Chylomicron-sized lipid particles are formed in the setting of apolipoprotein B deficiency

Robert L. Hamilton,[1,*,§] Jinny S. Wong,[*] Candace M. Cham,[†] Lars Bo Nielsen,[†] and Stephen G. Young[*,†,]**

Cardiovascular Research Institute;* Gladstone Institute of Cardiovascular Disease,† and Departments of Anatomy§ and Medicine,** University of California, San Francisco, CA 94143-0452

Abstract **The mechanisms for packaging large quantities of neutral lipids into apolipoprotein (apo) B-containing lipoproteins (chylomicrons or VLDL) are incompletely understood. However, several lines of evidence have suggested that the addition of core lipids to apoB to form a lipoprotein particle within the endoplasmic reticulum (ER) may involve two steps: first, the addition of small amounts of core lipids to membrane-bound apoB, generating a lipid-poor, small apoB-containing particle, and second, the fusion of that particle with a larger, independently formed triglyceride-rich and apoB-free "lipid particle." We sought to test this two-step hypothesis of apoB core lipidation by using electron microscopy to compare chylomicron assembly in mice that are genetically deficient in the ability to synthesize apoB in the intestine to control mice. In 19-day gestational mice (fasting setting) that were deficient in intestinal apoB synthesis, chylomicron-sized lipid particles in the lumen of the enterocyte ER were even more abundant and were 2- to 3-fold larger than those in the enterocytes of normal control mice. However, there were fewer lipid-staining particles in the Golgi apparatus, and many fewer particles in the extracellular space, compared with normal control mice. In both types of newborn suckling mice, much larger lipid particles were assembled within the lumen of the ER. They were however, less abundant and rarely reached the Golgi apparatus in fatty enterocytes of intestines deficient in apoB synthesis. These observations provide in vivo evidence that chylomicron formation could involve the synthesis of apoB-free triglyceride-rich particles within the endoplasmic reticulum (ER) lumen, and that the transport of these lipid particles out of the ER to Golgi apparatus and interstitium is facilitated by the acquisition of apoB.—**Hamilton, R. L., J. S. Wong, C. M. Cham, L. B. Nielsen, and S. G. Young. **Chylomicron-sized lipid particles are formed in the setting of apolipoprotein B deficiency. *J. Lipid Res.* 1998.** 39: **1543–1557.**

Supplementary key words absorptive enterocytes • lipoproteins • intestinal fat absorption • endoplasmic reticulum • Golgi apparatus • cytosolic lipid droplets

Gage and Fish (1) established the concept that dietary fats, and not proteins or carbohydrates, caused the appearance of "a multitude of glancing particles which look like motes in a sunbeam" when intestinal lymph or peripheral blood was viewed with the aid of a "dark-field ultramicroscope." They termed these lipid-containing particles "chylomicrons," from the Greek chylos for chyle and mikron for any minute object. Since these seminal studies of fat absorption were published nearly 75 years ago, we have learned that the core of chylomicron particles contains large amounts of triglycerides and that the surface protein components of chylomicrons (now called apolipoproteins) play critical roles in the assembly, secretion, and metabolism of these particles (2).

Apolipoprotein B (apoB) is known to have a particularly critical role in the assembly of the triglyceride-rich lipoproteins. ApoB-48 is used for chylomicron assembly in intestinal enterocytes (2–4). ApoB-100 is used for VLDL assembly in visceral yolk sac endoderm (5); and either apoB-100 or apoB-48 is used for VLDL formation in hepatocytes (2–4). We have been interested in understanding the assembly of the triglyceride-rich lipoproteins, particularly the mechanisms by which the triglyceride core is added to apoB during the assembly of these lipoproteins.

Previously, we described the absence of apoB on lipid particles of VLDL size within the smooth endoplasmic reticulum (SER) of rat hepatocytes assessed by immunoelectron microscopy (6). These findings, taken together with subsequent kinetic studies of VLDL assembly and secretion (7–11) have led to the hypothesis that the production of nascent VLDL involves the synthesis of a triglyceride-rich (and apoB-free) lipid particle in the SER, which joins a lipid-poor apoB-particle formed in the rough ER (RER) before the completed lipoprotein particle is secreted from the cell. We call this the "two-step hypothesis" for apoB core lipidation (12, 13) (**Fig. 1**). In the current study, we sought to further assess this two-step hypothesis,

Abbreviations: apoB, apolipoprotein B; ER, endoplasmic reticulum; SER, smooth ER; RER, rough ER; MTP, microsomal triglyceride transfer protein; TG, triglycerides; VLDL, very low density lipoproteins; HDL, high density lipoproteins.

[1] To whom correspondence should be addressed.

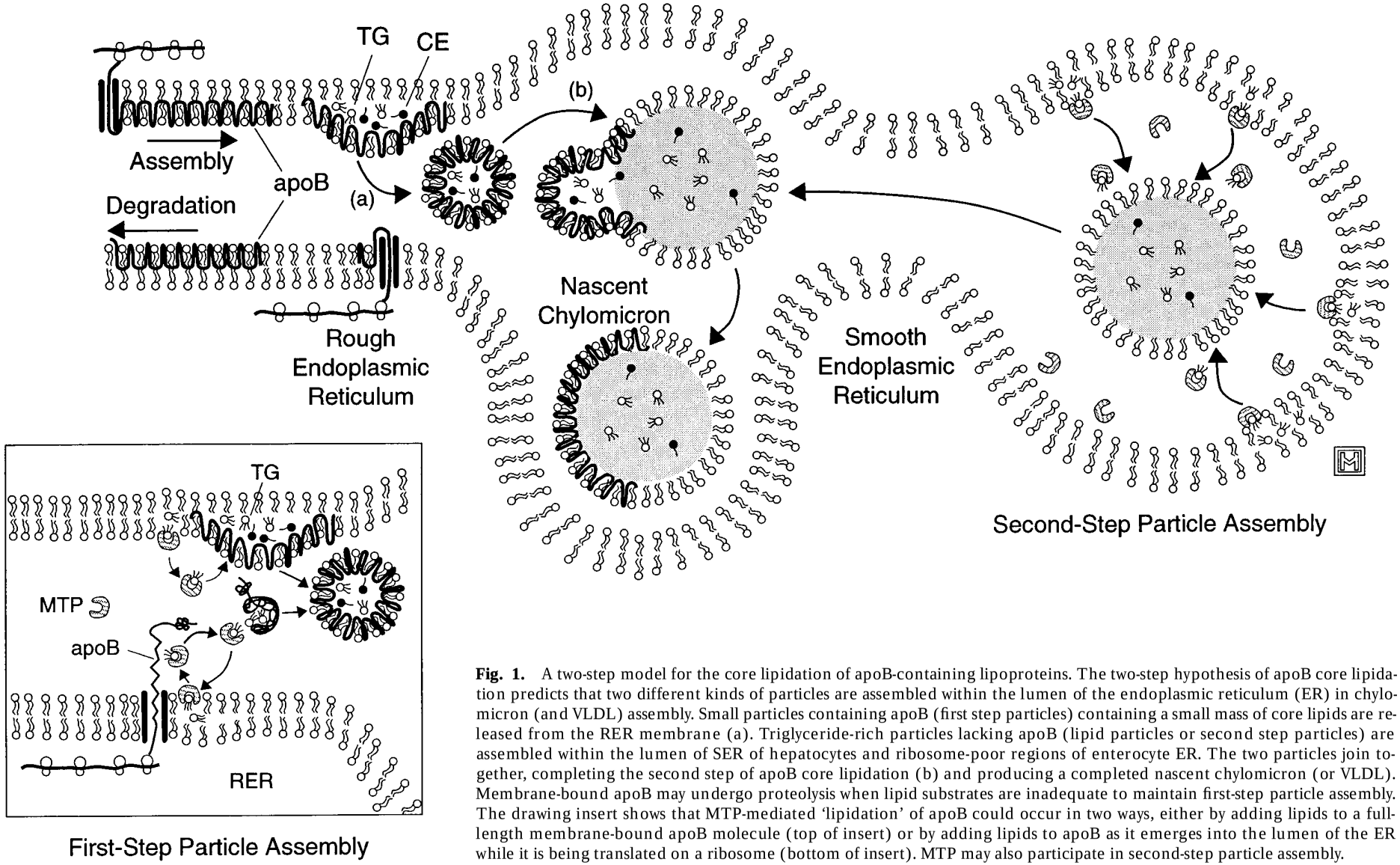


Fig. 1. A two-step model for the core lipidation of apoB-containing lipoproteins. The two-step hypothesis of apoB core lipidation predicts that two different kinds of particles are assembled within the lumen of the endoplasmic reticulum (ER) in chylomicron (and VLDL) assembly. Small particles containing apoB (first step particles) containing a small mass of core lipids are released from the RER membrane (a). Triglyceride-rich particles lacking apoB (lipid particles or second step particles) are assembled within the lumen of SER of hepatocytes and ribosome-poor regions of enterocyte ER. The two particles join together, completing the second step of apoB core lipidation (b) and producing a completed nascent chylomicron (or VLDL). Membrane-bound apoB may undergo proteolysis when lipid substrates are inadequate to maintain first-step particle assembly. The drawing insert shows that MTP-mediated 'lipidation' of apoB could occur in two ways, either by adding lipids to a full-length membrane-bound apoB molecule (top of insert) or by adding lipids to apoB as it emerges into the lumen of the ER while it is being translated on a ribosome (bottom of insert). MTP may also participate in second-step particle assembly.

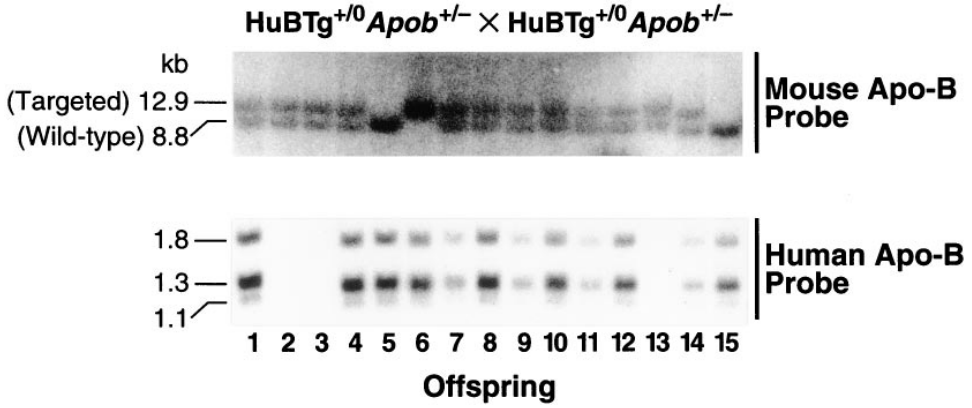


Fig. 2. Southern blot analysis of mouse DNA, using probes that allow the detection of the targeted (knockout) allele of the mouse apoB gene, and the presence of the human apoB transgene. In this experiment, a single runt (a human apoB transgenic mouse carrying two copies of the targeted allele) was identified (lane 6).

and in particular to determine whether the second-step triglyceride (TG)-rich particles can form in the ER lumen independently of apoB. To approach this issue experimentally, we have used electron microscopy to characterize chylomicron assembly in mice that are deficient in intestinal apoB synthesis.

Our laboratories have generated mice deficient in intestinal apoB synthesis by mating human apoB transgenic mice (in which the transgene was expressed only in the yolk sac and liver, but not in the intestine) with apoB knockout mice (14, 15). The human apoB transgenic mice that were homozygous for the knockout mutation ($HuBTg^{+/o}Apob^{-/-}$) develop normally in utero, and at birth are normal in size, weight, and physical appearance. However, because these mice made no apoB (mouse or human) in the intestine, they could not synthesize chylomicrons and developed fat malabsorption and vitamin E deficiency. Within 24 h of birth, these mice developed a white, protuberant abdomen caused by a massive accumulation of intracellular fat within the intestinal epithelium, from the duodenum to the colon (15). Within 2 days of birth, marked growth retardation was evident. Because of their small size, we have dubbed these mice 'runts.'

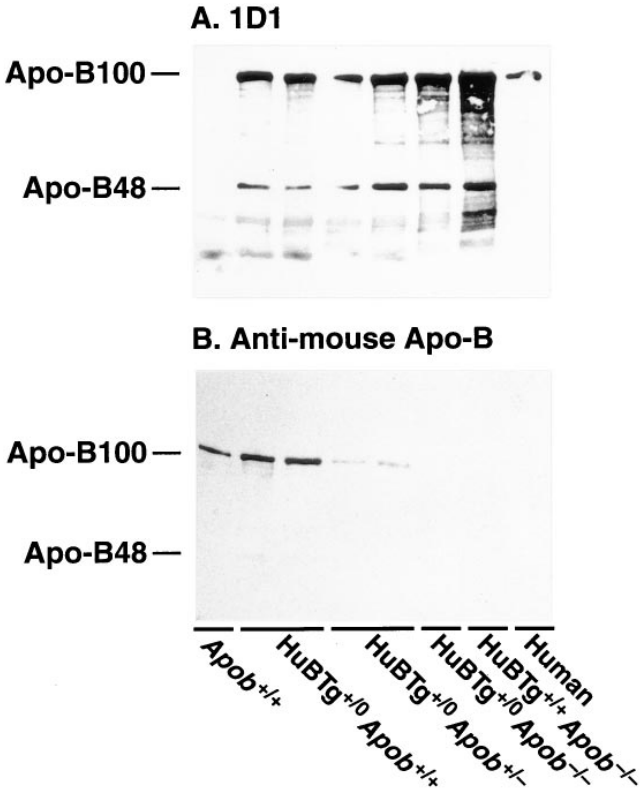


Fig. 3. Western blot analysis of the plasma of 'runts' and control littermates, using antibodies specific for human and mouse apoB. (Panel A) Western blot of mouse plasma that had been size fractionated on a 4% polyacrylamide/SDS gel. The blot was incubated with monoclonal antibody 1D1, which is specific for human apoB (21, 22). (Panel B) Western blot of mouse plasma that had been size fractionated on a 4% polyacrylamide/SDS gel. The blot was incubated with a rabbit antiserum to mouse apoB. Because the antiserum was generated in a human apoB transgenic rabbit (20, 29), it was specific for mouse apoB.

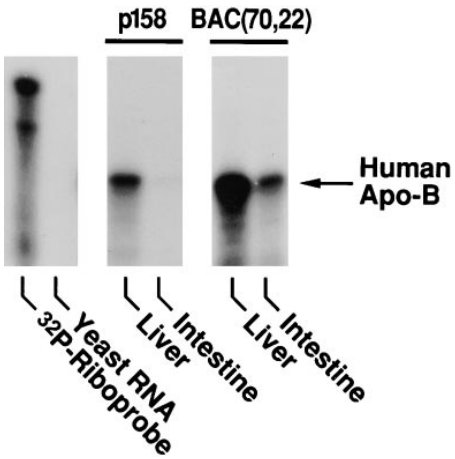
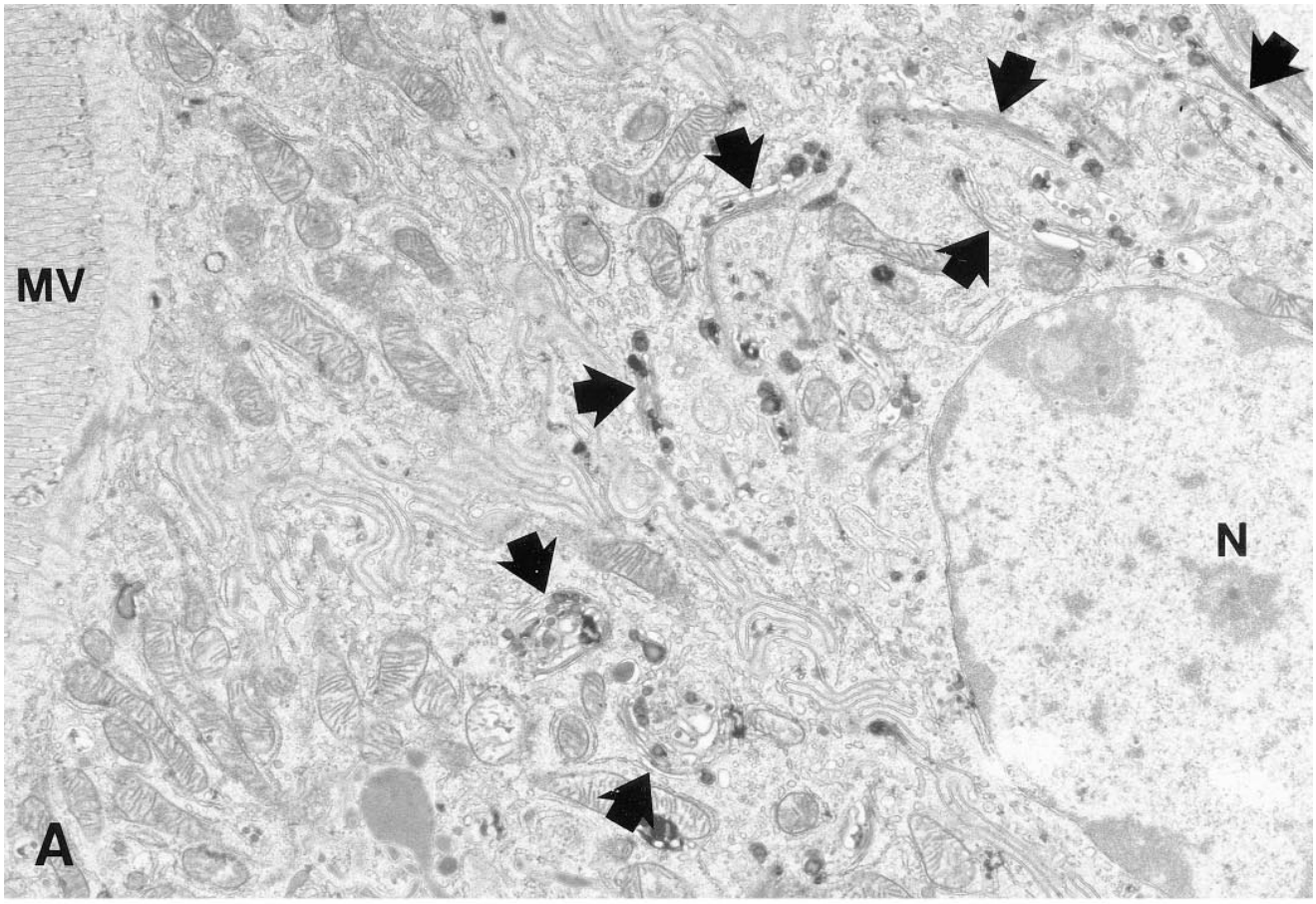


Fig. 4. RNase protection assay for human apoB with liver and intestinal RNA from the a 19-day embryo carrying the p158 human apoB transgene and a 19-day embryo carrying the BAC(70,22) transgene.



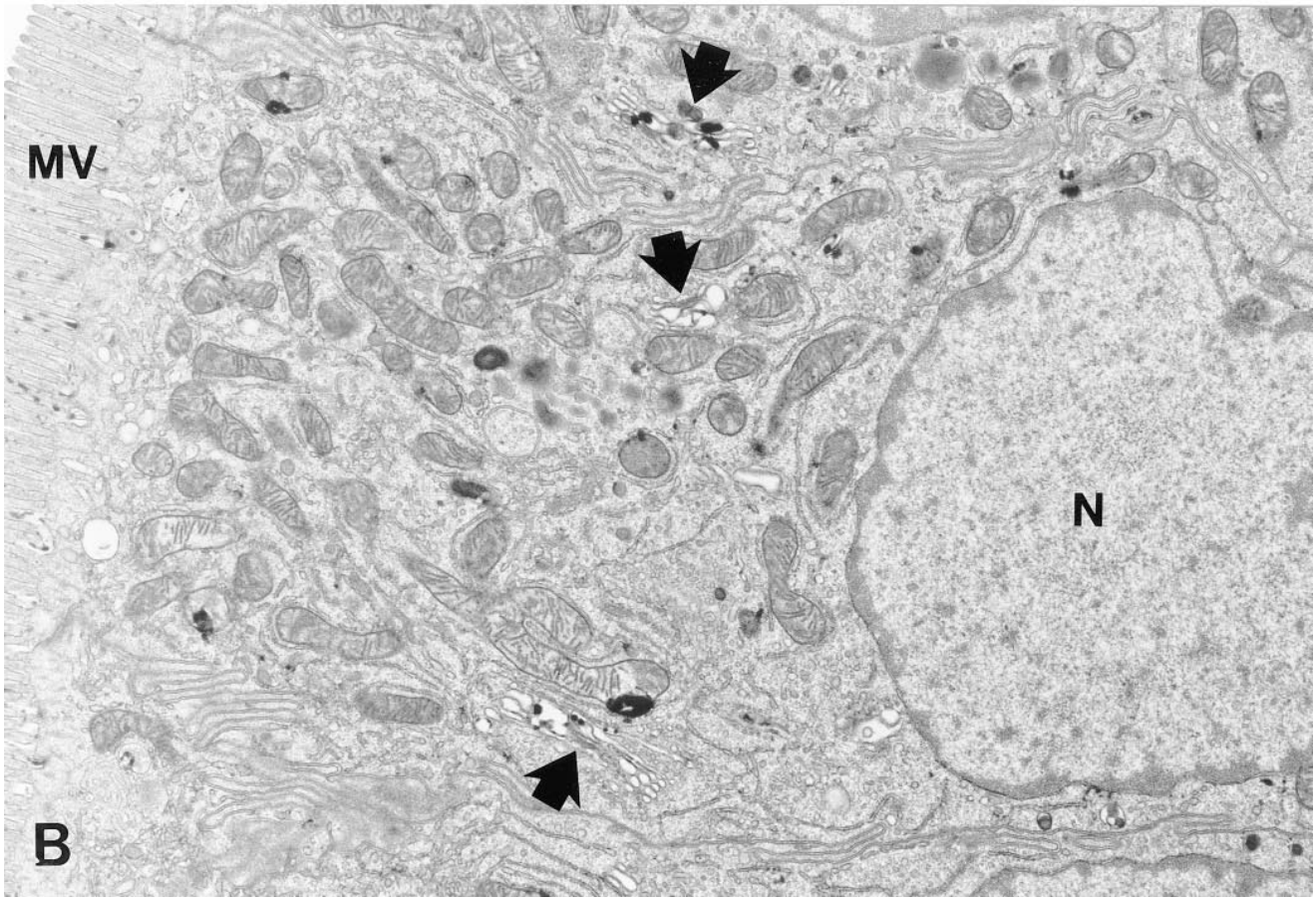


Fig. 5. Electron micrographs of supranuclear area of duodenal absorptive enterocytes of control (panel A) and runt (panel B) mice at 19 days of gestation. In both panels A and B, arrows point to Golgi apparatus stacks concentrated below the microvilli (MV) apical to the nucleus (N). In control enterocytes, many Golgi stacks contain intensely stained nascent chylomicrons, whereas in runt enterocytes, most Golgi stacks appear to be empty or contain very few lipid particles of nascent chylomicron size. This difference between the appearance of the Golgi apparatus of the enterocytes of runts and controls was consistently observed. Some of the enterocyte Golgi compartments of control embryos contained fewer particles than shown in this micrograph. A thorough analysis of enterocytes at this low magnification of five different runt intestines infrequently demonstrated several lipid particles that appeared to be associated with a single Golgi stack of membranes. (×12,000)

Our ultrastructural analysis of the absorptive enterocytes of the 2-week-old runts revealed that the villus enterocytes were essentially "filled" with cytosolic lipid droplets; the cells had little ER, and no membrane-bound triglyceride-rich particles were observed (15). Ultrastructural studies by others have revealed that the duodenal enterocytes of the villus tips of 10 day suckling rats are fatty and largely lacking in chylomicrons, whereas mid-villus enterocytes are not fatty and contain nascent chylomicrons within the secretory organelles (16). Based on these studies, we hypothesized that an ultrastructural analysis of the runts' intestines might yield valuable insights into lipoprotein assembly if the analyses could be performed prior to the massive accumulation of cytosolic fat droplets.

In this study, we examined the ultrastructure of runt duodenal enterocytes at 19 days of gestation (i.e. in the fasting setting) when apoB expression and lipoprotein secretion are established, but before the mice have taken their first fatty meal. We also examined the duodenum of runts during the first few hours after birth (within hours of their first fatty meal). The principal goal of these studies was to determine whether TG-rich particles are formed in the enterocyte ER lumen in the setting of apoB deficiency.

MATERIALS AND METHODS

ApoB knockout mice

ApoB knockout mice were generated previously by gene-targeting in mouse embryonic stem cells (14). In the absence of the human apoB transgene, mice that were homozygous for the apoB knockout mutation ($Apob^{-/-}$) died early during embryonic development, likely because the absence of VLDL synthesis in the yolk sac interferes with the delivery of lipid nutrients to the developing embryo (5).

Human apoB transgenic mice and mice deficient in apoB expression in the intestine

To generate mice deficient in intestinal apoB, the apoB knockout mice were mated with human apoB transgenic mice in which the transgene was expressed in the liver and yolk sac, but not in the intestines (17–19). For these studies, we used human apoB transgenic mice ($HuBTg^{+/o}$, line 1102) generated with the P1 bacteriophage clone, p158 (18). The p158 human apoB transgene was not expressed in the intestines because it lacks the DNA sequences that control the intestinal expression of the apoB gene (18–20). To obtain mice deficient in intestinal apoB synthesis, we intercrossed $HuBTg^{+/o}Apob^{+/-}$ mice to obtain human apoB transgenic mice that were homozygous for the mouse apoB knockout mutation ($HuBTg^{+/o}Apob^{-/-}$ or $HuBTg^{+/+}Apob^{-/-}$).

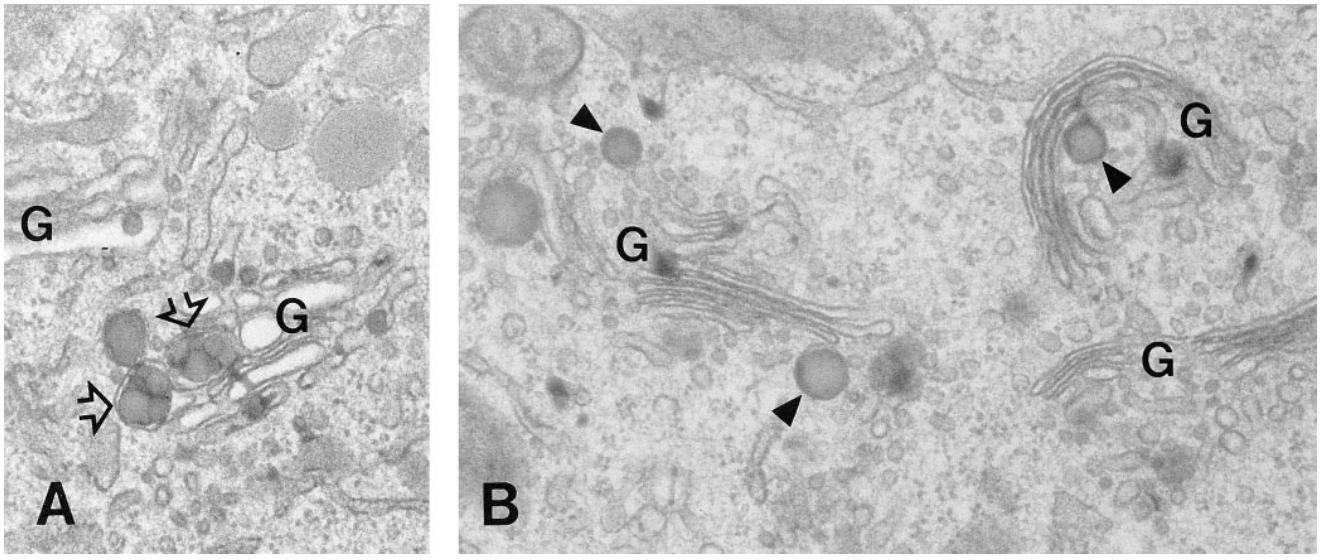


Fig. 6. Electron micrographs contrasting distinctive ultrastructural characteristics of Golgi membrane compartments (G) of 19-day gestational absorptive enterocytes of control (panel A) and runt (panel B) mice. In controls, clusters of smaller-sized nascent chylomicrons (open arrows) are observed within Golgi forming secretory vesicles (panel A). In contrast, runt Golgi membranes either lack lipid particles or manifest a single larger-sized (Table 1) lipid particle (arrowheads) adjacent to but not clearly within the membranes comprising a single Golgi stack. Tightly packed Golgi stacks, often in horseshoe configurations (upper right of panel B), were a common ultrastructural characteristic of runt enterocytes. (×36,000)

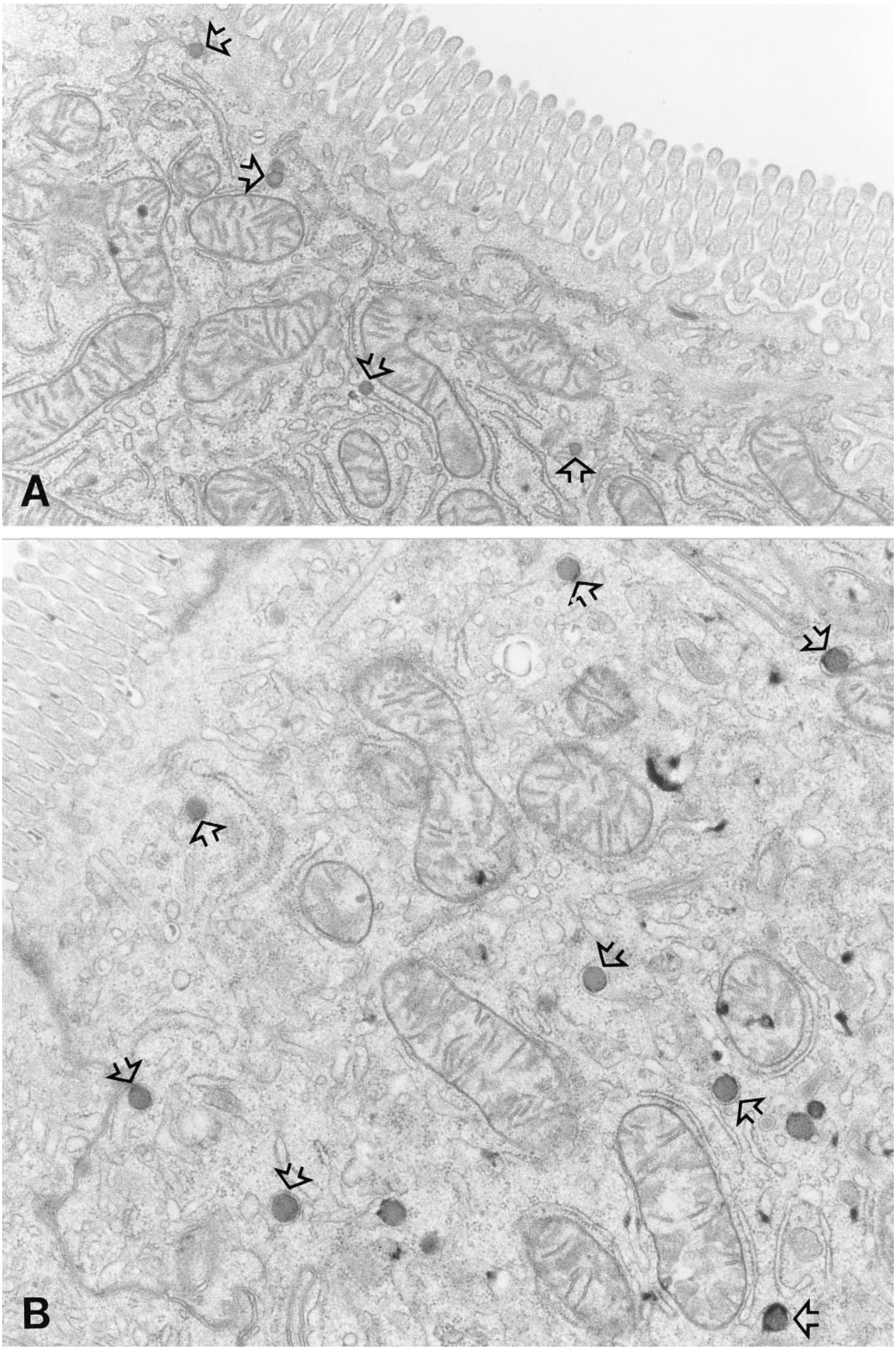


Fig. 7. Electron micrographs of putative second-step triglyceride-rich particles within the endoplasmic reticulum (open arrows) of the apical cytoplasm below the microvilli (MV) of both control (panel A) and runt (panel B) duodenal enterocytes at 19 days of gestation. Note that the 'lipid particles' are clearly within the membrane-bound lumenal compartment of the endoplasmic reticulum, and that they are larger and more numerous in the runt enterocytes compared to controls (Table 1). (×36,000)

The lethal developmental abnormalities observed in *Apob*$^{-/-}$ mice could be rescued with p158 because that clone confers human apoB expression in the yolk sac during development (14). However, because the HuBTg$^{+/o}$*Apob*$^{-/-}$ mice could not make apoB (mouse or human) in the intestine, they developed severe fat malabsorption and vitamin E deficiency and grew very slowly (15). Because of their small size, those mice have been dubbed 'runts' in our laboratory. For simplicity's sake, we have also referred to them as runts in this paper.

In this paper, we have characterized runts and littermate control mice after 19 days of gestation, and within a few hours after birth. The genotypes of the control mice consisted of HuBTg$^{+/o}$ *Apob*$^{+/-}$, HuBTg$^{+/o}$*Apob*$^{+/+}$, and *Apob*$^{+/-}$ mice. To obtain 19-day gestational mice, timed matings of HuBTg$^{+/o}$*Apob*$^{+/-}$ mice were performed as previously described (5). The 19-day embryos were genotyped by Southern blot analysis of tail DNA. The DNA probes used to detect the presence of the apoB knockout mutation and to detect the presence of the human apoB transgene have been described previously (14, 18).

Western blots and RNase protection assays

To assess the presence of mouse and human apoB in the plasma of runts and control mice, 2 ml of plasma was size-fractionated on a 4% polyacrylamide-SDS gel; the separated proteins were then transferred to a sheet of nitrocellulose membrane for Western blot analysis with a rabbit antiserum specific for mouse apoB (20) or a monoclonal antibody (1D1) (21, 22) specific for human apoB. Antiserum binding was detected with a peroxidase-labeled goat anti-rabbit or goat anti-mouse Ig and the enhanced chemiluminescense detection reagents (Amersham, Arlington Heights, IL).

To detect the presence of human apoB mRNA in mouse tissues, RNA was prepared from both liver and duodenum of p158-transgenic mice. As controls for these experiments, we examined the liver and intestinal RNA from human apoB transgenic mice and 19-day embryos generated with a 145-kb bacterial artificial chromosome clone, BAC(70,22) (19). BAC(70,22) contained 70 kb of 5′ flanking sequences and therefore contained all of the sequences controlling appropriate intestinal expression of the human apoB gene (19). RNase protection assays of apoB mRNA levels were performed using a 121-bp fragment of exon 1. The RNase protection experiments were performed with the RPA II ribonuclease protection assay kit (Ambion, Austin, TX) according to the manufacturer's instructions. Electrophoresis of protected RNA fragments was performed on 6% polyacrylamide gels containing 7 mol/L urea (Novex, San Diego, CA). The dried gels were exposed to autoradiographic film.

Preparation of mouse tissues for electron microscopy

Pregnant mice at 19 days of gestation were anesthetized by an intraperitoneal injection of pentobarbital (~2 mg/25 gm body weight). A midline abdominal incision was made, and the uterine wall was opened around individual fetuses. As each pregnancy produced 5–10 pups, fixation and surgical removal of individual fetal mouse duodenal and upper jejunal tissue took several hours. During this time, the mother was kept under a warming lamp and carefully monitored, a gauze sponge drenched in warm saline covering the incision. Each pup was removed from the uterine cavity, its tail removed for genotyping, its abdominal wall incised, and its viscera exposed. Under a dissecting microscope, a 27-gauge needle was inserted into the duodenal lumen, and the small intestine was flooded with a fixative of 1.5% glutaraldehyde, 4% polyvinylpyrolidone, and 0.5% calcium chloride in 0.1 m sodium cacodylate buffer, pH 7.4 (5, 15) from a syringe. Simultaneously, fixative was delivered drop-wise for 10 min onto the serosal surface, submerging the intestine in a pool of fixative in the abdominal cavity.

For early suckling mouse intestines, pups born the previous night were genotyped and their intestines were fixed during the afternoon according to the techniques described above. Thus, these pups had been suckling for approximately 5–12 h.

To stain the core lipids of lipoproteins, we used a modified version of the imidazole-buffered osmium tetroxide procedure described by Angermüller and Fahimi (23). Depending on the anticipated fat content of the tissue, we used 2–4 different concentrations of imidazole, ranging from 0.02 m to 0.3 m. We also added a 2% aqueous uranyl acetate block stain for 1 h at 4°C before dehydration and embedding in Epon (DuPont, Wilmington, DE). Thin sections were stained for 5 min with 0.8% aqueous lead citrate and photographed in a Siemens 101 electron microscope (Siemens CTI Corp., Knoxville, TN).

To be certain that electron microscopic images were representative of a particular mouse genotype, a minimum of three pups of each genotype and at each time point were examined. In addition, 2–6 blocks of tissue from each mouse pup were thin sectioned and photographed. Finally, duodenal and upper jejunal enterocytes were examined throughout the tip, middle, and basal regions of the villi in all cases. Although we present for illustration only selected fields that the authors agreed were representative of each situation, more than 800 electron micrographs were printed and examined for this project.

RESULTS

Analysis of human and mouse apoB expression in the intestines

'Runts,' which are homozygous for the apoB knockout mutation and carry the p158 human apoB transgene, were identified by Southern blot analysis (**Fig. 2**). We have shown that mouse apoB is not expressed in embryos that are homozygous for the apoB knockout mutation (14, 15, 19). We could not detect the presence of mouse

TABLE 1. Mean diameters of lipid-staining particles in the duodenal absorptive enterocytes of 19-day mouse duodenum

	Control Mice	Runts
	Å	Å
Endoplasmic reticulum	725 ± 235 (n = 100)	1772 ± 627[a] (n = 170)
Golgi apparatus	601 ± 188 (n = 112)	1580 ± 488[a] (n = 88)
Extracellular spaces	526 ± 134 (n = 140)	1182 ± 428[a] (n = 26)

Particles sizes are shown in angstroms (means ± standard deviations).
[a]Different from controls, $P < 0.0001$.

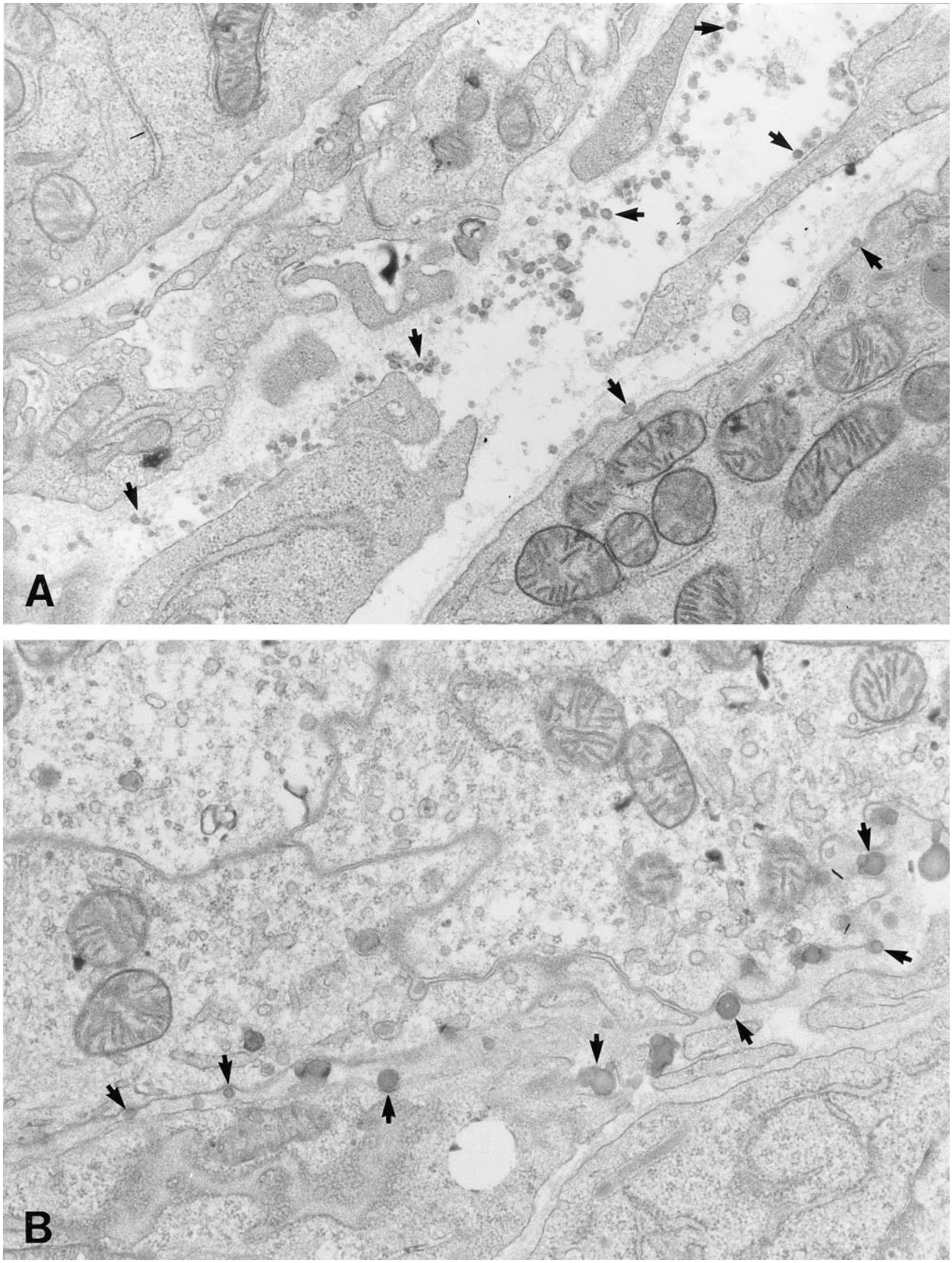


Fig. 8. Electron micrographs of nascent chylomicrons (arrows) secreted into the extracellular spaces by control (panel A) and runt (panel B) duodenal absorptive enterocytes at 19 days of gestation. Note the larger diameters of the lipid particles in the extracellular compartment of the runt duodenum compared to those produced by the duodenum of control mice (Table 1). (×24,000)

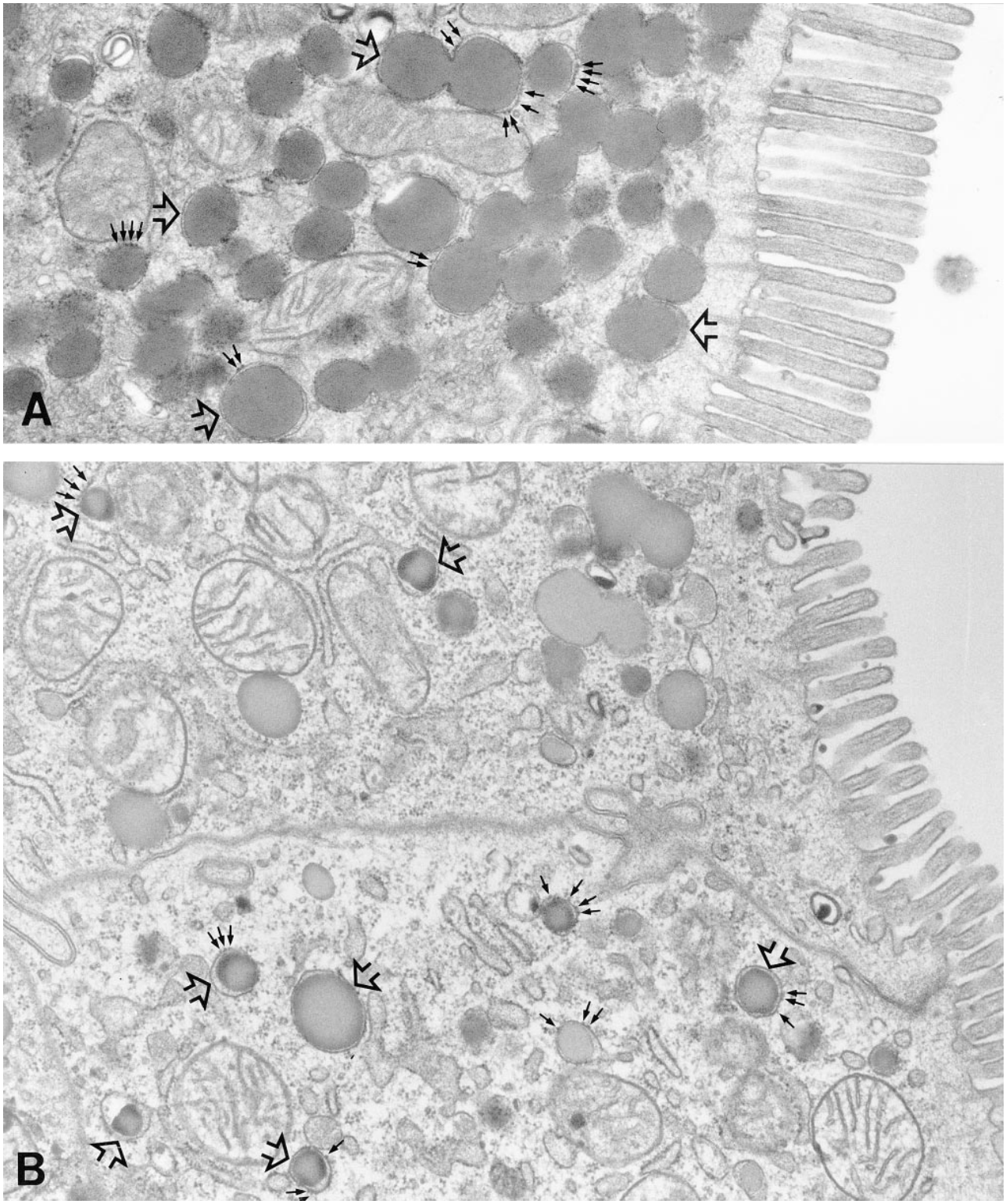


Fig. 9. Electron micrographs of very large putative second-step triglyceride-rich particles assembled within the lumenal spaces of duodenal enterocyte endoplasmic reticulum (open arrows) during the first hours of suckling of both control (panel A) and runt (panel B) newborn mice. Note that some of these lipid particles appear within the transitional rough to smooth membrane compartment (top left and top center of panel B). The membrane surrounding these lipid particles typically contains a few ribosomes (arrows). Although these large putative second-step triglyceride-rich particles (4,000–6,000 Å) are clearly assembled within runt enterocytes as well as in control enterocytes, they are typically many fewer in runts during the first hours of suckling. (×24,000)

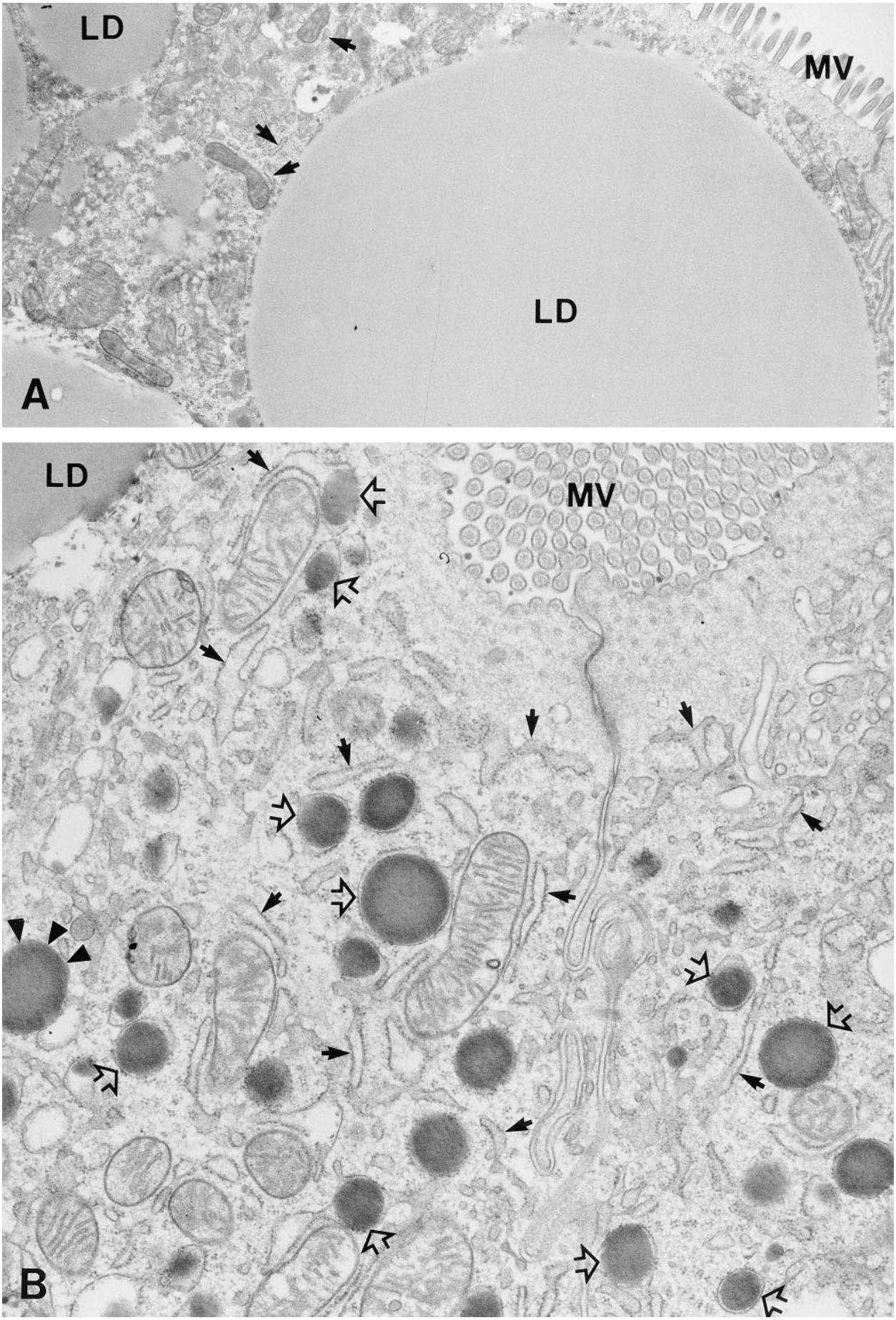


Fig. 10. Electron micrographs contrasting apical regions of villus tip (panel A, ×12,000) and villus side (panel B, ×24,000) enterocytes of newborn suckling runts. The microvilli (MV) identify the apex of these enterocytes. Villus tip enterocytes (panel A) were characterized by many large (5–10 micron) cytosolic lipid droplets (LD), decreased amount of endoplasmic reticulum (arrows), and the complete absence of second-step lipid particles within the lumen of the endoplasmic reticulum. Enterocytes further down the villus sides (panel B) of newborn suckling duodenum were characterized by abundant endoplasmic reticulum (arrows) often containing second-step lumenal particles (open arrows) and a variable amount of cytosolic lipid droplets (LD). Some of the lipid particles derived from the endoplasmic reticulum appear to have lost their surrounding membrane, thereby contributing to the pool of cytoplasmic lipid droplets (arrowheads).

apoB in the plasma of adult 'runts' by Western blot analysis (**Fig. 3**). We also could not detect mouse apoB-100 in the plasma of adult runts with a monoclonal antibody-based RIA specific for mouse apoB-100 (data not shown). We have also shown that human apoB is essentially undetectable in the intestines of adult human apoB transgenic mice generated with the P1 bacteriophage clone, p158 (19, 24). In this study, we performed RNase protection assays to determine whether human apoB gene expression is also absent in the intestines of 19-day gestational transgenic mice generated with p158. As shown in **Fig. 4** of the 19-day p158-transgenic embryos, human apoB expression was robust in the liver but was either undetectable or extremely faint in the intestines. Similarly, in adult p158 human apoB transgenic mice, human apoB expression was robust in the liver but was extremely faint or undetectable in the intestine (data not shown). As controls for these experiments, we analyzed human apoB expression in 19-day gestational BAC(70,22) transgenic mice. BAC(70,22) contains the distant 5′ intestinal enhancer element and confers full expression of the apoB gene to the intestine (19). In the BAC(70,22) mice, there was robust expression of the human apoB gene in both the liver and intestine (Fig. 4).

Ultrastructure of the Golgi apparatus of duodenal absorptive enterocytes of 19-day gestational mice

We analyzed lipoprotein assembly in the villus enterocytes of 'runts' at 19 days of gestation because of prior data indicating that lipoprotein secretion would be developed at that time point.[2] We concentrated our analyses on the duodenal villi distal to the entrance of the common bile duct and the upper jejunum. Triglyceride-rich lipoprotein assembly and secretion occurs in the apical portion of absorptive enterocytes between the nucleus and microvillus border. As shown in **Fig. 5**, low magnification electron micrographs revealed a consistent difference between control and runt enterocytes. In the absorptive enterocytes of control mice, we often observed large numbers of nascent chylomicrons within numerous supranuclear Golgi compartments. In contrast, the Golgi compartments of runt absorptive enterocytes often completely lacked lipid-staining particles (Fig. 5B). However, Golgi areas infrequently contained several lipid-staining particles (Fig. 5B, top), but generally only one or two large particles of chylomicron size were observed. These larger lipid particles in runt enterocytes were most commonly found singly, and were closely adjacent to, but not clearly within the Golgi stack (**Figs. 6A** and **6B**). In contrast, smaller chylomicrons were often clustered together clearly within forming Golgi secretory vesicles in control enterocytes (Fig. 6A).

Ultrastructure of the endoplasmic reticulum (ER) of absorptive enterocytes of 19-day gestational mice

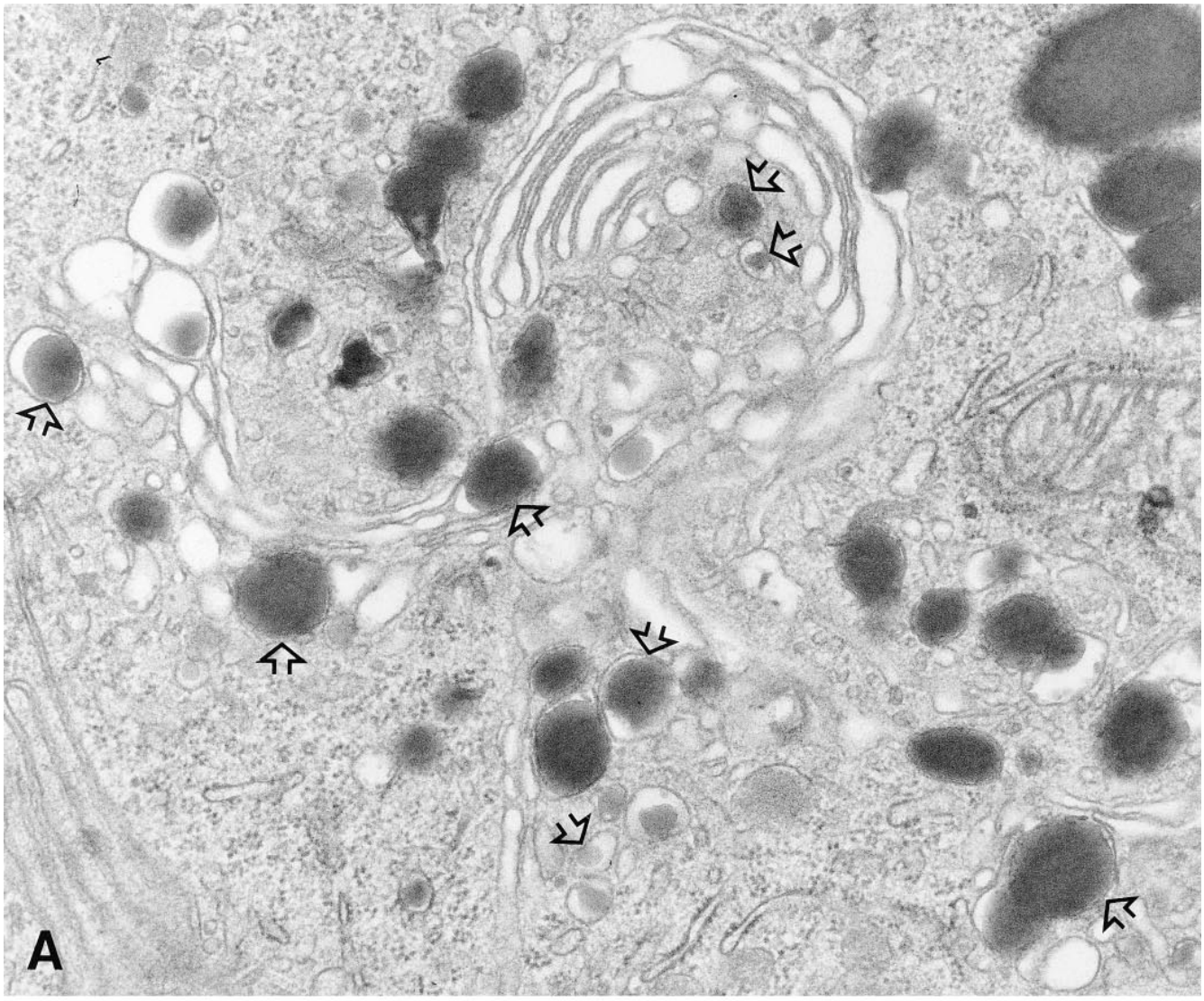
In the apical cytoplasm beneath the microvillus border, lipid-staining particles of chylomicron size were evident within the lumenal spaces of the membranes of the ER of both control and runt duodenal absorptive enterocytes (**Fig. 7**). However, the ultrastructural characteristics of ER lipid-staining particles were distinctly different in two respects. First, the ER lipid particles from the runts were clearly more abundant and easier to find; and second, the particles were always substantially larger than those observed in controls (Fig. 7). The mean diameter of the lipid-staining particles within the lumen of the ER of the runts was more than double the diameter of the ER lipid particles in the 'apoB-expressing' control enterocytes (1,772 ± 627 Å in the runts versus 725 ± 235 Å in control mice) (**Table 1**).

Ultrastructural analysis of lipoproteins within the extracellular space of 19-day gestational mice

Numerous lipid-staining particles of nascent chylomicron size were frequently observed between adjacent absorptive enterocytes of control mice (**Fig. 8A**). In contrast, the lipid-staining particles were much less frequently observed in the extracellular spaces of the runts. However, whenever lipid-staining particles were observed in the extracellular spaces, they were invariably larger than those observed in controls. Figure 8B shows an unusually large number of large-sized extracellular lipid particles in runt intestine. (Although most of the other micrographs revealed few or no extracellular lipoproteins, this image was chosen to demonstrate the larger size of lipid-staining particles in the extracellular spaces of the runt intestines.)

Thus, the diameters of lipid-staining particles in the ER, Golgi apparatus, and interstitium were uniformly smaller in controls compared to runts (Table 1). As we were only able to identify and measure a few particles in the extracellular spaces of runts' intestines, it is difficult to draw firm conclusions regarding the comparison of particles sizes in the extracellular space.

[2] We chose the 19-day time-point because intestinal villi develop in rats on the 18th day of gestation and lipoprotein particle assembly/secretion is first observed on the 19th day of gestation (28).



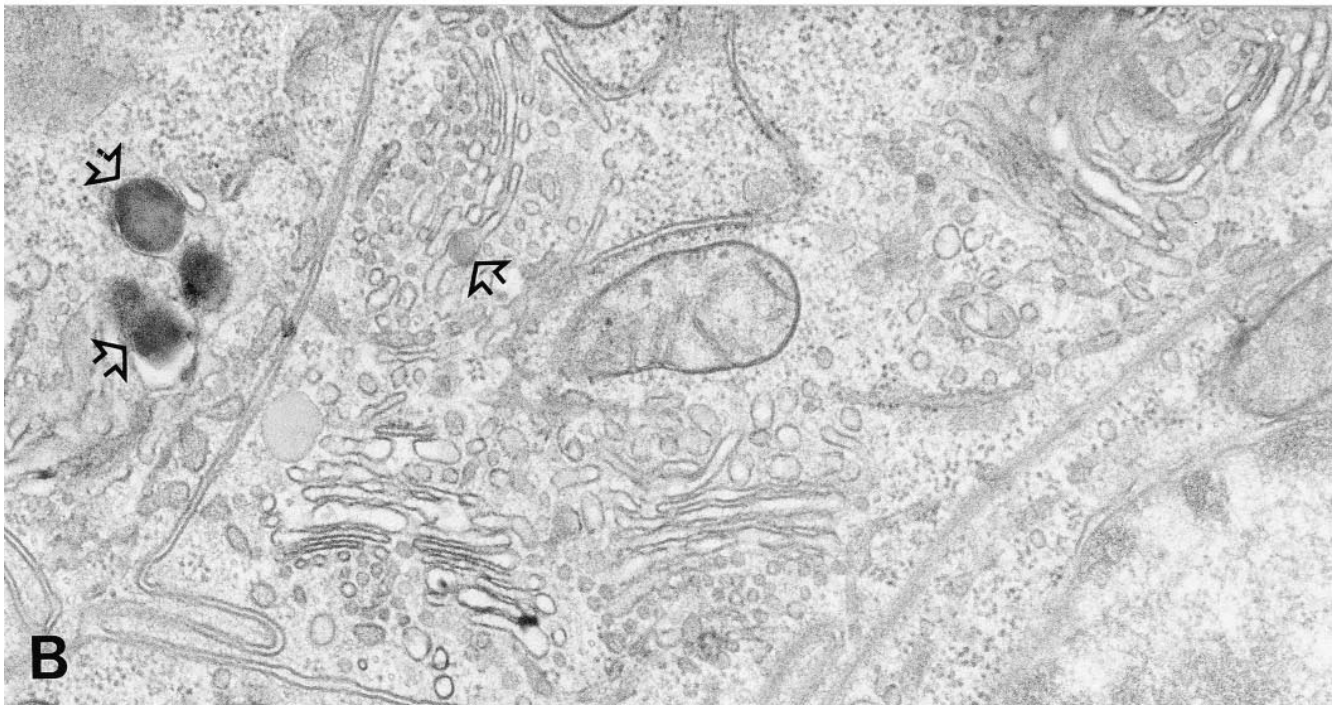


Fig. 11. Electron micrographs of very large nascent chylomicrons within membrane-bound compartments of the Golgi apparatus (open arrows) of control (panel A) and runt (panel B) duodenal enterocytes of newborn suckling mice. Note that either no or very few nascent chylomicrons are found within Golgi compartments of runt enterocytes, whereas they are often numerous in Golgi compartments of control mice in this early suckling period. (×36,000)

Ultrastructure of the ER and Golgi apparatus of absorptive enterocytes of newborn suckling mice

The apical cytoplasm of duodenal enterocytes of control newborn suckling mice is characterized by the presence of numerous lipid-staining particles within the lumenal spaces of the ER (**Fig. 9A**). These TG-rich particles are 4- to 8-times greater in diameter (4,000 Å to 6,000 Å) than those of the control intestine at 19 days of gestation (~750 Å) (contrast Figs. 7A and 9A). In the newborn control mice, the duodenal enterocytes at the villus tips sometimes also contained large cytosolic lipid droplets in addition to the lipid particles within the lumenal spaces of the ER and Golgi apparatus (data not shown). However, in newborn runts, the villus tip enterocytes were almost always fatty. Apical cytoplasm was filled with very large (5–10 micron) cytosolic lipid droplets and very few cytosolic organelles remained (**Fig. 10A**). In particular, the quantity of ER was greatly reduced in the villus tip enterocytes of runts, and it was difficult to find ER that contained lipid particles. Thus, the ultrastructural appearance of the villus tip enterocytes of newborn suckling runts was closely similar to enterocytes that we observed previously in the 2-week-old suckling runts (15). Interestingly, enterocytes along the sides of the villi in the newborn suckling runts (away from the villus tips) exhibited a different ultrastructural appearance. Although cytosolic lipid droplets were present to a variable extent, these runt enterocytes often contained abundant ER membranes with content lipid particles in the lumenal space (Figs. 9B and 10B). These TG-rich particles within the lumen of the ER of suckling runt enterocytes were less numerous than in the control enterocytes, but were of comparable size (compare Figs. 9A and 9B). Large lipid-staining particles were often observed singly within the membrane bound lumenal spaces of the Golgi apparatus of enterocytes of control newborn suckling mice (**Fig. 11A**), indicating that they are nascent chylomicrons bound for the extracellular spaces. In contrast, most Golgi compartments of runt suckling absorptive enterocytes lacked these nascent chylomicron particles, although a few single lipid particles were sometimes observed (Fig. 11B).

DISCUSSION

We reported previously that lipid-staining particles of VLDL size within the lumenal spaces of the SER of rat hepatocytes lacked apoB, as judged by immunoelectron microscopy (6). In those studies, the ER apoB was localized to the RER, where no lipid particles of VLDL size were observed. These ultrastructural observations led to the concept that apoB obtains its core lipids in two separate steps during the assembly of TG-rich lipoproteins, as depicted in Fig. 1. The basic features of this hypothesis are that apoB-free TG-rich particles are generated within the ER lumenal space, and that these particles then join with a small, lipid-poor apoB-containing particle before exiting the cell. Within the past few years, this concept has been supported by pulse-chase studies of lipoprotein assembly in cultured rat McA-RH7777 hepatoma cells. These studies demonstrated that small apoB-containing HDL particles within the lumen of the ER could be transformed, apparently in a single rapid step, into VLDL particles simply by incubating the cells with oleate (8–11).

We elected to study runt (HuBTg$^{+/o}$*Apob*$^{-/-}$) mice at 19 days of gestation because our prior studies had shown that it was next to impossible to examine the ultrastructure of chylomicron assembly during the late suckling phase, in which the absorptive enterocytes of runts were virtually filled with cytosolic fat droplets, and the quantity of ER within these cells was markedly reduced or even absent.[3] At 19 days of gestation, the runts and control mice were of identical size. The maturity of the absorptive enterocytes in runts and controls was evidently equivalent, as judged by the appearance of the microvillus border and by the fact that they apparently contained equivalent amounts of ER and other organelles within the apical portions of the enterocytes. However, with respect to the ultrastructure of chylomicron assembly, the control enterocytes and runt enterocytes were different. First, the number of lipid-staining particles within the lumen of the ER was often greater in the runt enterocytes compared with the controls. Second, the lipid-staining particles were always substantially larger in the runt enterocytes. Third, the number of the larger lipid-staining particles in the Golgi apparatus of runt enterocytes was substantially reduced. Fourth, these larger lipid particles were often adjacent to and apparently not within the Golgi stacks, and fifth, the number of lipid-staining particles outside of runt enterocytes was strikingly reduced. Whereas we often observed numerous smaller-sized lipid particles in extracellular compartments of the control animals, we found only

[3] The reason for the striking reduction of runt enterocyte ER in the presence of massive amounts of cytosolic fat is not known. During the first hours of suckling, the villus tip cells had a massive accumulation of cytosolic fat droplets, much reduced amounts of ER, and no ER lipid particles. The mid-villus enterocytes of runts were often not as fatty and had abundant lipid particles within the ER lumenal space, suggesting that the cellular machinery for generating lipoproteins may be down-regulated by excessive cytosolic fat. Interestingly, the extent of Golgi membranes did not appear to be significantly reduced in runt absorptive enterocytes containing massive amounts of cytosolic fat droplets.

a few instances in which the larger lipid particles were evident outside of enterocytes in the runts.

How should this series of ultrastructural findings be interpreted? The greatly decreased numbers of lipid-staining particles in the extracellular spaces outside of the runt enterocytes compared to controls was the most constant finding. This is certainly consistent with the long-standing concept that apoB synthesis is required for secretion of lipoproteins from cells (2–4). An extremely sensitive RNase protection assay suggested that trace levels of apoB synthesis may well exist within the absorptive enterocytes of the 19-day runts (as a result of transgene leakiness), and we therefore believe it is possible that the rare extracellular particles that we observed in the runts could actually contain a molecule of apoB. However, the appearance of the ER in runt enterocytes is particularly interesting. In the ER, we found increased numbers of lipid particles, despite markedly decreased numbers of particles in the Golgi apparatus and extracellular spaces. We believe that this observation supports the two-step assembly model of apoB core lipidation for lipoprotein synthesis. Our explanation for these observations is that the formation of lipid particles within the ER does not require apoB synthesis, but that their transport out of the cell is greatly facilitated by apoB synthesis. We propose that the vast majority of the lipid particles within the ER represent the apoB-free "second-step" particles (depicted in Fig. 1). These putative second-step particles were also substantially larger in the enterocytes of runts than control mice. We suggest that the much larger size of these particles is due to a retarded rate of transport through the ER compartment as a consequence of apoB deficiency. In other words, we suggest that the lipid particles are assembled in the ER in the absence of apoB, and that an apoB molecule is responsible for allowing them to be swept towards and into the Golgi compartment and out of the cell. When an apoB molecule does not come along (or comes along very rarely), as in the case of the runts, the ER lipid particles continue to accumulate lipids within the ER lumen and become larger.

Our ultrastructural observations show that many fewer of these large lipid particles in runts appear to 'leak' through the Golgi apparatus to the extracellular spaces compared to wild-type intestine, which deserves special comment. Adult runts that survived suckling and thrived on low fat chow diet had extremely low plasma levels of vitamin E and mildly reduced levels of essential fatty acids (15). It seems possible that a 'leakage' of small numbers of lipid particles throughout the length of the small intestine may be sufficient to ensure some fat absorption, preventing overt manifestations of vitamin E and essential fatty acid deficiency. Interestingly, this phenotype in the runts is quite similar to that in humans with abetalipoproteinemia, which is characterized by trace levels of apoB-containing lipoproteins in the plasma (25) and only a minimal reduction in plasma levels of essential fatty acids (26). We suspect that the 'leakage' of a comparatively small number of lipid particles into the bloodstream in abetalipoproteinemia may help to explain the absence of essential fatty acid deficiency in this disorder, and may also explain why these individuals on a low fat diet respond favorably to high doses of orally administered vitamin E (2–4, 25).

From a teleological standpoint, a two-step process for adding core lipids to chylomicrons makes sense, especially as a diet rich in fats has little or no effect on apoB expression itself. When dietary fats are abundant (e.g., the suckling phase), the lipid particles within the lumen of the ER can become larger, leading to larger chylomicron particles. If the 'core' diameter of first-step apoB-containing particles is ~100 Å, the amount of core volume would be increased by 1000-fold by adding a second-step particle that was ~1000 Å in diameter and by 8000-fold if the second-step particle were ~2000 Å (13). Thus, the ability to modulate the size of lipid particles allows an organism to increase the size of apoB-containing TG-rich lipoproteins when nutrients are plentiful and to decrease their size during periods of fasting. We suspect that the ability to add additional lipids to apoB-containing lipoproteins may prove to be unique to the intestine and the liver. For example, we have not observed lipid particles within the ER of yolk sac visceral endoderm cells from *Apob*$^{-/-}$ mice (5). However, this epithelial cell contains only RER and is devoid of SER.

Whereas the present studies provide in vivo support for the two-step hypothesis of apoB core lipidation, we emphasize that many questions remain regarding the molecular mechanisms for this process, particularly the molecules involved in the formation of the ER lipid particles. For example, hypothesized gene products that would presumably be needed for the generation of apoB-free lipid particles within the lumen of the ER have not yet been identified. Also, the precise role of microsomal triglyceride transfer protein (MTP) in the core lipidation of apoB-containing lipoproteins is not established. It seems likely that MTP is essential for the generation of 'first step' particles, inasmuch as virtually no apoB-containing lipoproteins are secreted from cells in the absence of MTP (27). However, the role of MTP in the second step of apoB core lipidation requires further study. In pulse-chase studies of apoB assembly in McA-RH7777 cells, one research group concluded that MTP inhibitor drugs had little effect on the addition of bulk lipids to dense apoB48-containing particles (first-step particles) within the lumen of the ER, implying that MTP might not play a critical role in the second step (10). However, this conclusion has been disputed by another group of investigators who also used pulse-chase studies to study lipoprotein assembly in McA-RH7777 cells (11).

In the future, we predict that ultrastructural studies of yolk sac, liver, or intestinal cells may help to delineate the role of MTP in lipoprotein assembly. We have recently generated and characterized MTP-deficient mice (30) with phenotypes analogous to the apoB knockout mice (5, 14). Homozygotes died early in development, the visceral yolk sac endoderm was fatty and lacked ultrastructural evidence of VLDL assembly/secretion, and the embryos manifested neurodevelopmental abnormalities. We suspect that tissue-specific knockout strategies or transgene rescue strategies (akin to the one used in the current

study) will allow us to be able to study the ultrastructural characteristics of lipoprotein assembly in tissues lacking MTP.

Supported by National Institutes of Health grants HL41633, HL14237PO1, HL14237, and a Grant-in-Aid from the American Heart Association, California Affiliate. L.B.N. was a recipient of a postdoctoral fellowship award from the American Heart Association, California Affiliate.

Manuscript received 12 February 1998.

REFERENCES

1. Gage, S. H., and P. A. Fish. 1924. Fat digestion, absorption, and assimilation in man and animals as determined by the dark-field microscope and a fat-soluble dye. *Am. J. Anat.* **34:** 1–85.
2. Havel, R. J., and J. P. Kane. 1995. Introduction: Structure and metabolism of plasma lipoproteins. *In* The Metabolic and Molecular Bases of Inherited Disease. 7th edition. Vol. 2. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, New York. 1841–1851.
3. Kane, J. P., and R. J. Havel. 1995. Disorders of the biogenesis and secretion of lipoproteins containing the B apolipoproteins. *In* The Metabolic Bases of Inherited Disease. 7th edition. Vol. 2. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, New York. 1853-1885.
4. Young, S. G. 1990. Recent progress in understanding apolipoprotein B. *Circulation*. **82:** 1574–1594.
5. Farese, R. V., Jr., S. Cases, S. L. Ruland, H. J. Kayden, J. S. Wong, S. G. Young, and R. L. Hamilton. 1996. A novel function for apolipoprotein B: lipoprotein synthesis in the yolk sac is critical for maternal–fetal lipid transport in mice. *J. Lipid Res.* **37:** 347–360.
6. Alexander, C. A., R. L. Hamilton, and R. J. Havel. 1976. Subcellular localization of B apoprotein of plasma lipoproteins in rat liver. *J. Cell Biol.* **69:** 241–263.
7. Swift, L. L. 1995. Assembly of very low density lipoproteins in rat liver: A study of nascent particles recovered from the rough endoplasmic reticulum. *J. Lipid Res.* **36:** 395–406.
8. Borén, J., S. Rustaeus, and S-O. Olofsson. 1994. Studies on the assembly of apolipoprotein B-100- and B-48-containing very low density lipoproteins in McA-RH7777 cells. *J. Biol. Chem.* **269:** 25879–25888.
9. Rustaeus, S., K. Lindberg, J. Borén, and S-O. Olofsson. 1995. Brefeldin A reversibly inhibits the assembly of apoB-containing lipoproteins in McA-RH7777 cells. *J. Biol. Chem.* **270:** 28879–28886.
10. Gordon, D. A., H. Jamil, R. E. Gregg, S-O. Olofsson, and J. Borén. 1996. Inhibition of the microsomal triglyceride transfer protein blocks the first step of apolipoprotein B lipoprotein assembly but not the addition of bulk core lipids in the second step. *J. Biol. Chem.* **271:** 33047–33053.
11. Wang, Y., R. S. McLeod, and Z. Yao. 1997. Normal activity of microsomal triglyceride transfer protein is required for the oleate-induced secretion of very low density lipoproteins containing apolipoprotein B from McA-RH7777 cells. *J. Biol. Chem.* **272:** 12272–12278.
12. Hamilton, R. L. 1994. Apolipoprotein-B-containing plasma lipoproteins in health and in disease. *Trends Cardiovasc. Med.* **4:** 131–139.
13. Hamilton, R. L., S. K. Erickson, and R. J. Havel. 1995. Nascent VLDL assembly occurs in two steps in the endoplasmic reticulum (ER) of hepatocytes. *In* Atherosclerosis. X. F. P. Woodford, J. Davignon, and A. Sniderman, editors. Elsevier Science, Amsterdam. 414–418.
14. Farese, R. V., Jr., S. L. Ruland, L. M. Flynn, R. P. Stokowski, and S. G. Young. 1995. Knockout of the mouse apolipoprotein B gene results in embryonic lethality in homozygotes and protection against diet-induced hypercholesterolemia in heterozygotes. *Proc. Natl. Acad. Sci. USA.* **92:** 1774–1778.
15. Young, S. G., C. M. Cham, R. E. Pitas, B. J. Burri, A. Connolly, L. Flynn, A. S. Pappu, J. S. Wong, R. L. Hamilton, and R. V. Farese, Jr. 1995. A genetic model for absent chylomicron formation: mice producing apolipoprotein B in the liver, but not in the intestine. *J. Clin. Invest.* **96:** 2932–2946.
16. Berendsen, P. B., and E. J. Blanchette-Mackie. 1979. Milk lipid absorption and chylomicron production in the suckling rat. *Anat. Rec.* **195:** 397–414.
17. Young, S. G., R. V. Farese, Jr., V. R. Pierotti, S. Taylor, D. S. Grass, and M. F. Linton. 1994. Transgenic mice expressing human $apoB_{100}$ and $apoB_{48}$. *Curr. Opin. Lipidol.* **5:** 94–101.
18. Linton, M. F., R. V. Farese, Jr., G. Chiesa, D. S. Grass, P. Chin, R. E. Hammer, H. H. Hobbs, and S. G. Young. 1993. Transgenic mice expressing high plasma concentrations of human apolipoprotein B100 and lipoprotein[a]. *J. Clin. Invest.* **92:** 3029–3037.
19. Nielsen, L. B., S. P. A. McCormick, V. Pierotti, C. Tam, M. D. Gunn, H. Shizuya, and S. G. Young. 1998. Human apolipoprotein B transgenic mice generated with 207-kb and 145-kb bacterial artificial chromosomes: evidence that a distant 5′ element confers appropriate transgene expression in the intestine. *J. Biol. Chem.* In press.
20. McCormick, S. P. A., J. K. Ng, M. Véniant, J. Borén, V. Pierotti, L. M. Flynn, D. S. Grass, A. Connolly, and S. G. Young. 1996. Transgenic mice that overexpress mouse apolipoprotein B. Evidence that the DNA sequences controlling intestinal expression of the apolipoprotein B gene are distant from the structural gene. *J. Biol. Chem.* **271:** 11963–11970.
21. Marcel, Y. L., M. Hogue, R. Theolis, Jr., and R. W. Milne. 1982. Mapping of antigenic determinants of human apolipoprotein B using monoclonal antibodies against low density lipoproteins. *J. Biol. Chem.* **257:** 13165-13168.
22. Pease, R. J., R. W. Milne, W. K. Jessup, A. Law, P. Provost, J-C. Fruchart, R. T. Dean, Y. L. Marcel, and J. Scott. 1990. Use of bacterial expression cloning to localize the epitopes for a series of monoclonal antibodies against apolipoprotein B100. *J. Biol. Chem.* **265:** 553-568.
23. Angermüller, S., and H. D. Fahimi. 1982. Imidazole-buffered osmium tetroxide: an excellent stain for visualization of lipids in transmission electron microscopy. *Histochem. J.* **14:** 823–835.
24. Purcell-Huynh, D. A., R. V. Farese, Jr., D. F. Johnson, L. M. Flynn, V. Pierotti, D. L. Newland, M. F. Linton, D. A. Sanan, and S. G. Young. 1995. Transgenic mice expressing high levels of human apolipoprotein B develop severe atherosclerotic lesions in response to a high-fat diet. *J. Clin. Invest.* **95:** 2246–2257.
25. Aguie, G. A., D. J. Rader, V. Clavey, M. G. Traber, G. Torpier, H. J. Kayden, J. C. Fruchart, H. B. Brewer, Jr., and G. Castro. 1995. Lipoproteins containing apolipoprotein B isolated from patients with abetalipoproteinemia and homozygous hypobetalipoproteinemia: identification and characterization. *Atherosclerosis.* **118:** 183–191.
26. Kayden, H. J. 1980. Is essential fatty acid deficiency part of the syndrome of abetalipoproteinemia? *Nutr. Rev.* **38:** 244–246.
27. Gordon, D. A. 1997. Recent advances in elucidating the role of the microsomal triglyceride transfer protein in apolipoprotein B lipoprotein assembly. *Curr. Opin. Lipidol.* **8:** 131–137.
28. Mak, K. M., and J. S. Trier. 1975. Lipoprotein particles in the jejunal mucosa of fetal rats. *Dev. Biol.* **43:** 204–211.
29. Fan, J., S. P. A. McCormick, R. M. Krauss, S. Taylor, R. Quan, J. M. Taylor, and S. G. Young. 1995. Overexpression of human apolipoprotein B-100 in transgenic rabbits results in increased levels of LDL and decreased levels of HDL. *Arterioscler. Thromb. Vasc. Biol.* **15:** 1889–1899.
30. Raabe, M., L. M. Flynn, C. H. Zlot, J. S. Wong, M. M. Véniant, R. L. Hamilton, and S. G. Young. 1998. Knockout of the abetalipoproteinemia gene in mice: reduced lipoprotein secretion in heterozygotes and embryonic lethality in homozygotes. *Proc. Natl. Acad. Sci. USA.* In press.