Rat McA-RH7777 cells efficiently assemble rat apolipoprotein B-48 or larger fragments into VLDL but not human apolipoprotein B of any size

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Abstract **Studies of truncated apoB peptides in human subjects with familial hypobetalipoproteinemia, as well as of puromycin-generated spectra of nascent apoB peptides in rat and hamster liver, suggest that a minimum size is required for N-terminal fragments of apoB to be efficiently assembled into full-sized VLDL. We report here results of experiments undertaken to examine this phenomenon in greater detail by expressing individual carboxyl-truncated human apoB constructs in McArdle cells. Thus, apoB-29, -32, -37, -42, -47, -53, -70 and full length apoB-100 were transiently expressed in rat McA-RH7777 hepatoma cells, or human apoB-31 and apoB-53 were stably expressed in the same cells, and the secreted VLDL particles were characterized by kinetic gradient ultracentrifugal flotation. Calibration with rat plasma VLDL subfractions showed that about 90 and 50%, respectively, of lipoprotein particles containing endogenous rat B-100 and B-48 floated between fractions 2–8 of the 11-fraction gradient. This corresponds to the normal VLDL diameter range of about 47 to 28 nm, with the remaining half of rat B-48 recovered as HDL particles in the 1.1 g/ml range. In contrast, regardless of their size, only 2–5% of any of the truncated human apoB peptides expressed in these cells was recovered in the VLDL region of the gradient. The remaining 95+% of the lipoproteins were found as high density particles; as previously found in other systems the densities of the latter were inversely related to their peptide chain-length. Furthermore, transiently expressed full-length human apoB-100 was inefficiently secreted as VLDL by these cells, with the remainder appearing as LDL-sized particles. Thus, although we showed that McA-RH7777 cells secreted endogenous rat apoB as normal-sized VLDL, we found them unsuitable for our original purpose of using human apoB fragments to further define effects of apoB size on VLDL assembly. These cells appeared unable to efficiently use any size of human apoB for that process. Pulse-labeled untransfected McA-RH7777 cells chased in the presence of puromycin did, however, show a sharp decline in VLDL assembly efficiency for endogenous nascent rat apoB peptides shorter than B-48, similar to that originally found in normal rat liver.**—Xiao, Q., J. Elovson, and V. N. Schumaker. **Rat McA-RH7777 cells efficiently assemble rat apolipoprotein B-48 or larger fragments into VLDL but not human apolipoprotein B of any size.** ***J. Lipid Res.*** **2000.** 41: **116–125.**

Supplementary key words VLDL kinetic gradients • truncated human apoB peptides • puromycin-generated rat apoB peptides • apoB-48 minimum size requirement

Hepatic very low density lipoproteins (VLDL) are the triglyceride-rich precursors of plasma low density lipoproteins (LDL). As high plasma LDL levels are a major risk factor for cardiovascular disease, it is important to delineate the hepatic processes involved in VLDL assembly. Apolipoprotein B is the only protein on VLDL known to be essential for assembly. ApoB-100 is a large protein with 4,536 residues, while apoB-48 corresponds to the amino-terminal 48% of apoB-100 and contains 2152 residues (1). Both proteins are encoded by the same gene, and apoB-48 is produced by a postranscriptional modification of apoB-100 mRNA that converts glutamine codon 2153, CAA, to a stop condon, UAA (2, 3). In rats and mice both apoB-100 and apoB-48 are produced in liver and secreted as VLDL (4).

VLDL assembly has been under active investigation recently, and kinetic data obtained from cell culture and rat liver studies has suggested that the assembly of apoB-100-containing particles and apoB-48-containing particles follow broadly similar pathways, with at least the latter proceeding in two discrete steps (5–12). The first step involves the co-translational association of elongating nascent apoB peptides with increasing amount of triglyceride, so that upon completion of translation the size of the nascent primary particle is directly proportional to the length of the resident apoB (13–18). Actually, data from studies in HepG2 cells have shown that the length of the apoB protein

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; IDL, intermediate density lipoproteins; MTP, microsomal triglyceride transfer protein; apoB, apolipoprotein B; ALLN, N-acetyl-leucyl-leucyl-norleucinal; DMEM, Dulbecco's modified Eagle's medium; SDS-PAGE, 5%-polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl sulfide; CMV, cytomegalovirus.

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equals the circumference of the core of the primary particle (16), suggesting that apoB wraps once about the core. Microsomal triglyceride transfer protein (MTP) clearly is required for normal VLDL assembly (11, 17–20).

A second assembly step converts the primary precursor particle to the final product, i.e., nascent VLDL. The mechanism by which the primary particles take up large amounts of lipid in this second step is still unclear, but the size distribution of the markedly polydisperse products does not differ between those containing B-100 or B-48. Whether MTP plays a role in the second step is under debate, and evidence from both sides has been reported (11, 12, 19, 20). It has been suggested that a protein factor in addition to MTP may be involved in the second step (11).

Two lines of observations suggest that a minimum size be required for N-terminal fragments of apoB to efficiently participate in second-step assembly into full-sized VLDL. Studies of human subjects with familial hypobetalipoproteinemia (21–25), showed that truncated apoB corresponding to apoB-86, -50, -46, -40 and -39 all were found in the VLDL density range, albeit with increasing proportions of the shorter peptides in denser particles. In contrast, apoB-32.5, -31, and -27 were found only in the HDL density range, suggesting an inability to undergo second-step assembly for these very short peptides. In another approach, we have used puromycin-generated spectra of nascent apoB peptides in rat and hamster liver/hepatocytes to obtain evidence that N-terminal apoB peptides shorter than B-48 are much less efficiently assembled into very low density lipoprotein (VLDL) than are B-48 and larger entities (J. Elovson, unpublished observations).

We have attempted to examine this phenomenon more directly by characterizing presumed VLDL particles secreted by rat McA-RH7777 hepatoma cells both transiently and stably expressing individual carboxyl-truncated human apoB constructs. McA-RH7777 cells have been used previously to study VLDL assembly, as they are the only established hepatoma line assumed to be able to perform the second, as well as first, assembly step, albeit with varying efficiency depending on culture conditions (8), particularly in regard to B-48. However, these studies have typically equated VLDL with whatever floated to the top of isopycnic sucrose gradients with upper limiting densities of 1.01–1.03 g/ml, a range that includes IDL and VLDL particles much smaller than those normally found in rat plasma VLDL. To avoid such ambiguities, we have adopted a kinetic gradient ultracentrifugation procedure to explicitly separate and determine the size of VLDL particles, and apply it to the question whether truncated human apoB peptides can be used to establish a minimum apoB length requirement for VLDL assembly in McA-RH7777 cells.

EXPERIMENTAL PROCEDURES

Materials

Dulbecco's modified Eagle's medium (DMEM) and all other reagents for cell culture were purchased from Life Technologies, Inc. (Gaithersburg, MD). [^{35}S]methionine was purchased from DuPont NEN (Boston, MA). The cysteine protease inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN) was purchased from Boehringer Mannheim (Indianapolis, IN). Puromycin, phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, pepstatin, soybean trypsin inhibitor, and fatty acid-free bovine serum albumin were obtained from Sigma (St. Louis, MO). All chemicals used for SDS-polyacrylamide gel electrophoresis and the nitrocellulose membrane for immunoblot analysis were obtained from Bio-Rad (Hercules, CA). Peroxidase-conjugated goat anti-mouse antibody and the ECL Western blotting detection system were purchased from Amersham (Arlington Heights, IL). Centricon tubes were purchased from Amico (Beverly, MA). Monoclonal antibodies 1D1 (directed against N-terminal epitopes between residues 474–539), and $B_{sol}16$ (directed against C-terminal epitopes between residues 4154–4189), were generous gifts from Drs. R. W. Milne and Y. L. Marcel (Ottawa Heart Institute). Affinity-purified rabbit antibodies to rat apoB were obtained as described (26). Restriction enzymes and other DNA modification enzymes were purchased from New England Biolabs (Beverly, MA).

Preparation of human plasma VLDL

Blood was drawn from a normolipemic donor after a 12-h fast, and plasma was isolated after centrifugation in a table-top ultracentrifuge. EDTA, sodium azide, and gentamycin sulfate were added to 20 ml of plasma to final concentrations of 0.04%, 0.05%, and 0.005%, respectively. The plasma VLDL was obtained by centrifugation at 45,000 rpm at 20°C for 24 h, using a Beckman Ti-75 rotor and Beckman LE-80K ultracentrifuge (Beckman, Palo Alto, CA). VLDL was recovered with a pipette from the top 2 ml of each tube.

Kinetic gradient ultracentrifugation of human VLDL

Human VLDL was subfractionated by kinetic gradient ultracentrifugational flotation. A 1-ml sample of human VLDL, 0.6 mg protein/ml, adjusted with NaBr to solvent density of 1.32 g/ml and containing 1% Ficoll (Sigma, St. Louis, MO) to stabilize the interface, was layered under a 10-ml linear sodium bromide gradient, density 1.25–1.30 g/ml, and centrifuged in a Beckman SW41 rotor at 18,000 rpm and 20°C for 90 min. The gradient was collected by pipetting eleven 1-ml fractions from the top.

Analytical ultracentrifugation of human VLDL fractions

Each kinetic gradient fraction was concentrated 10-fold by Centricon-30 centrifugation (Amico, Beverly, MA) and divided into three aliquots. The three samples were brought to a protein concentration of 0.15 mg/ml and final NaBr solvent densities of 1.06 g/ml, 1.12 g/ml, and 1.18 g/ml, respectively, and simultaneously subjected to velocity analytical ultracentrifugation at 20°C, using regular cells in the An 60 Ti rotor in a Beckman Optima XL-A (Beckman, Palo Alto, CA) ultracentrifuge. Two consecutive centrifugations were performed at speeds ranging from 8,000 to 20,000 rpm depending upon particle size, with samples redispersed between the runs by gentle shaking of the ultracentrifuge cells, which had been removed from the rotor. Sedimentation coefficients were calculated from a plot of the center of the symmetrical boundaries, r, as ln r versus time, t. The buoyant densities of VLDL from each fraction were determined from the density-intercept of an ηs vs. ρ plot. The flotation rates were converted to standard s_f^0 values, defined as the negative sedimentation coefficient in an aqueous NaCl solvent of density 1.063g/ml and η = 0.01028 poise at infinite dilution. VLDL molecular weights were calculated by solving two simultaneous equations for molecular weight, M, and particle radius, R:

$$s = M(1-\bar{v}\rho)/(N6\pi\eta Rf/f_0) \qquad \textit{Eq. 1}$$

$$(4/3)\pi R^3 = M\bar{v}/N \qquad \textit{Eq. 2}$$

where $\bar{v}$, the partial specific volume, is taken as the reciprocal of the buoyant density of the lipoprotein. The solvent density, ρ, equals 1.063 g/ml, and the viscosity, η, equals 0.01028 poise. N is Avogadro's number. The value for the frictional ratio, f/f_0, was assumed to be 1.08 for VLDL particles.

Construction of carboxyl-terminal truncated apoB expression plasmids

All expression plasmids were prepared using the mammalian expression vector pcDNA1 (Invitrogen, Carlsbad, CA). Truncated apoB cDNAs were prepared using pRSV/B48 (27) and PB100 (28) as starting material. pB100 was a generous gift from Dr. Zemin Yao (Lipoprotein and Atherosclerosis Group, University of Ottawa Heart Institute, Ottawa, Canada). The plasmid pcDNA1/B70 (nucleotides 1–9983 of the apoB-100 cDNA) was generated by excision of the NotI to AvrII fragment from pB100 and ligation into pcDNA1 that had been digested with NotI and XbaI. The plasmid pcDNA1/B53 (nucleotides 1–7334 of apoB) was generated from pB53. To make pB53, we first cleaved pB100 with KpnI and MluI (nucleotide 7011 of apoB) to get the pB50 fragment (including the pCMV5 vector and nucleotides 1–7011 of apoB), and we then generated a PCR fragment (nucleotides 6929–7334 of apoB) using two primers (the upper primer was 5′-GCAGCTTAAGAGACACATACAGAA, and the lower primer was 5′-CGCGGTACCTTCTTCACAGCATCATCAATAAAT-3′). The PCR fragment was digested with KpnI and MluI and ligated into pB50 to generated pB53. The plasmid pcDNA1/B53 was generated by excision of the NotI to SmaI fragment (nucleotides 1–7334 of apoB) from pB53 and ligation into pcDNA1 that has been cleaved with XbaI, end-filled with Klenow fragment, and subsequently cleaved with NotI. The plasmid, pcDNA1/B47 (nucleotides 1–6506 of apoB-100 cDNA), was generated by cutting out EcoRI to EcoRI (polylinker and nucleotide 6506 of apoB) from pRSV/B48 and ligating it into pcDNA1 that has been linearized with EcoRI. The plasmid pcDNA1/B42 (nucleotides 1–5848 of apoB) was prepared by cleaving pRSV/B48 with ClaI (nucleotide 5848 of apoB), end-filling with Klenow fragment, and subsequently cleaving with EcoRI (polylinker), and then ligating the EcoRI to ClaI fragment into pcDNA1 that has been digested with EcoRI and EcoRV. The plasmid pcDNA1/B37 (nucleotides 1–5289 of apoB) was generated by cutting out the NotI to SalI (nucleotide 5289 of apoB and polylinker) fragment from pcDNA1/B42, end-filling and religating the remaining vector. The plasmid pcDNA1/B32 (nucleotides 1–4499 of apoB) was generated by cutting out the NsiI to NsiI (nucleotide 4499 of apoB and polylinker) fragment and religating the remaining vector. The plasmid pcDNA1/B29 (nucleotide 1–4123 of apoB) was generated by cutting out the XhoI to XhoI (nucleotides 4123 of apoB and polylinker) fragment from pcDNA1/B47 and religating the remaining vector.

Cell culture and transfection

McA-RH7777 cells (ATCC CRL 1601) were obtained from the American Type Culture Collection (Rockville, MD). McA-RH7777 cells stably transformed with pB31 and pB53 (referred to as B31 and B53, respectively) were previously described (29). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum, re-fed every other day, and split 1:20 in the same medium every 7 days. McA-RH7777 cells were transiently transfected for 16 h using standard DNA-$Ca_3(PO_4)_2$ coprecipitation procedure (27). After 2 washes with phosphate-buffered saline (PBS), transfected cells were incubated for another 48 h in oleate-supplemented medium (DMEM + 20% FBS + 0.36 mm oleate + 0.5% BSA), prior to metabolic labeling, or to having the conditioned fresh medium collected for analysis by Western blotting.

Metabolic labeling

On the day of labeling, transfected or untransfected McA-RH7777 cells were washed twice with PBS and incubated in methionine-free DMEM for 3 h. They were then pulse-labeled for 3 h in 4 ml of methionine-free, oleate-supplemented medium containing 0.4 mCi of [^{35}S]methionine. The pulse medium was collected into 10 mm EDTA, 0.5% sodium azide, 10 mm phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml trypsin inhibitor, 40 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1.92 μg/ml ALLN. Total lipoproteins were isolated from the media as described (29), and subjected to kinetic gradient ultracentrifugation.

Kinetic gradient ultracentrifugation of lipoproteins secreted by McA-RH7777 cells

[^{35}S]methionine-labeled lipoproteins, or unlabeled lipoproteins from 48-h conditioned media, were concentrated, brought to NaBr solvent density of 1.32 g/ml, and separated by flotation through a linear 1.25–1.30 g/ml NaBr gradient at 18,000 rpm for 90 min in an SW 41 rotor at 20°C, as described above for human VLDL. ApoB-containing particles from each fraction were precipitated by adding 1.5 mg of fumed-silica (Cab-O-Sil, Sigma) (30), the apoB eluted from the silica in 30 μl SDS-gel sample buffer containing 4% SDS/2% beta-mercaptoethanol, and resolved on 5% SDS-polyacrylamide gels. [^{35}S]methionine-labeled peptides were visualized and quantitated on the Phospho-Imager using the Image-QuaNT software (Molecular Dynamics, Sunnyvale, CA). Unlabeled apoB peptides were analyzed by immunoblotting as described (29), using monoclonal antibodies 1D1 or B_{sol}[16] to detect N- or C-terminal epitopes, respectively.

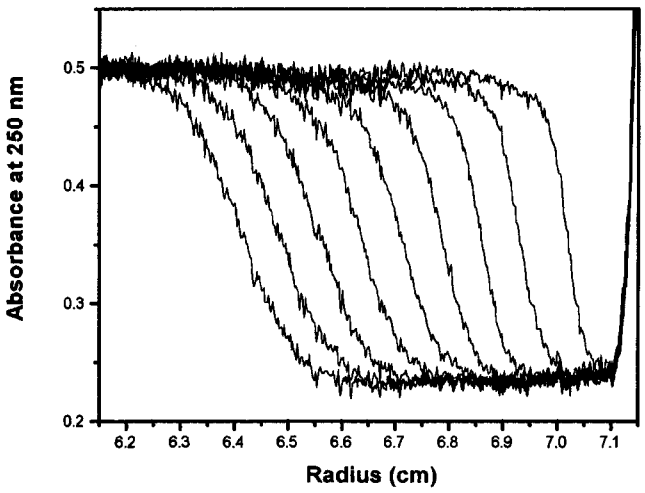

Fig. 1. Consecutive ultracentrifuge scans of a fraction of human VLDL. Triglyceride-rich lipoproteins from a normolipemic donor were obtained by centrifugation of plasma at 45,000 rpm at 20°C for 24 h, after which the top 2 ml was removed from each tube. The VLDL were then subfractionated by flotation in a kinetic gradient at 18,000 rpm and 20°C for 90 min. This gradient was then separated into 11 fractions by pipetting from the top as described under Experimental Procedures. Each of the 11 fractions was then examined in an analytical ultracentrifuge at 250 nm at three densities. Shown are the serial scans taken at 4-min intervals during a single ultracentrifuge run of fraction 4 at a single density, d 1.063 g/ml, typical of the remaining ultracentrifuge runs for VLDL fractions 4–8, which showed exhibiting sharp boundaries and flat plateaus. Fractions 2 and 3 also had sharp boundaries, but some heterogeneity at the leading edges. Flotation was from right to left.

The density of the primary particles secreted by the transfected cells was determined by isopycnic density centrifugation as previously described (16).

Puromycin treatment of McA-RH7777 cells

This was conducted essentially as described (23). McA-RH7777 cells, grown to near-confluence (>80%) on 100-mm plates, were labeled as described above with 0.40 mCi of [^{35}S]methionine for 7.5 min, followed by addition of 2000-fold excess unlabeled methionine and 300 μm puromycin to stop further [^{35}S]methionine incorporation and to release polysomal pulse-labeled nascent apoB peptides. The cells were immediately rinsed once with complete DMEM containing 150 μm puromycin, 15% fetal bovine serum, 0.36 mm oleate, and 0.5% BSA, and further incubated in this chase medium for 3 h at 37°C. Negative controls were carried out without the addition of puromycin. The chase media were collected and total lipoproteins were isolated as described above. For the immunoprecipitation (29) experiment in Fig. 7, VLDL was first isolated by the same procedure from the unadjusted culture medium, and the denser primary particles were separately collected by a second flotation of the VLDL infranatant adjusted to d 1.22 g/ml. The fractions were adjusted to 1 m NaCl, lysed in 2% Triton X-100/1% sodium sarcosylate/0.2% SDS in the presence of protease inhibitors as above, and incubated overnight with non-immune serum. After removing non-specific materials by absorption to *Staphylococcus aureus* cells, the precleared lysates were reincubated overnight with excess rabbit anti-rat apoB antibody, and specific immune-complexes recovered by a second addition of *Staphylococcus aureus* cells. The cells were washed twice with in 0.2% Triton X-100/0.1% sodium sarcosylate/0.02% SDS, boiled in sample buffer containing 1% SDS, and the released apoB peptides were separated by SDS-PAGE.

RESULTS

Calibration of kinetic gradient ultracentrifugation using human VLDL

Human VLDL were separated by velocity flotation through a NaBr density gradient. The procedure separates VLDL particles essentially according to size, as any small differences in particle densities are insignificant compared to the very high average density of the gradient used. The human VLDL introduced at the bottom of the gradient (fraction 11) floated upwards and were distributed throughout the gradient with the peak at fraction 7. Fractions 4–8 produced sharp boundaries with flat plateaus in the analytical ultracentrifuge, indicating that the VLDL and remnants in these fractions were relatively uniform in size. A typical fraction, fraction 4, is shown in **Fig. 1**. Fractions 2 and 3 showed sharp boundaries that blended into the plateaus, indicating some heterogeneity at the leading edges. **Figure 2** shows the determination of the molec-

TABLE 1. Molecular and hydrodynamic properties of human VLDL fractions

VLDL Fraction	s_f^0	$\bar{v}$ (ml/g)	VLDL Mol. Wt. (Mda)	Buoyant Density	Particle Diameter
				g/ml	*nm*
2	132.3	1.057	32.0	0.946	47.4
3	114.6	1.046	29.8	0.956	46.2
4	93.1	1.042	23.3	0.960	42.6
5	74.6	1.037	17.8	0.964	38.8
6	56.2	1.030	13.1	0.971	35.0
7	42.0	1.016	10.7	0.984	32.6
8	30.3	1.010	7.5	0.990	28.8

s_f^0 is the concentration-corrected average flotation coefficient for the fraction determined in an aqueous NaCl solvent with density 1.063 g/ml and viscosity 0.01026 Poise at 26°C. $\bar{v}$ is taken as the reciprocal of buoyant density determined from ηs vs. ρ plot.

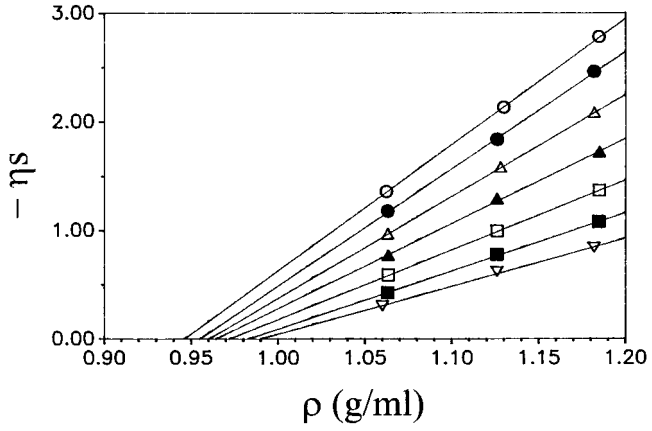

Fig. 2. Buoyant density determination for human VLDL fractions 2–8. The product of solvent viscosity, η; (in units of Poise, P), and flotation coefficient, -s (in units of Svedbergs, S), was plotted as a function of solvent density, ρ. The horizontal intercept, -ηs = 0, yields the buoyant density of the lipoproteins. Fraction 2, ○; fraction 3, ●; fraction 4, △; fraction 5, ▲; fraction 6, □; fraction 7, ■; fraction 8, ▽. All measurements are duplicates with single symbols representing closely overlapping values.

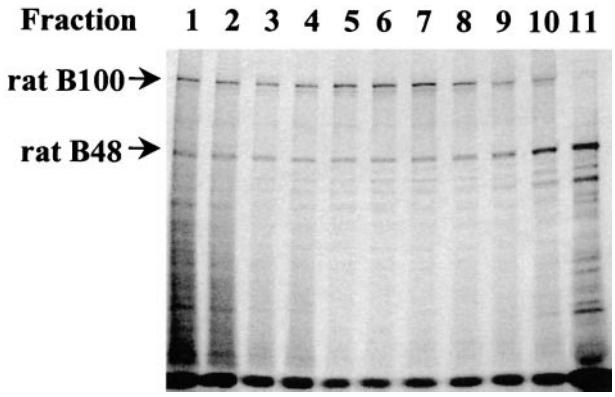

Fig. 3. Kinetic gradient ultracentrifugation of endogenous apoB-containing lipoproteins present in the culture medium of McA-RH7777 cells. McA-RH7777 cells were incubated with [^{35}S] methionine (in culture medium) for 3 h. The culture medium was collected, and the total lipoprotein was isolated from the culture medium by ultracentrifugation (see Experimental Procedures). The lipoproteins were subfractionated by kinetic gradient ultracentrifugation, and the whole gradient was separated into 11 fractions by pipetting from the top. The apoB-100 and apoB-48 were recovered from each fraction by absorption onto Cab-O-Sil, and they were subjected to electrophoresis on 5% SDS-PAGE and visualized on a Phosphor-Imager. The bands corresponding to apoB-100 and apoB-48 were identified by comparing their positions to protein molecular weight markers run on the same gel. This experiment was repeated three times.

TABLE 2. Efficiency of assembly of endogenous rat apoB-100 and apoB-48 into VLDL in McA-RH7777 cells

Rat ApoB Size	% of ApoB in VLDL	% of ApoB in Primary Particles
B-100	90 ± 3	10 ± 3
B-48	50 ± 15	50 ± 15

Each experiment was repeated 3 times.

ular weight and the buoyant densities for the human VLDL fractions 2–8. Using the average flotation coefficients and buoyant densities for each fraction, the average molecular weights and particle radii can be calculated from equations 1 and 2, as described in Experimental Procedures. The results, shown in **Table 1**, define fractions 1 to 8 as containing VLDL particles.

McA-RH7777 cells can assemble VLDL efficiently

To determine the efficiency with which endogenous apoB proteins assemble VLDL, McA-RH7777 cells were labeled for 3 h with [^{35}S]methionine. The total lipoproteins secreted into the media were recovered by ultracentrifugation and separated by kinetic flotation as described above for human VLDL. Lipoproteins were recovered from each fraction by absorption onto fumed-silica and the distribution of apoB radioactivity was determined by SDS-polyacrylamide gel electrophoresis and Phosphor-Imager analysis (**Fig. 3**). The relative assembly efficiencies for apoB-100 and -48 were defined as the percentages of their total radioactivities recovered in VLDL (fractions 1–8). The results of several experiments are shown in **Table 2**. It can be seen that oleate-supplemented McA-RH7777 cells secreted 90% of their endogenous rat apoB-100 on lipoproteins that floated as VLDL (fractions 1–8), with the remaining 10% trailing into the IDL-LDL range of the gradient (fractions 9–11). On the other hand, only about one half of endogenous apoB-48 was secreted on lipoproteins that floated as VLDL, with the other half remaining in the bottom two fractions of the gradient, as expected for apoB-48-containing primary particles secreted without traversing the second assembly step. The bands below apoB-48 are most likely products of apoB degradation occurring after lipoprotein assembly, as they also appear in the VLDL fractions.

Thus, we conclude that oleate-supplemented McA-RH 7777 cells can assemble endogenous rat apoB-100 and apoB-48 into VLDL with about 90% and 50% relative efficiencies, respectively.

Human apoB proteins transiently expressed in McA-RH7777 cells are assembled into VLDL with low efficiency

We next turned to our original question of whether expression of truncated human apoB peptides in McA-RH7777 cells can be used to establish a minimum apoB length requirement for VLDL assembly. As described under Experimental Procedures, a CMV promoter-driven vector expressing human apoB-100 was used to generate a series of plasmids containing the cDNAs for human apoB-29, apoB-32, apoB-37, apoB-42, apoB-47, apoB-53 and apoB-70. The sizes and amino acid numbers of these apoB constructs are shown in **Fig. 4**. Transiently transfected McA-RH7777 cells were labeled with [^{35}S]methionine, the conditioned media were collected and the secreted lipoproteins were analyzed as described for untransfected cells. The images of the kinetic gradients, and the corresponding percentages of the total radioactivities of each truncated apoB peptide recovered in the VLDL fractions, are shown in **Fig. 5** and **Table 3**, respectively. It can be seen that small amounts of the C-terminally truncated human apoB-containing lipoproteins were found throughout the gradients; however, in each instance the vast majority corresponded to primary particles (fractions 9–11), with only about 2–5% floating as VLDL (fractions 1–8). Thus, while McA-RH7777 cells can incorporate C-terminally truncated human apoB into VLDL, the process was of much lower efficiency than that for endogenous rat apoB-100 and apoB-48. The many-fold greater secretion of total human apoB compared to endogenous rat apoB presumably resulted from multiple copies of plasmids entering the cells during transfection. In contrast to a study reported by another research group (10), we did not observe loss of VLDL formation when human apoB was truncated to human apoB-29. All C-terminally truncated human apoB proteins examined, including apoB-70, B-53, B-47, B-42, B-37, B-32, as well as B-29, assembled VLDL with similar

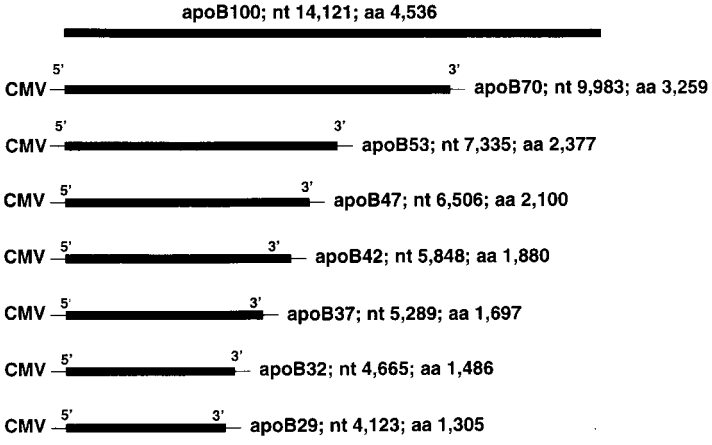

Fig. 4. ApoB constructs used to transfect McA-RH7777 cells. PcDNA1, a CMV-based eukaryotic expression vector, was used to express constructs containing the apoB coding sequence corresponding to apoB-100, B-70, B-53, B-47, B-42, B-37, B-32, and B-29. The corresponding number of nucleotides and amino acids is also given.

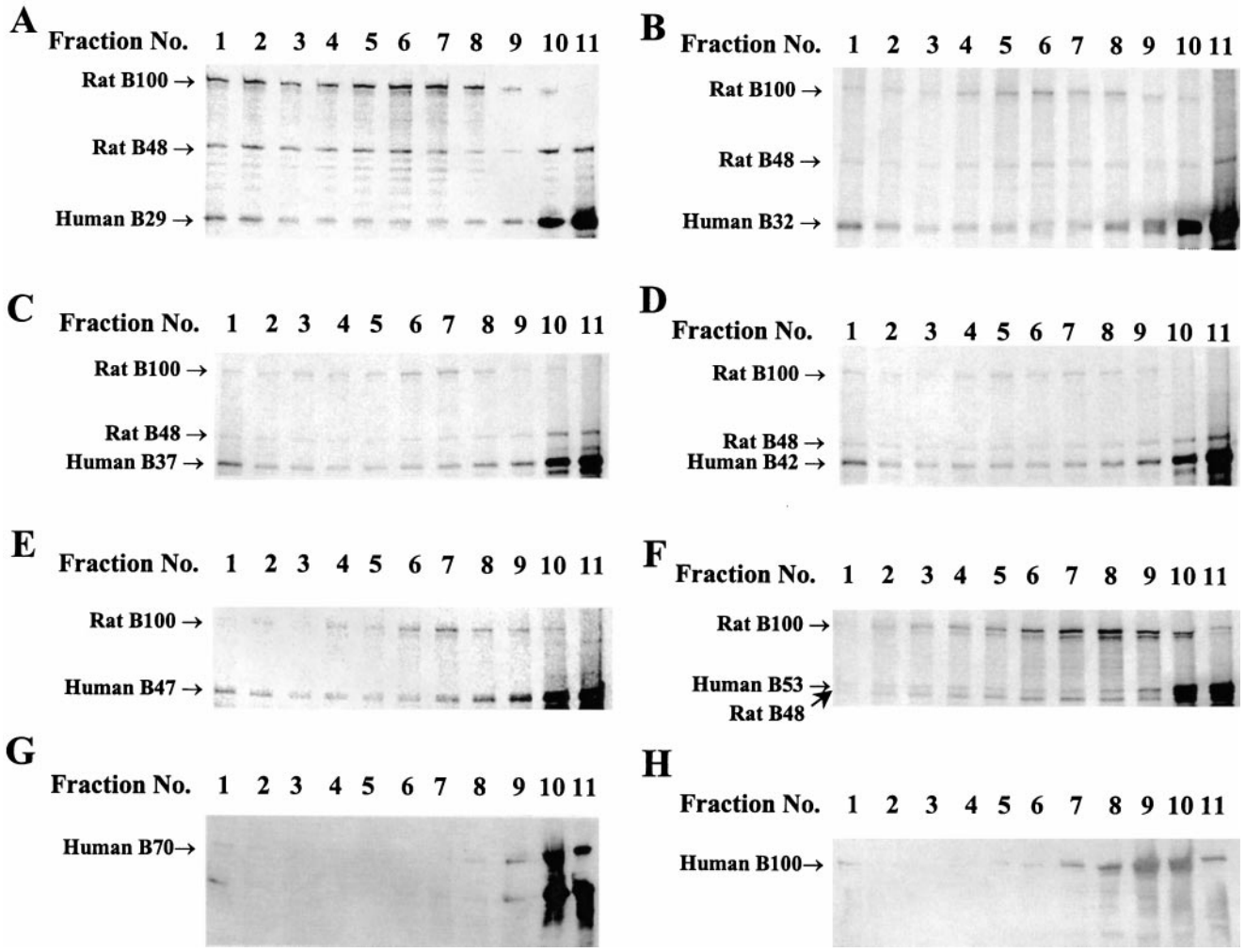

Fig. 5. Kinetic gradient ultracentrifugation of various C-terminally truncated human apoB-containing lipoproteins present in the culture medium of transiently transfected McA-RH7777 cells. After transient transfection with the human apoB constructs, McA-RH7777 cells were either incubated for 3 h in [^{35}S]methionine–containing media (panels A–F) or for 2 days in unlabeled media (panels G, H). The media were collected, and the total lipoproteins were isolated by ultracentrifugation (see Experimental Procedures). The lipoproteins were subfractionated by kinetic gradient ultracentrifugation and 11 equal fractions were collected from the top. ApoB proteins were recovered from each fraction by absorption onto Cab-O-Sil and subjected to electrophoresis on 5% SDS-PAGE. ^{35}S-labeled apoB peptides (panels A–F) were visualized on the Phosphor-Imager, and the bands corresponding to endogenous rat apoB-100 and apoB-48 were identified by comparing their positions to protein molecular weight markers run on the same gel. The expressed human apoB protein was identified by its position on the gel relative to apoB-100 and apoB-48. After transfection with constructs expressing human apoB-70 (panel G), or apoB-100 (panel H), peptides were visualized by Western blotting and probing with human apoB-specific monoclonal antibodies 1D1 (N-terminal epitope between residues 474–539), and $B_{sol}16$ (C-terminal epitopes between residues 4154–4189), respectively. Panels show results from McA-RH7777 cells transiently transfected with A: PcDNA1/B29; B: PcDNA1/B32; C: PcDNA1/B37; D: PcDNA1/B42; E: PcDNA1/B47; F: PcDNA1/B53; G: PcDNA1/B70; H: PB100.

low efficiencies regardless of length. In fact, as shown in Fig 5H, cells transfected with the full-length construct similarly secreted human apoB-100 almost entirely as LDL-sized primary particles (centered on fractions 8–10), with only trace amounts entering the VLDL-proper region of the gradient. Thus, we did not observe any significant differences in the uniformly low incorporation into VLDL of all human apoB proteins between B-29 and B-100.

As determined by isopycnic gradient centrifugation, the densities of the human apoB-containing lipoproteins denser than VLDL were in good agreement with those reported in previous studies (**Table 4**) of McA-RH7777 and HepG2 cells (16).

Human apoB proteins stably expressed in McA-RH7777 cells are also assembled into VLDL with low efficiency

It seemed possible that transient transfection could produce a huge expression of apoB protein confined to a small subset of cells, overwhelming the ability of these cells to form VLDL from the apoB, and thereby producing instead a large number of small, dense, LDL-sized particles. We have tested this possibility with two stably transfected cell lines that produced human apoB-31 and human apoB-53 (and apoB-48) proteins. If transient transfection created a preponderance of small, dense particles, we should see a different result when we used stably transfected cells. The results are shown in **Fig. 6**. In the upper

TABLE 3. The efficiency of assembly of different C-terminally truncated human apoB peptides into VLDL in transiently transfected McA-RH7777 cells

Human ApoB Size	% of ApoB in VLDL	% of ApoB in Primary Particles
B-70	2	98
B-53	3	97
B-47	5	95
B-42	3	97
B-37	4	96
B-32	4	96
B-29	5	95

panel, human apoB-48, presumably produced by enzymatic cleavage of apoB-53, appeared along with apoB-53 in fractions 10 and 11, when assayed by immunoblotting with monoclonal antibody 1D1, which did not recognize rat apoB. Aside from a trace in fraction 9, more rapidly floating VLDL were not visualized, and clearly they did not contain more human apoB. The enhanced sensitivity of [^{35}S]methionine blotting was used to visualize the human apoB-31; again, this protein appeared in fractions 10 and 11, with only a small amount, perhaps along with a contaminating band, in the other fractions. Again, there was only a small amount of protein present in these other fractions. Thus, we see essentially the same picture with the stably transfected apoB as with the transiently transfected species shown above. The production of an abundance of the small, dense species by the stably transfected cells would seem to rule out the contrasting hypothesis that the abundance of small particles is a consequence of the transient transfection.

Puromycin treatment of McA-RH7777 cells

The unanticipated failure of McA-RH7777 cells to efficiently incorporate any size of human apoB into VLDL obviously rendered moot the use of such fragments to further define effects of apoB size on VLDL assembly in these cells. It appeared worthwhile, nonetheless, to use these cells to examine this effect in regard to the spectrum of endogenous nascent rat apoB peptides generated by puromycin treatment. Untransfected McA-RH7777 cells were pulsed with [^{35}S]methionine for 7.5 min, chased in the presence of puromycin for 3 h, and the secreted apoB-containing lipoproteins were analyzed as described in Experimental Procedures. Addition of puromycin to the chase medium immediately after the pulse of [^{35}S]methionine generated a "line spectrum" of apoB fragment sizes (presumably reflecting ribosomal pausing during translation). **Figure 7** compares the apoB peptide spectrum of unfractionated VLDL with that of denser primary particles in samples of both control and puromycin-treated cells. The fluorogram in Fig. 7 shows that, in addition to the apoB-100 and apoB-48 secreted by control cells (lane 4), cells chased in puromycin also secrete dense primary particles containing several prominent nascent apoB peptides smaller than B-48 (lane 3). Significantly, however, none of these shorter peptides are assembled into the VLDL secreted by puromycin-treated cells, which instead display a number of prominent nascent apo-B peptides larger than apoB-48 (lane 1), which are not seen in VLDL from control cells (lane 2). Thus, only puromycin-generated nascent apoB peptides larger than B-48 achieved second-step assembly into VLDL during the 3-h chase, while peptides shorter than B-48, being excluded from this pathway, were secreted as primary particles.

These results were confirmed in a second experiments where the lipoproteins secreted by puromycin-treated cells were further analyzed by kinetic gradient ultracentrifugation (**Fig. 8**). As shown in panel A, VLDL particles in fractions 1–8 contain a spectrum of bands between apoB-100 and apoB-48, which may be presumed to represent nascent peptides rather than proteolytic degradation products, as they are absent from the corresponding fractions from cells not treated with puromycin (Fig. 3), while nascent peptides smaller than apoB-48 again were almost exclusively found on primary particles in the bottom two fractions. The cut-off at apoB-48 is graphically illustrated in panel B, which superimposes the scans of lanes 5 (VLDL) and 11 (primary particles). Thus, in contrast, the situation for human apoB, the second step of VLDL assembly in McA-RH7777 cells, discriminates against endogenous rat apoB peptides smaller than apoB-48.

TABLE 4. The buoyant densities of different C-terminally truncated human apoB-containing primary particles secreted by transiently transfected McA-RH7777 cells

ApoB Size	Buoyant Density
	g/ml
B-53	1.105
B-47	1.119
B-42	1.131
B-37	1.140
B-32	1.149
B-29	1.161

DISCUSSION

In this paper, we use kinetic gradient ultracentrifugation to characterize and separate VLDL particles. The procedure is sensitive and can be applied to studies in cell culture. Lipoprotein solutions are adjusted to 1.32 g/ml with NaBr, loaded under a linear sodium bromide gradient, density 1.25 g/ml to 1.30 g/ml, and centrifuged in the SW41 rotor at 18,000 rpm for 90 min. Lipoproteins float towards the top at rates proportional to their flotation coefficients, and the conditions are chosen so as to distribute VLDL according to their particle size in the top 8 out of a total of 11 fractions. Lipoproteins from each fraction can be recovered by immunoprecipitation or adsorption to fumed silica, run on SDS-PAGE, and quantitated on the Phosphor-Imager. We alluded earlier to the problems of interpretation in previous studies of VLDL assembly in McA-RH7777 cells, where VLDL typically were

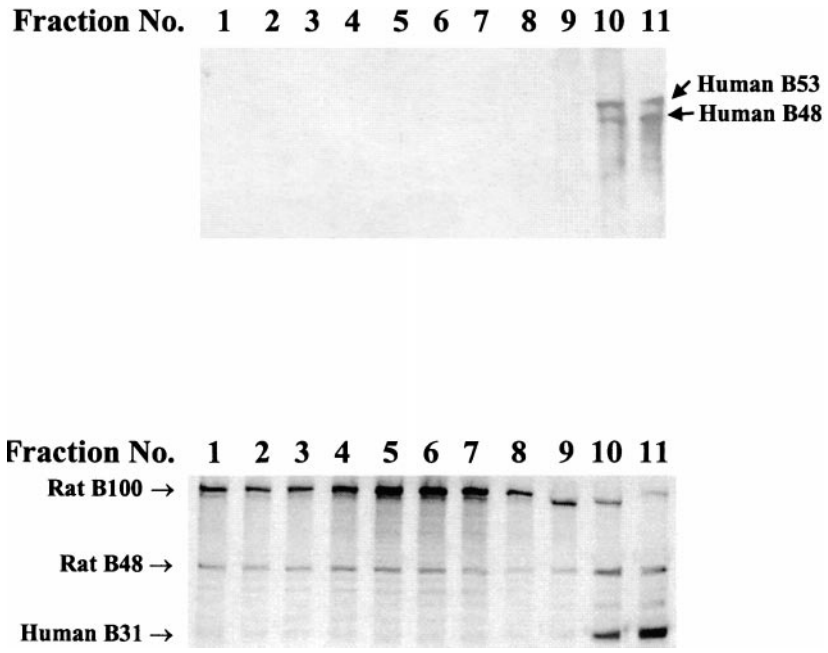

Fig. 6. Kinetic gradient ultracentrifugation of the lipoproteins secreted by stably transfected McArdle cells. The upper figure shows lipoproteins isolated from the culture medium of McA-RH7777 cells stably expressing human B53, as detected on an immunoblot using monoclonal antibody 1D1, which does not recognize rat apoB. The lower figure shows [^{35}S]methionine-labeled apoB peptides isolated from the culture medium of McA-RH7777 cells stably expressing human B31.

equated with whatever floated to the top of isopycnic sucrose gradients, i.e., a range of particles that would also include IDL as well as VLDL particles much smaller than those normally found in rat plasma. In contrast, using our procedure, we now unambiguously show that oleate-supplemented McA-RH7777 cells secrete about 90% and 50%, respectively, of apoB-100 and of apoB-48 as true VLDL particles.

We next applied the kinetic gradient ultracentrifugation procedure to our original purpose of expressing a series of C-terminally truncated human apoB peptides in McA-RH7777 cells, in order to determine whether the second step of VLDL assembly could be shown to require a minimum length of N-terminal sequence (31). However, as detailed above, this approach was rendered moot by the fact that McA-RH7777 cells turned out to be unable to efficiently incorporate any human apoB peptide, between B-29 and full-sized B-100, into VLDL, regardless of size. Other than obviously being disappointing, this result was also unexpected, as a recent study (10) reported that permanently transfected McA-RH7777 cells secreted human B-48, B-42, B-37, and B-34, but not apoB-29, in part as VLDL, as well as on denser primary particles. Although we cannot definitely account for these different results, they are likely due to the different methods of analysis used, specifically the ambiguities attending the defining of "VLDL" as anything which floated to the top of isopycnic sucrose gradients.

It should be pointed out that there is no assurance that even the 2–5% of the various human apoB peptides in our experiments that appeared in the VLDL region of the gradient did so as the result of having undergone true second-step VLDL assembly. Elimination of the trivial alternative explanation, i.e., their "piggy-backing" by nonspecific adsorption to endogenous rat apoB-containing VLDL, requires demonstration by species-specific immuno-absorption that the rat and human apoB peptides reside on separate particles (29). Although we have previously demonstrated this to be the case for a different human apoB construct (29), the amounts of materials available in the present study were insufficient for such analysis.

It is difficult to account for the inability of McA-RH7777 cells to efficiently process human apoB through the second step of VLDL assembly. It might be thought that the marked overexpression of the human apoB proteins in our transiently transfected McA-RH7777 cells could overwhelm their total capacity for second step assembly, perhaps inherently limited by their ability to supply sufficient triglycerides for that process. However, two lines of experimental evidence argue against this possibility. First, in contrast to a previous report (32), overexpression of human apoB in our McA-RH7777 cells did not change the efficiency with which they assembled their endogenous apoB-100 and apoB-48 into VLDL. Clearly, lipid availability, or any other non-species-specific limiting factor, should affect assembly of rat and human apoB to the same degree, which was not observed. The second line of evidence comes from our experiments with stably transfected McA-RH7777 cell lines which express human apoB-53 and human apoB-31 peptides at much lower levels, comparable to those of their endogenous rat apoB; yet, these low levels of human apoB peptides are as inefficiently assembled into VLDL as are the same peptides massively expressed in transiently transfected cells.

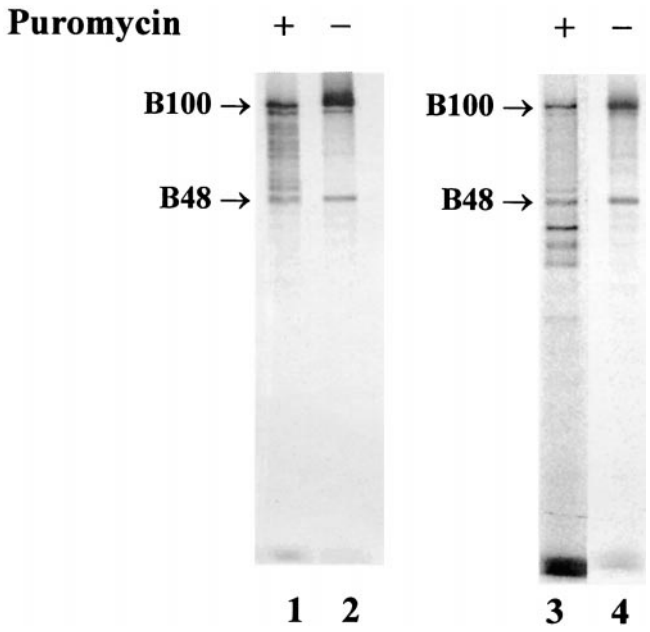

Fig. 7. SDS-PAGE analysis of [^{35}S]methionine-labeled apoB peptides in VLDL and denser precursor particles secreted by McA-RH7777 cells in pulse-chase experiments performed in the presence or absence of puromycin. McA-RH7777 cells were pulsed for 7.5 min with [^{35}S]methionine, followed by 3 h chases in unlabeled medium with or without added puromycin as described under Experimental Procedures. The chase media were collected and VLDL and primary particles were recovered by sequential flotation at d 1.006 g/ml and d 1.22 g/ml. ApoB peptides were recovered from each fraction by immunoprecipitation, separated by 5% SDS-PAGE, and visualized on the Phosphor-Imager. This experiment was performed twice with similar results. Lanes 1 and 2: VLDL with and without puromycin. Lanes 3 and 4: primary particles with and without puromycin.

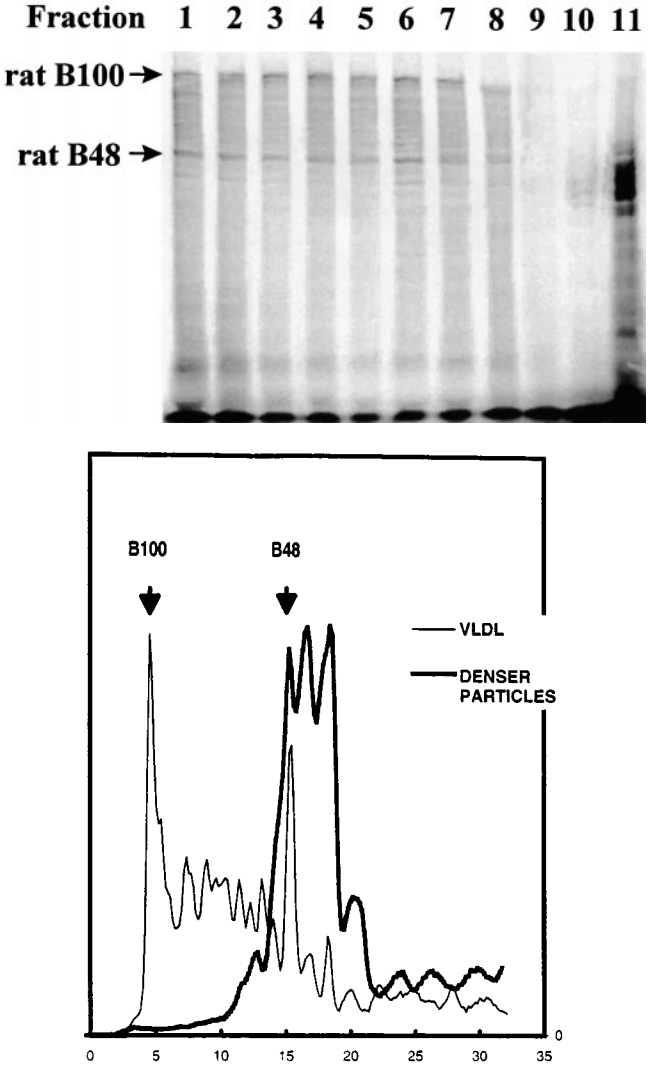

Fig. 8. Kinetic gradient ultracentrifugation of [^{35}S]methionine-labeled apoB-containing particles secreted by McA-RH7777 cells in pulse-chase experiments performed in the presence of puromycin. McA-RH7777 cells were pulsed for 7.5 min with [^{35}S]methionine, followed by 3 h chase in unlabeled medium with puromycin as described under Experimental Procedures. The chase medium was collected, and lipoproteins were recovered by flotation at d 1.22 g/ml further subfractionated by kinetic gradient ultracentrifugation, as described in the legend of Fig. 5. Panel A shows the corresponding autoradiograph. Panel B superimposes scans of lanes 5 (midpoint VLDL) and 11 (precursor particles) of panel A. Thin line: lane 5; thick line: lane 11. This experiment was performed twice with similar results.

Another obvious possibility would be species differences, i.e., if the rat assembly machinery did not recognize human apoB sequences well. One candidate protein could be MTP, if it indeed is required in the second step of VLDL assembly (11, 17–20), or other unknown protein factors suggested to be required for the second step (11). On the other hand, any simple arguments about rat–human species incompatibilities would seem to be seriously weakened by the well-established ability of transgenic mice to assemble human apoB normally into VLDL. It is, however, worth remembering that McA-RH7777 cells are of hepatoma origin, and are shown here to assemble endogenous rat B-48 less efficiently into VLDL than B-100, which has not been demonstrated to be the case for mouse (or rat) liver in vivo. It is, therefore, conceivable that rat–human interspecies incompatabilities in second-step VLDL assembly could be aggravated in these transformed cells, as compared to mouse–human interactions in normal mouse liver.

We have recently reported that a McA-RH7777 permanent cell line expressing B-18/95 (a fusion of apoB-18 with the C-terminal 80–95% of the human apoB sequence) secreted significant amounts of this peptide as VLDL (29). How could this phenomenon be reconciled with our present findings? One possible explanation was that the fusion might interrupt the native apoB tertiary structure in the junction region, creating an improperly folded region of exposed hydrophobic residues, which might artifactually direct apoB-18/95 through the second assembly step.

To conclude, the results of the puromycin experiment reported here confirmed those of our unpublished experiments in normal rat liver, which showed that apoB peptides smaller than apoB-48 were not efficiently assembled into VLDL. These results raised the obvious question: does this simply represent a requirement for a minimum length of apoB peptide, or does the sequence towards the

C-terminus of apoB-48 contain particular structural motifs essential for the second step of VLDL assembly? We undertook the transfection experiments in McA-RH7777 cells reported here with the expectation of confirming the apoB-48 cut-off point using human apoB peptides as a preliminary to expressing other variously deleted, substituted, and rearranged constructs to address this question directly. With the unanticipated failure of this cell culture system, it may be necessary to use transgenic mice for such experiments, with their demonstrated ability to assemble human apoB-100 and -48 into normal VLDL.

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