at d < 1.063 g/ml (the nonspecific protein is not lipid-associated and has a density greater than 1.063 g/ml) and placed onto HepG2 cells that had been preincubated with BSA (C) or with VLDL for 20 h (the VLDL-containing medium had been washed out immediately before the addition of d < 1.063 g/ml fraction). Incubation was continued for 60 min, and the amount of apoB in the medium before (B) and after incubation (both C and VLDL) was determined by immunoprecipitation. The results from duplicate wells are presented.

VLDL treatment increases apoB mRNA content

To further define the mechanism by which apoB synthesis was increased by VLDL treatment, apoB mRNA levels were determined. HepG2 cells preincubated for 20 h with serum-free MEM, in the absence or presence of VLDL, were extracted with Trisolv reagent (Biotex, CA) and total RNA was prepared. Northern blotting and solution hybridization were carried out as described in Experimental Procedures. We were unable to observe significant differences in apoB mRNA levels between control and VLDL-treated cells by Northern blotting (**Fig. 9**). With the more sensitive solution hybridization/RNase protection assay, however, we consistently observed a 20–30% increase in apoB mRNA in VLDL-treated HepG2 cells compared to BSA-treated cells ($P < 0.05$) (**Table 3**). This increase in apoB mRNA paralleled the increase in apoB synthesis that we had observed. When Sandoz 58-035 was included in VLDL incubation, apoB mRNA level was unchanged compared to VLDL alone. As had been reported by other investigators (11, 12, 32),

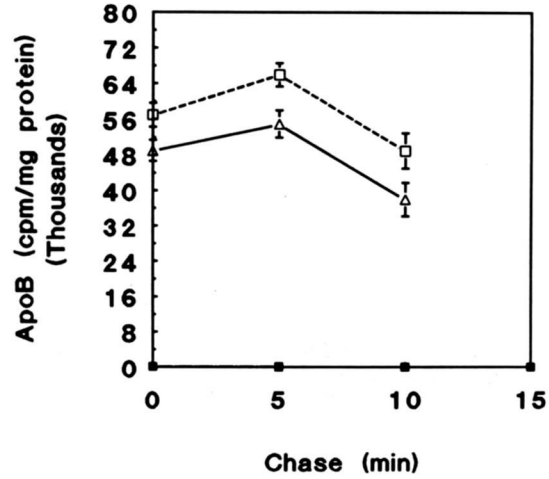

Fig. 8. VLDL treatment increases apoB synthesis. HepG2 cells were preincubated with 1.5% BSA (△) or VLDL (□) for 20 h, labeled for 5 min, and chased for various periods (0, 5, and 10 min). Cellular apoB was determined by immunoprecipitation. The data represent the mean ± SE of triplicate dishes at each time point. At all time points, apoB radioactivities recovered from VLDL-treated cells are significantly greater than that from control cells ($P < 0.05$). This experiment was repeated three times with identical results.

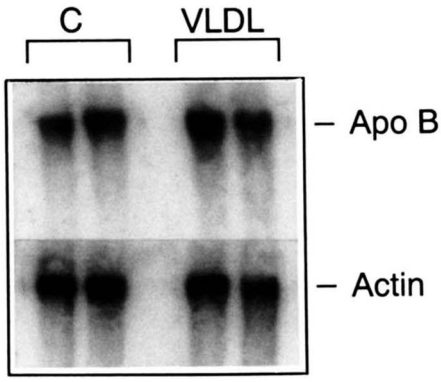

Fig. 9. Effect of VLDL treatment on apoB mRNA content in HepG2 cells. HepG2 cells were incubated in serum-free medium containing 1.5% BSA (C) or VLDL for 20 h. Total RNA was prepared as described in Experimental Procedures. Ten μg total RNA was run on 0.8% agarose denaturing gels containing formadehyde, and then transferred to nitrocellular membranes by capillary blotting. Prehybridization and hybridization with ^{32}P-labeled apoB cDNA (1.6 kb) or ^{32}P-labeled α-actin were both performed at 42°C for 16 h. The membranes were placed into cassettes and exposed to film for 24 h. There was no discernible effect of VLDL on apoB mRNA. Total mRNA recovery was the same in both control and VLDL-treated cells, as judged by actin mRNA.

OA treatment had no effects on apoB mRNA levels. Indeed, treatment of HepG2 cells with VLDL plus OA increased apoB mRNA to levels comparable to those seen with VLDL alone (data not shown).

DISCUSSION

The results of the present studies indicate that exogenous VLDL stimulates the assembly and secretion of apoB-containing lipoproteins by two mechanisms. First, VLDL appears to protect nascent apoB from intracellular degradation; this effect is similar to that which we observed for OA (13), and seems to account for most of the increased secretion of apoB. Second, incubation of HepG2 cells with VLDL stimulates apoB synthesis; these effects of VLDL are pretranslational and modest in our cell culture system. Thus, although our findings generally agree with the widely accepted concept that apoB secretion from cultured cells is regulated posttranslationally, the results suggest that pretranslational regulation of apoB secretion could be significant under certain conditions or in other systems.

The conclusion that the major effect of VLDL on apoB secretion in HepG2 cells is similar to that of OA is based on the following observations. First, both OA and VLDL must be present continuously in the medium in order to stimulate apoB secretion; once OA or VLDL is removed from medium, their effect on apoB secretion disappears rapidly. We previously observed a lag time of about 40 min for the appearance and the disappearance of OA's effect on apoB secretion (13). In the present studies we found a similar time course for the effect of VLDL. Second, both VLDL and OA protect newly synthesized apoB from intracellular degradation. From the results obtained with pulse-chase experiments, it is obvious that the stimulatory effect of VLDL on apoB secretion is due principally to its ability to protect apoB from intracellular degradation. Finally, Triacsin D, which inhibits TG synthesis significantly, blocks most of the effects of both VLDL and OA on apoB secretion. In the presence of Triacsin D, neither VLDL nor OA protect newly synthesized apoB from rapid, intracellular degradation.

TABLE 3. Effect of VLDL treatment on apoB mRNA content (cpm riboprobe hybridized/μg RNA).

	BSA (%)	VLDL (%)	VLDL/58-035 (%)	OA (%)
Exp. 1	277.6 (100.0)	349.3 (125.8)	342.4 (123.3)	281.4 (101.4)
Exp. 2	476.4 ± 9.3 (100.0)	625.7 ± 13.1 (131.3)	Not done	496.6 ± 11.2 (104.2)
Exp. 3	371.2 ± 22.4 (100.0)	442.8 ± 12.0 (119.3)	480.4 ± 56.0 (129.6)	428.8 ± 13.3 (115.2)
Exp. 4	239.6 (100.0)	307.2 (128.2)	Not done	Not done
Exp. 5	173.7 ± 4.5 (100.0)	215.0 ± 8.8 (123.8)	203.3 ± 16.4 (117.0)	182.1 ± 6.3 (104.8)
% Control Mean ± SD (%)	100.0	125.7 ± 6.3[a]	123.3 ± 6.1[a]	106.4 ± 6.2

HepG2 cells were preincubated in serum-free medium containing BSA (1.5%), BSA with VLDL (with and without 2 μg/ml Sandoz 58-035) for 20 h, or OA for 1 h. Total RNA was prepared and solution hybridization/RNase protection was carried out as described in Experimental Procedures. Five experiments are listed individually. Results are reported as cpm per μg of total RNA. Where the data are cpm ± SD, the data are the mean of duplicate or triplicate wells. Each experimental result is also presented as a % in relation to the BSA alone group done at the same time (100%). The mean percent changes in mRNA for each stimulus compared to BSA alone are presented at the bottom of the table as mean ± SD.

[a] $P < 0.05$ (VLDL or VLDL/58-035 vs. BSA).

Although VLDL uptake by cells delivers TG, cholesterol, and phospholipids (along with several proteins), we believe that it is the TG, or more likely TG-derived free fatty acids, that play the key role in the post-translational regulation of apoB secretion. Support for this view derives from our previous work that demonstrated that OA increased TG synthesis by more than 5-fold, but only increased cholesterol and CE synthesis modestly (13). In the present study, VLDL treatment increased TG mass by 3-fold, but also increased CE mass by 2.6-fold. Several lines of evidence, however, indicated that the VLDL-associated increase in CE mass did not result in changes in apoB secretion. In the experiment with Triacsin D, the VLDL-stimulated increase in TG mass was inhibited by 60% without any change in the VLDL-stimulated increase in CE mass. Despite a lack of effect on CE mass, Triacsin D reversed most of the protective effect of VLDL on apoB, and apoB secretion was reduced greatly. Our results are in accord with those from Arbeeny et al. (33) who reported that inhibition of fatty acid synthesis decreased hepatic synthesis of TG and secretion of both VLDL-TG and VLDL apoB-100 in the hamster, while cholesterol synthesis was unaffected.

The strongest evidence against a key role for VLDL-delivered cholesterol in the regulation of apoB secretion comes from our ACAT inhibitor experiments. Sandoz 58-035, which effectively inhibited the formation of CE during incubation of the HepG2 cells with VLDL, had no effect on either intracellular degradation or secretion of apoB. These findings contrast with those of Cianflone et al. (34), who reported that in HepG2 cells it was CE, and not TG, that was the major regulator of apoB secretion. This discrepancy is difficult to explain, but it should be noted that in their studies (34), the changes in apoB secretion (measured by mass assays of the media) associated with perturbations of CE mass or synthesis, were small (less than 30%).

The protective effect of both OA and VLDL on apoB was observed after the cells were pulsed for 10 min and chased for 20 min, suggesting that VLDL and OA are affecting apoB at the same, early stage in the secretary pathway. We previously reported that the ER is the site where early apoB degradation occurs (21). Presumably, either VLDL or OA, by increasing TG synthesis and thereby making more TG available for association with nascent apoB, can facilitate the assembly of apoB lipoproteins and reduce the accessibility of apoB to one or more proteases associated with the ER (21).

The mechanism by which exogenous VLDL increases the intracellular lipid content of HepG2 cells is probably multi-staged. Presumably, VLDL particles are taken up via receptor-mediated pathways and then hydrolyzed in lysosomes. The fatty acids and glycerol derived from hydrolyzed TG are reutilized to synthesize TG and phospholipids, while VLDL-derived free cholesterol (including that from lysosomal hydrolysis of VLDL CE) can be either distributed to plasma and intracellular membranes or re-esterified. In addition to intracellular hydrolysis, however, lipolysis of VLDL TG can occur in the medium. Indeed, Evans et al. (35) recently reported that large hypertriglyceridemic VLDL (S_f 60–400) failed to augment the total cholesterol, CE, and TG content of HepG2 cells after a 24-h incubation unless the VLDL were co-incubated with bovine milk lipoprotein lipase. In the present studies, we used a more heterogenous VLDL (the entire $d < 1.006$ g/ml fraction), which included small VLDL particles. These small particles could have been taken directly into the cells via receptors, and/or lipolyzed in the medium before uptake. While we do not know if large VLDL particles were lipolyzed in our media, we have found that the TG component of nascent lipoprotein particles secreted from HepG2 cells are rapidly hydrolyzed into diacylglycerol, monoacylglycerol, or fatty acids and glycerol. Moreover, we find that HepG2 cell conditioned medium is able to lipolyze VLDL-TG in vitro (X. Wu and H. N. Ginsberg, unpublished data). Arrol et al. (36) also reported that a large percentage of HepG2 cell-secreted TG is hydrolyzed in culture medium. Hepatic lipase secreted by HepG2 (37) cells could be responsible for lipoprotein-TG lipolysis in the medium.

Our studies suggest that VLDL treatment also stimulates apoB secretion from HepG2 cells by a second mechanism: increased apoB synthesis. We consistently observed that VLDL treatment not only protected apoB from intracellular degradation, but also increased the incorporation of [^{3}H]leucine into apoB. Additionally, in repeated experiments, the apoB mRNA level was found to be increased by 20–30%. The effect of VLDL on apoB mRNA content contrasted with the well-described lack of effect of OA on apoB mRNA (9–11, 32), which we confirmed.

Despite the body of data indicating that apoB secretion is regulated at posttranslational level, several groups have reported that it can also be regulated at transcriptional or pretranslational levels. For example, 25-OH cholesterol increased apoB mRNA levels by 50% in HepG2 cells (14), EPA decreased apoB mRNA levels to 25% of control in CaCo2 cells (32), and thyroid hormone increased apoB mRNA levels by 30% in HepG2 cells (38). Additionally, a study in mice by Srivastava et al. (7) suggested that hepatic apoB mRNA content could be changed by diet. The lack of an effect of short-term incubations with OA on apoB mRNA levels, in contrast to the effect of VLDL, suggests that increased TG synthesis and mass do not effect apoB mRNA. On the other hand, the report by Dashti (14), indicating that 25-OH cholesterol increases apoB mRNA (a finding that we have confirmed), suggests that VLDL-derived cholesterol might be involved at the level of pretranslation. We were unable to demonstrate differences between apoB mRNA levels after incubation of cells with VLDL plus the ACAT inhibitor compared to

VLDL alone, despite the fact that cell free cholesterol content increases further and CE content decreases significantly in the presence of the ACAT inhibitor. Obviously, more work is required to fully define the regulation of apoB secretion at the pretranslational level.

The current results, together with those from several other laboratories (23, 24, 36) indicate that the delivery of fatty acids and/or TG to hepatocytes by TG-rich lipoproteins can stimulate the assembly and secretion of nascent apoB-containing lipoproteins. One could speculate that in situations where lipoprotein-TG was not efficiently cleared from peripheral plasma by lipoprotein lipase-mediated hydrolysis, the resulting TG-rich remnant lipoproteins could be catabolized in the hepatic vascular bed and stimulate the assembly and secretion of nascent apoB-containing lipoproteins. This scheme might be of particular importance during the postprandial period, where any deficiency in lipoprotein lipase-mediated clearance of chylomicron-TG could be the stimulus for increased VLDL concentrations in the fasting state. Additionally, our finding that incubation of HepG2 cells with VLDL, in addition to affecting apoB secretion posttranslationally, may increase apoB gene expression or stability of apoB mRNA, suggests a second mechanism whereby inefficient TG disposal in the periphery could result in chronic overproduction of apoB-containing lipoproteins. It will be important to demonstrate similar findings in human hepatocytes, and to determine the basis for the effect of VLDL on apoB mRNA.

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