Apolipoprotein B-related gene expression and ultrastructural characteristics of lipoprotein secretion in mouse yolk sac during embryonic development

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Abstract **In mice, the yolk sac appears to play a crucial role in nourishing the developing embryo, especially during embryonic days (E) 7–10. Lipoprotein synthesis and secretion may be essential for this function: embryos lacking apolipoprotein (apo) B or microsomal triglyceride transfer protein (MTP), both of which participate in the assembly of triglyceride-rich lipoproteins, are apparently defective in their ability to export lipoproteins from yolk sac endoderm cells and die during mid-gestation. We therefore analyzed the embryonic expression of apoB, MTP, and α-tocopherol transfer protein (α-TTP), which have been associated with the assembly and secretion of apoB-containing lipoproteins in the adult liver, at different developmental time points. MTP expression or activity was found in the yolk sac and fetal liver, and low levels of activity were detected in E18.5 placentas. α-TTP mRNA and protein were detectable in the fetal liver, but not in the yolk sac or placenta. Ultrastructural analysis of yolk sac visceral endoderm cells demonstrated nascent VLDL within the luminal spaces of the rough endoplasmic reticulum and Golgi apparatus at E7.5 and E8.5. The particles were reduced in diameter at E13.5 and reduced in number at E18.5–19. The data support the hypothesis that the yolk sac plays a vital role in providing lipids and lipid-soluble nutrients to embryos during the early phases (E7–10) of mouse development.**—Terasawa, Y., S. J. Cases, J. S. Wong, H. Jamil, S. Jothi, M. G. Traber, L. Packer, D. A. Gordon, R. L. Hamilton, and R. V. Farese, Jr. **Apolipoprotein B-related gene expression and ultrastructural chracteristics of lipoprotein secretion in mouse yolk sac during embryonic development.** ***J. Lipid Res.*** **1999.** 40: **1967–1977.**

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Vertebrate animals have evolved widely different strategies to nourish developing embryos (1). All species possess a yolk sac, but its function and morphology vary greatly. In many species, the yolk sac encloses and subsequently absorbs a yolk containing a variety of proteinaceous and lipid substances, which provide structural materials and fuel for the developing embryo. In addition, blood islands found in the yolk sac play a role in early hematopoeisis. Several mammals, including rodents, rabbits, moles, shrews, armadillos, and some bats, have yolk sacs whose absorptive surface is partially or completely "inverted" (1). In such cases, the absorptive surface faces the surrounding uterine decidual tissue, where it is thought to be important in the absorption of maternal nutrients during the early phases of development. This nutritive function precedes and is probably prerequisite for the establishment of the embryo's circulation and chorioallantoic placentation, which subsequently contribute to both nutrient and respiratory functions. In mice, the yolk sac develops around embryonic day (E) 7 and probably plays a vital nutritive role until at least E9–10, when placental function is established.

Recent evidence has indicated that lipoprotein synthesis and secretion may be important functions of the mouse yolk sac visceral endoderm. Mouse embryos lacking apolipoprotein (apo) B, the chief structural component of triglyceride-rich lipoproteins, usually die around E8.5–10.5 (2, 3), and the few that survive to later stages are often exencephalic (2). Further, gene-targeted embryos capable of synthesizing only low levels of truncated apoB species often have neural tube–related developmental de-

Abbreviations: apo, apolipoprotein; bp, base pairs; E, embryonic day; ER, endoplasmic reticulum; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; MTP, microsomal triglyceride transfer protein; nt, nucleotide; PCR, polymerase chain reaction; RER, rough endoplasmic reticulum; RT, reverse transcriptase; α-TTP, α-tocopherol transfer protein; VLDL, very low density lipoprotein.

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fects, including exencephaly and hydrocephaly (4–6). A similar phenotype was observed decades ago in rat embryos deficient in vitamin E (7–9). Indeed, α-tocopherol is undetectable in E9.5 mouse embryos lacking apoB (10).

These observations led us to hypothesize that the yolk sac endoderm synthesizes and secretes apoB-containing lipoproteins during the early stages of embryonic development. To test this hypothesis, we recently performed an ultrastructural analysis of yolk sac endodermal cells from E9.5 embryos and demonstrated very low density lipoprotein (VLDL)-sized lipoprotein particles in the endoplasmic reticulum, Golgi apparatus, and secretory vesicles (10). In apoB-deficient embryos, these particles were absent in yolk sac endoderm cells, which therefore accumulated cytosolic lipid droplets at their basal surface. From these observations, we proposed a model (10) in which the absorptive surface of the yolk sac endoderm cells absorbs lipids and lipid-soluble nutrients from the maternal decidual tissue or maternal blood, hydrolyzes these lipids at the apical surface of the endoderm cells, and repackages both exogenously derived lipids and lipid-soluble nutrients and endogenously synthesized lipids into apoB-containing lipoproteins. These lipoproteins are then secreted from the basal surface of endoderm cells for transport to the developing embryo, presumably by direct diffusion or via the vitelline circulation.

This model of yolk sac-mediated lipoprotein secretion predicted that other molecules crucial to the proper assembly of apoB-containing lipoproteins are essential for embryonic development in mice. This prediction was confirmed recently for microsomal triglyceride transport protein (MTP), a 96-kDa microsomal protein that is expressed abundantly in the liver and small intestine and is thought to participate in the lipidation of nascent apoB-containing particles (11). Human subjects with homozygous mutations in the MTP gene have abetalipoproteinemia (a near-total absence of apoB-containing lipoproteins in plasma) and can have severe neurologic disease due to vitamin E deficiency (12, 13). The recent demonstration that the mouse yolk sac expresses MTP at E10.5 and that mice homozygous for an MTP gene disruption die at ~E10.5 (14) underscores the importance of the synthesis and secretion of apoB-containing lipoproteins during early stages of mouse embryogenesis.

To better understand lipoprotein assembly and secretion by the yolk sac during embryonic development, we analyzed the expression of apoB, MTP, and α-tocopherol transfer protein (α-TTP), all of which are associated with the assembly and secretion of apoB-containing lipoproteins in the adult liver, at different time points of embryonic development. α-TTP is a 32-kDa protein expressed in hepatocytes of rats (15) and humans (16), where it is thought to facilitate the incorporation of α-tocopherol into apoB-containing nascent VLDL secreted into the plasma (17–19), although the mechanism for this does not appear to involve direct enrichment of nascent VLDL (20) (M. Traber and R. Hamilton, unpublished observations). Humans with homozygous mutations in the α-TTP gene have vitamin E deficiency and neurologic abnormalities (21–23). Because gene expression patterns in the adult liver and the visceral yolk sac endoderm are similar (24) and because the phenotype of apoB deficiency resembles that of vitamin E deficiency in rodents, we reasoned that α-TTP may be expressed in the yolk sac or other embryonic tissues during development. Finally, we examined the ultrastructural characteristics of the yolk sac endoderm by electron microscopy at early (E7.5–8.5), middle (E13.5), and late (E18.5) stages of development to better characterize this tissue with regard to the size and amounts of lipoproteins synthesized during development.

MATERIALS AND METHODS

Mice and embryos dissections

C57BL/6J mice were housed in a pathogen-free transgenic barrier facility with a 12-h light and 12-h dark cycle and had free access to rodent chow (Picolab Mouse Chow 20, Purina) and water. Embryonic tissues were obtained from timed matings (25). For expression studies in E7.5–8.5 embryos, whole embryos (including embryonic and extraembryonic tissues) were pooled and analyzed. For E9.5 and older embryos, yolk sac membranes (and other embryonic tissues) were isolated by dissection. Tissues for RNA and protein analysis were dissected into ice-cold magnesium- and calcium-free phosphate-buffered saline, snap-frozen in liquid nitrogen, and stored at −80°C until analysis.

RNA isolation

Total RNA was prepared from homogenized tissues (Polytron homogenizer, Brinkmann) with TRIzol Reagent (Life Technologies) according to the manufacturer's protocol.

Reverse transriptase-polymerase chain reaction

First-strand cDNA was synthesized from 10 μg of total RNA with random primers and a First Strand Synthesis kit (Stratagene). Polymerase chain reaction (PCR) was carried out with 5% of the first-strand reaction product and gene-specific primers. For mouse glyceraldehyde-3-phosphate dehydrogenase (G3PDH), the primers (10) amplify a 978-base pair (bp) product. For mouse apoB, the sense primer is located on exon 25, and the antisense primer is located on exon 26 (10); the amplified apoB product is 303 bp. For mouse MTP, the sense primer (5′-cgcgaat tcCAAAATTGAGCGGTCTGGAT-3′) on exon 5 and the antisense primer (5′-cgcgaattcTGACAGATGTGGCTTTTGAA-3′) on exon 6 (lowercase letters denote sequences containing an *Eco*RI restriction enzyme site for cloning purposes that are not present in the gene) amplify a 94-bp product. For α-TTP, the sense primer (5′-cgcgaattcGATTTCGATCTGGATCTGGC-3′) on exon 1 and antisense primer (5′-cgcgaattcCCACCTCCTGTACAATGAGC-3′) on exon 3 amplify a 283-bp product. For PCR of G3PDH or apoB, reactions were carried out for 35 cycles consisting of 30 s at 96°C, 1 min at 55°C, and 2 min at 72°C. MTP and α-TTP amplifications were carried out for 35 cycles of 30 s at 96°C, 1 min at 45°C, and 2 min at 72°C. Reactions (100 μl) contained 100 ng of each primer and 2.5 units of *Taq* polymerase and were performed in 80 mm Tris-HCl buffer, pH 9.0, containing 2.0 mm $MgCl_2$, 20 mm $(NH_4)_2SO_4$, and 20 mm NaCl. PCR products were analyzed by electrophoresis on 2% agarose gels.

Ribonuclease protection assays

Riboprobes were synthesized in a 20 μl reaction containing 2 μl of [α-^{32}P]UTP (10 mCi/ml, Amersham), 4 μl of 50 nm UTP (Pharmacia Biotech), 3 μl of 3.3 mm ATP, GTP, and CTP

(Pharmacia Biotech), 2 μl of 10× transcription buffer (Boehringer Mannheim), 1 μl of RNase inhibitor (Boehringer Mannheim), 1 μl of RNA polymerase (Boehringer Mannheim), and 200 ng of template in water. Reactions were incubated at 37°C for 1 h. RNase free DNase I (Boehringer Mannheim) (1 μl) was added, and the reaction was incubated at 37°C for 20 min. Labeled probes were purified on Probe Quant G-50 columns (Pharmacia Biotech). The mouse apoB probe is a 245-bp *Xba*I–*Msc*I fragment from the 5′ region of exon 26 (10). The *Xba*I-linearized template generates a riboprobe of 285 nucleotides (nt) and a protected apoB fragment of 245 nt. The mouse MTP probe is a 533-nt large subunit cDNA fragment from the 3′ end of the coding sequence that results in a protected fragment of 443 nt. The mouse α-TTP probe was a 283-nt fragment containing exons 1–3 [amplified by the primers described in the reverse transcriptase (RT)–PCR experiments]; the α-TTP probe size is 264 nt, and the protected fragment is 187 nt. The β-actin probe (provided by Dr. Martin Raabe, Gladstone Institute of Cardiovascular Disease) was a mouse β-actin cDNA fragment (nt 480–559, GenBank accession no. M18194) (14); the probe size is 120 nt, and the protected fragment is 80 nt.

Ribonuclease protection assays (RPAs) were performed with the RPA II kit (Ambion) according to the manufacturer's protocol. Briefly, 10 μg of total RNA was hybridized with empirically determined amounts of riboprobes (5×10^4 cpm/hybridization for apoB and β-actin; 1×10^5 cpm/hybridization for MTP and α-TTP) in combination overnight at 42°C. Nonhybridized RNA was digested by 1:100 RNase A/T1 for 30 min at 37°C. RNases were inactivated and the RNA was precipitated. Protected fragments were heat denatured at 90°C for 4 min in loading dye (solution E) and electrophoresed in a 7% polyacrylamide/8 m urea denaturing gel. Gels were dried on chromatography paper and exposed to X-ray film for various times. Hybridization signals were quantified with a phosphorimager (Fuji Medical Systems), and β-actin expression level was used as a control to standardize for RNA loading (14, 26).

Immunoblots and MTP assays

MTP immunoblotting was performed as described (11). Tissue homogenates (50 μg) were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. MTP was detected with affinity-purified antibodies that recognize the 96-kDa component of MTP (11). For α-TTP immunoblots, a rabbit antiserum was generated against the C-terminal 21 amino acids of the rat α-TTP sequence (15). This antiserum detected a ~30-kDa protein in mouse liver homogenates. Tissue samples (75 μg) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The blot was incubated first with a 1:1000 dilution of the antiserum and then with a goat anti-rabbit secondary antibody and detected with an enhanced chemiluminescence kit (Amersham).

MTP activity assays, based on rate of triglyceride transferred from donor small unilamellar vesicles to acceptor small unilamellar vesicles, were performed with 20–120 μg of protein as described (11).

Electron microscopy

Embryos were dissected into ice-cold 0.1 m sodium cacodylate buffer (pH 7.4), preserved immediately in fixative and stained with a modified (27) imidazole-buffered osmium tetroxide procedure that intensely stains unsaturated double bonds of tissue triglycerides as described (10, 27). For E7.5 and E8.5 time points, embryos were either dissected away from or left in the decidual

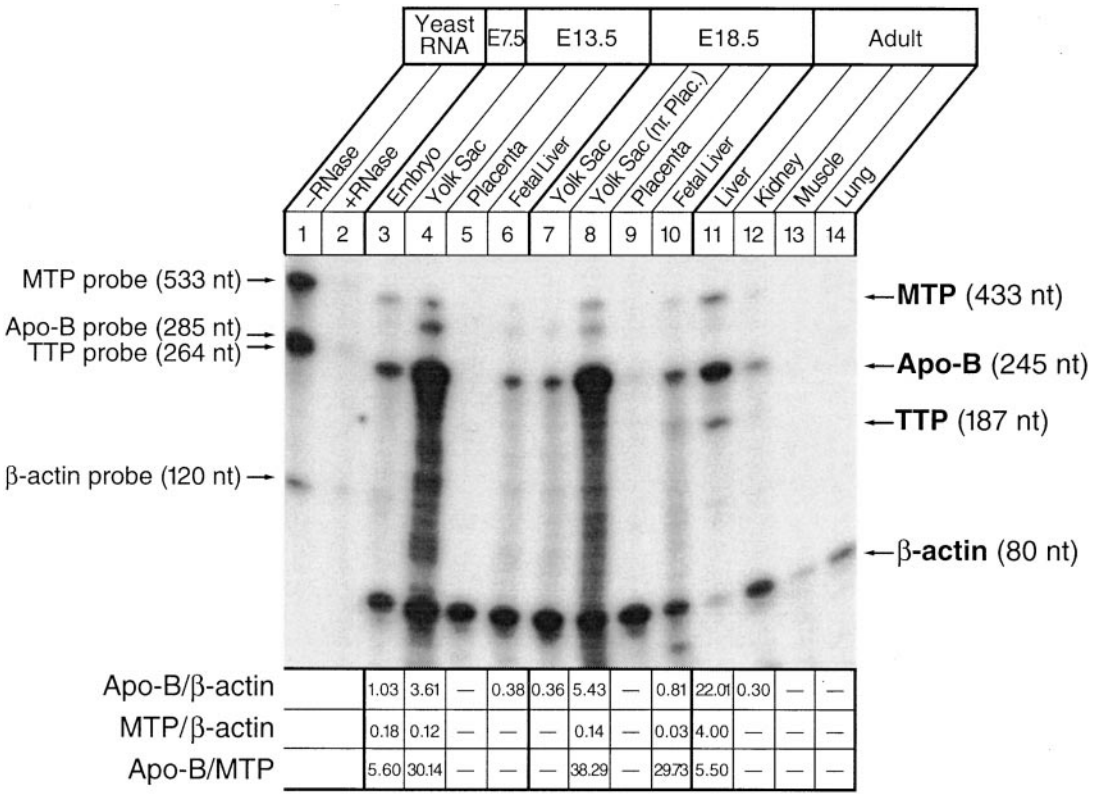

Fig. 1. Embryonic expression of apoB, MTP, and α-TTP mRNAs assessed by RPA. RNA samples (10 μg) were hybridized with ^{32}P-labeled apoB, MTP, α-TTP, and β-actin riboprobes as described in Materials and Methods. Ratios of mRNA expression as determined by phosphorimager analysis are shown below the gel. The experiment was repeated once with similar results; nr. Plac., near placenta.

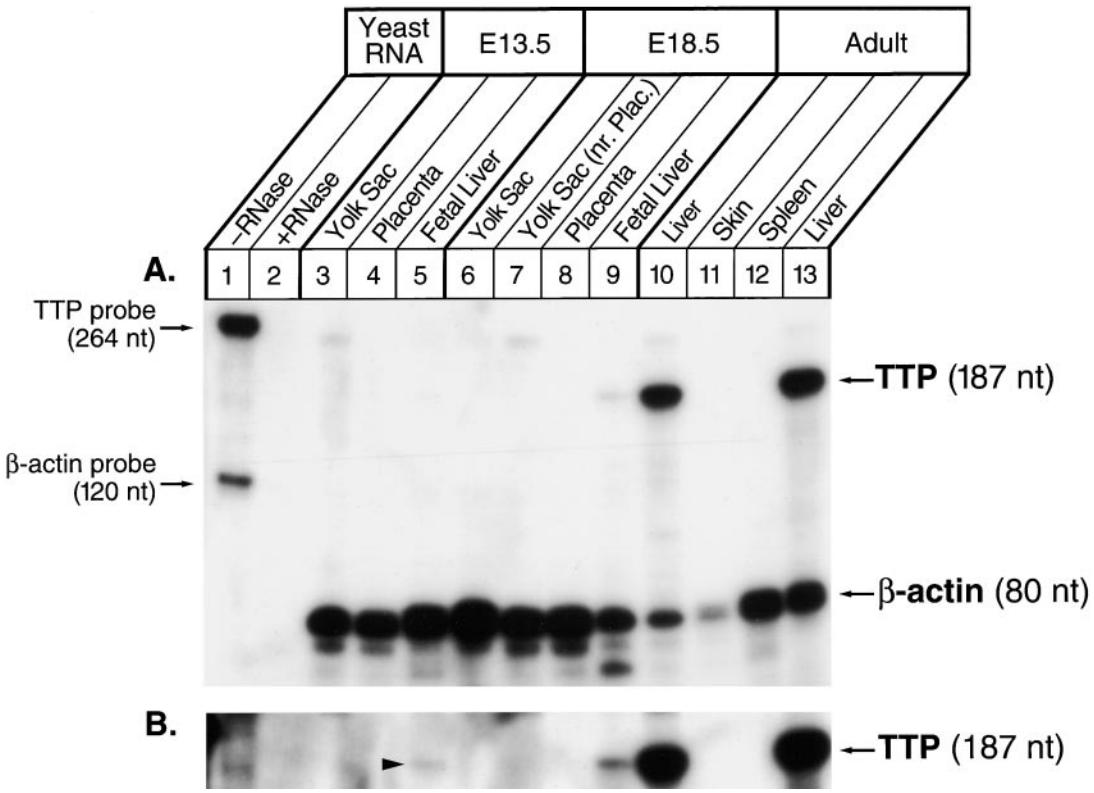

Fig. 2. Embryonic expression of α-TTP assessed by RPA. RNA samples (10 μg) were hybridized with ^{32}P-labeled α-TTP and β-actin riboprobes as described in Materials and Methods. (*A*) A 3-day exposure of the autoradiogram. (*B*) A 4-week exposure of the gel region containing protected fragment for α-TTP. Arrowheads indicate α-TTP protected fragments that are detectable in the embryonic liver. The experiment was repeated once with similar results; nr. Plac., near placenta.

tissue; in the latter case, a small incision was made in the deciduum to allow infiltration of the fixative.

RESULTS

Gene expression studies

In our initial gene expression studies, we performed RT-PCR assays for apoB, MTP, and α-TTP transcripts on cDNA generated from embryos at early (E7.5), middle (E13.5), or late (E18.5) stages of development. Because E7.5 embryos are so small, we analyzed expression from pooled samples of total embryos. At the E13.5 and E18.5 time points, we analyzed expression in the yolk sac, placenta, and embryonic liver. Using this sensitive method, we were able to amplify transcripts for each of the gene products at detectable levels for all of the tissues examined (not shown). The highest levels of apoB transcripts were in the yolk sac and liver; the highest levels of α-TTP were in fetal liver. MTP transcripts were detected in all tissues examined.

To better quantitate the expression levels, we performed RPAs of RNA from embryonic tissues at different developmental time points. ApoB expression was detected at E7.5 and was present at high levels in the yolk sac at E13.5 and E18.5 and at lower levels in embryonic liver at both time points (**Fig. 1**). At E18.5, apoB expression was higher in yolk sac samples obtained near the placenta than in more distant samples. Trace amounts of apoB expression were detected in E18.5 placenta**.** When standardized for RNA loading, apoB expression in the yolk sac was substantially less than that in adult liver but more than that in adult kidney.

The MTP expression pattern determined by RPA was similar to that for apoB (Fig. 1). MTP expression was detected at E7.5 and was present at higher levels in the yolk sac at E13.5 and E18.5 (especially near the placenta in the latter) and at lower levels in embryonic liver at both time points. MTP mRNA expression was not observed in the placenta. As was the case for apoB, MTP was expressed at lower levels in the yolk sac than in adult liver. α-TTP expression was detectable in E18.5 liver, but at a lower level than in adult liver (Fig. 1). α-TTP expression was not detectable in placenta. In the experiment shown in Fig. 1, we could not evaluate α-TTP expression in the yolk sac because of high levels of background from the apoB riboprobe. We therefore performed an RPA specifically to evaluate α-TTP expression (**Fig. 2**). A long exposure of the autoradiogram (Fig. 2B) showed low levels of α-TTP expression in the fetal liver at E13.5 and E18.5, but did not show expression in the yolk sac at any time point.

Immunoblots were performed to evaluate MTP and α-TTP protein expression. Immunoblotting with affinity-purified antibodies that recognize the 96-kDa component of

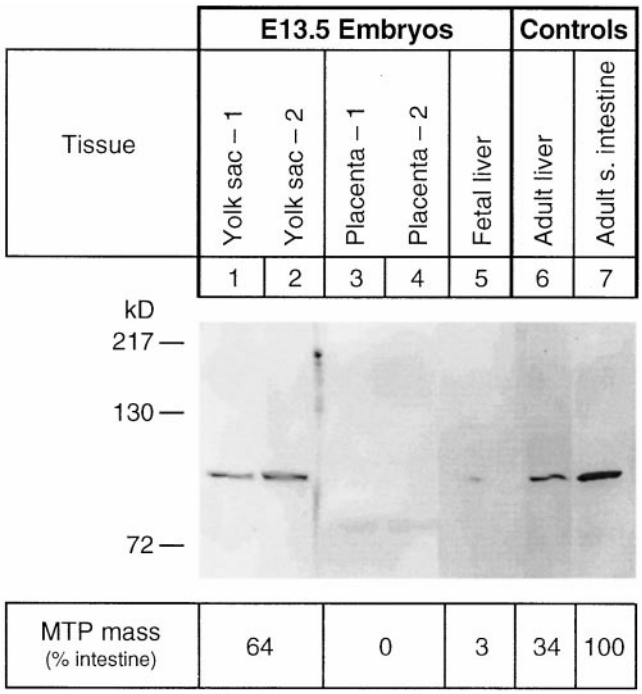

Fig. 3. MTP protein expression in E13.5 tissues assessed by immunoblot. Tissues were harvested from timed matings. Homogenate samples (50 μg) were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with an affinity-purified antibody that recognizes MTP (96-kDa) (11). The autoradiogram was analyzed by densitometry to determine the relative amounts of MTP protein as compared with the human small intestine control (lane 7).

MTP (11) demonstrated MTP protein in yolk sacs, but not placental tissue, of E13.5 embryos (**Fig. 3**). Small amounts of MTP protein were also detected in fetal livers from this time point (Fig. 3 and not shown). Quantitative analysis of these immunoblots demonstrated that the amount of MTP protein in the yolk sac was intermediate between the amounts normally found in adult mouse liver and small intestine. Immunoblotting with an antiserum against a 21–amino acid peptide from the rat α-TTP C-terminus demonstrated high expression levels of the ~30-kDa protein in samples from the adult mouse liver and low expression levels in E18.5

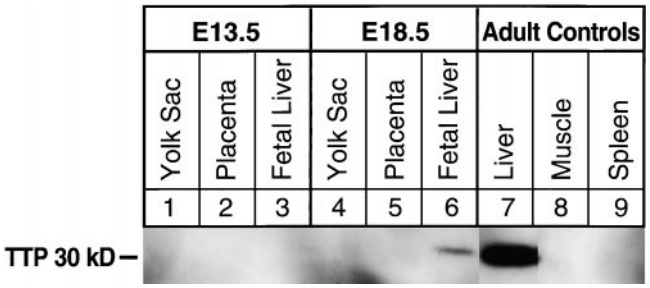

Fig. 4. α-TTP protein expression in embryonic tissues. Homogenate samples (75 μg) were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with an antiserum that recognizes α-TTP. Ponceau staining revealed similar amounts of separated proteins in each lane (not shown).

livers (**Fig. 4**). Trace levels of α-TTP expression were observed in E13.5 livers (not shown). α-TTP protein was not detected in the yolk sac or placenta at any time point.

To determine the tissue and temporal expression patterns of MTP activity, we performed MTP assays in embryonic tissues harvested at different developmental time points (**Fig. 5**). MTP activity was higher in total embryo tissues from E7.5 and E8.5, and in E9.5, E11.5, E13.5, and E18.5 yolk sacs, than in negative control tissues (such as skeletal muscle). At E18.5, MTP activity was higher in yolk sac obtained near the placenta than in samples obtained farther from the placenta (4.4 ± 0.3 vs. 3.1 ± 0.4, $n = 2$). MTP activity was also present in E13.5 and E18.5 liver. MTP activity levels in E13.5 placentas (0.9 ± 0.1) were similar to those in negative control tissues, but were slightly higher in E18.5 placentas (2.1 ± 0.5).

Ultrastructural analyses

Next, we performed electron microscopic studies on yolk sacs from E7.5, E8.5, E13.5, and E18.5–19 embryos. The ultrastructural analysis of E7.5 yolk sac endoderm revealed ample nascent VLDL assembly and secretion as evidenced by numerous lipid-staining particles, mostly 300–700 Å in diameter, within the secretory pathway (**Fig. 6**). These particles were present singly within the lumen of the rough endoplasmic reticulum (RER), whereas in the Golgi apparatus, they were tightly packed in clusters within forming secretory vesicles. At this early time point, not all of the visceral yolk sac endodermal cells appeared to be secreting nascent VLDL. By E8.5 however, virtually every visceral yolk sac endodermal cell examined contained abundant nascent VLDL particles within the lumen of the RER and Golgi apparatus, and most particles were of VLDL diameter (~300–800 Å) (**Fig. 7**).

At E13.5, nascent VLDL particles were substantially smaller in diameter than at E8.5 (~250–400 Å), but they appeared to be either as abundant or even more abundant, especially in cells adjacent to the placenta (**Fig. 8**). At E18.5–19 (**Fig. 9**), the particles were also smaller in diameter (~250–400 Å) and reduced in number, in that some visceral yolk sac endodermal cells, particularly those distant from the placenta, appeared to contain no, or few, identifiable lipoproteins. In endodermal cells near the placenta, clusters of small lipid-staining particles were observed in forming secretory vesicles in the Golgi apparatus; particles of the same diameters were also observed in the basal extracellular spaces at the junction of the yolk sac visceral and parietal endoderm (Fig. 9).

DISCUSSION

This study shows that two gene products previously associated with the adult hepatic assembly and secretion of apoB-containing lipoproteins, MTP and α-TTP, are also expressed in the developing mouse embryo. MTP was expressed at each developmental time point examined, with the highest levels in the yolk sac followed by fetal liver. α-TTP, in contrast, was expressed only in the fetal liver. The ultra-

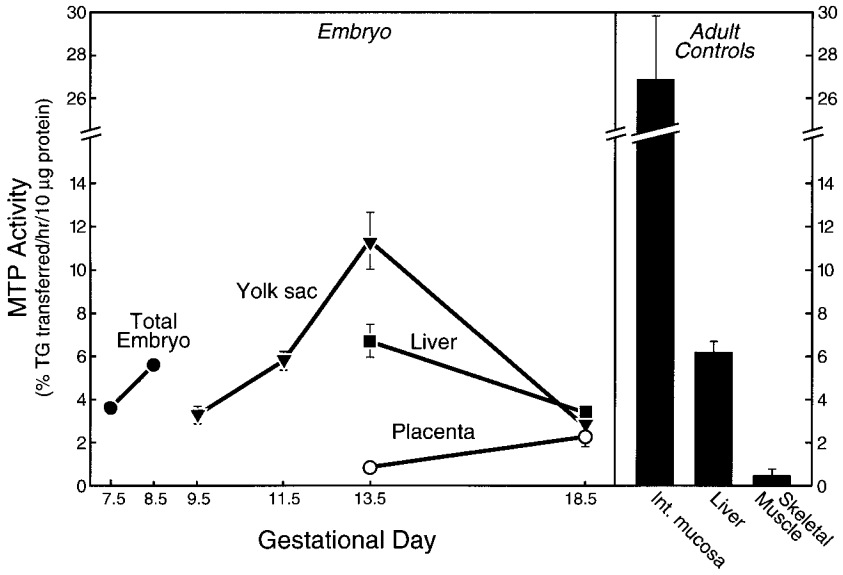

Fig. 5. MTP activity of tissues at various developmental time points. MTP activity (mean ± SE) is shown for embryonic tissues and positive (intestinal mucosa, liver) and negative (muscle) adult controls. The E7.5 and E8.5 time points represent 11 and 3 pooled total embryos, respectively. The data points for yolk sac and embryonic liver were determined on one to four samples of pooled tissues. E18.5 yolk sac represents activity in samples located far from the placenta. Placental MTP activity was determined on individual samples from E13.5 (n = 4) and E18.5 (n = 2) embryos. TG, triglyceride.

structural studies provided morphologic evidence that lipoprotein assembly and secretion by the yolk sac become active in most endodermal cells by E7.5 and are active in all visceral endodermal cells at E8.5, as judged by the abundance of VLDL-sized particles within the secretory organelles. By E13.5, lipoprotein secretion remains active with as many or perhaps even more particles in the secretory organelles, although the particles were of smaller size. At the latest stage of development studied (E18.5–19), more of the lipid-staining particles appeared to be smaller, and their numbers also appeared fewer, in large part because some endodermal cells distant from the placenta appeared to lack particle secretion. These data support the model we have proposed (10) in which lipoprotein synthesis and secretion by the yolk sac play vital nutritional roles in the early (E7–10) stages of mouse development.

Rodent embryos grow rapidly between E7 and E10, a time of development that largely precedes the establishment of a hemachorial placentation. During this early

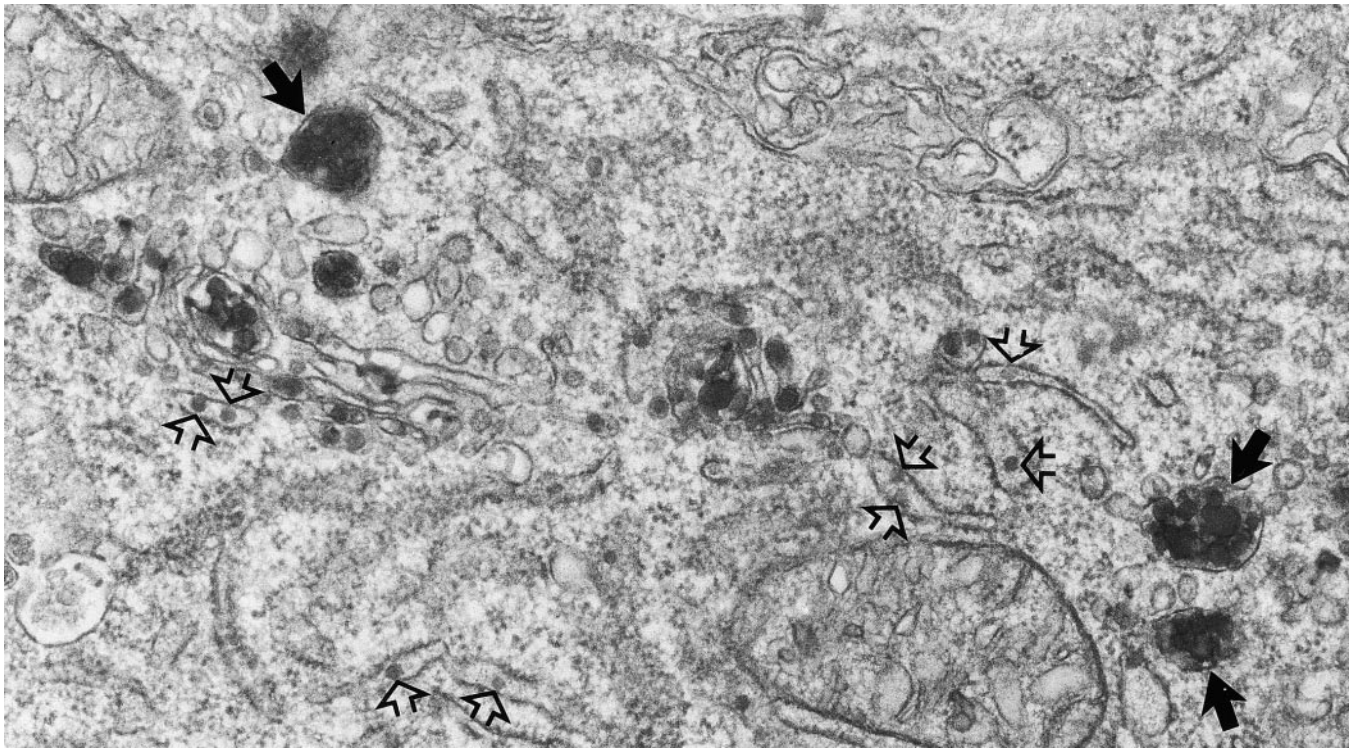

Fig. 6. Electron micrograph of E7.5 yolk sac visceral endoderm cells demonstrating the presence of lipid-staining particles of VLDL size (mostly 300–700 Å in diameter) singly within rough endoplasmic reticulum (RER) luminal spaces (open arrows) and as clusters tightly packed within forming secretory vesicles of the Golgi apparatus (solid arrows). Original magnification, ×36,000.

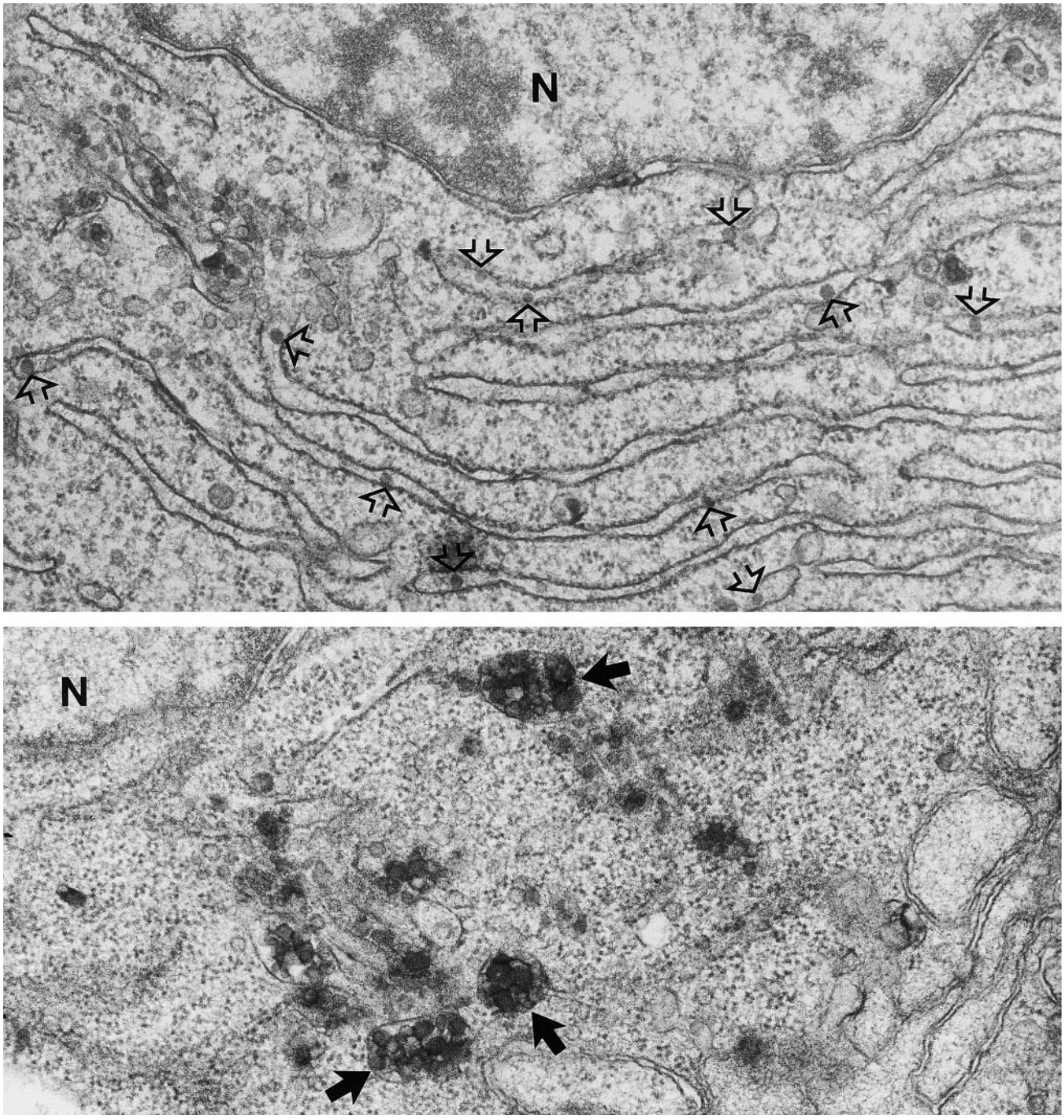

Fig. 7. Electron micrographs of E8.5 yolk sac visceral endoderm cells showing lipid-staining particles of VLDL size (mostly 300–800 Å) singly within RER cisternae (open arrows) and as tightly packed clusters within forming secretory vesicles (solid arrows) on the Golgi apparatus. The ultrastructural characteristics of yolk sac visceral endoderm VLDL secretion appear to be closely similar at E7.5, E8.5 and E9.5 (4). Original magnification, ×36,000.

period, the yolk sac visceral endoderm is thought to absorb nutrients for the developing embryo in a process similar to that in adult intestinal epithelium. This early function of the yolk sac is probably critical until the later establishment of a placental circulation. Because the pattern of gene expression in the yolk sac is similar to that of the liver (24), we reasoned that both MTP and α-TTP, gene products that have been associated with lipoprotein assembly and secretion in the liver, may be expressed in the yolk sac. MTP was indeed expressed in the yolk sac, as demonstrated by detection of expression at the mRNA, protein, and activity levels. This finding was in agreement with a recent observation by Raabe et al. (14) in their studies of MTP knockout mice. We also observed high levels of MTP protein and activity at the early and mid-stages of development, consistent with MTP's proposed role in providing lipidation of nascent apoB-containing lipoproteins (28).

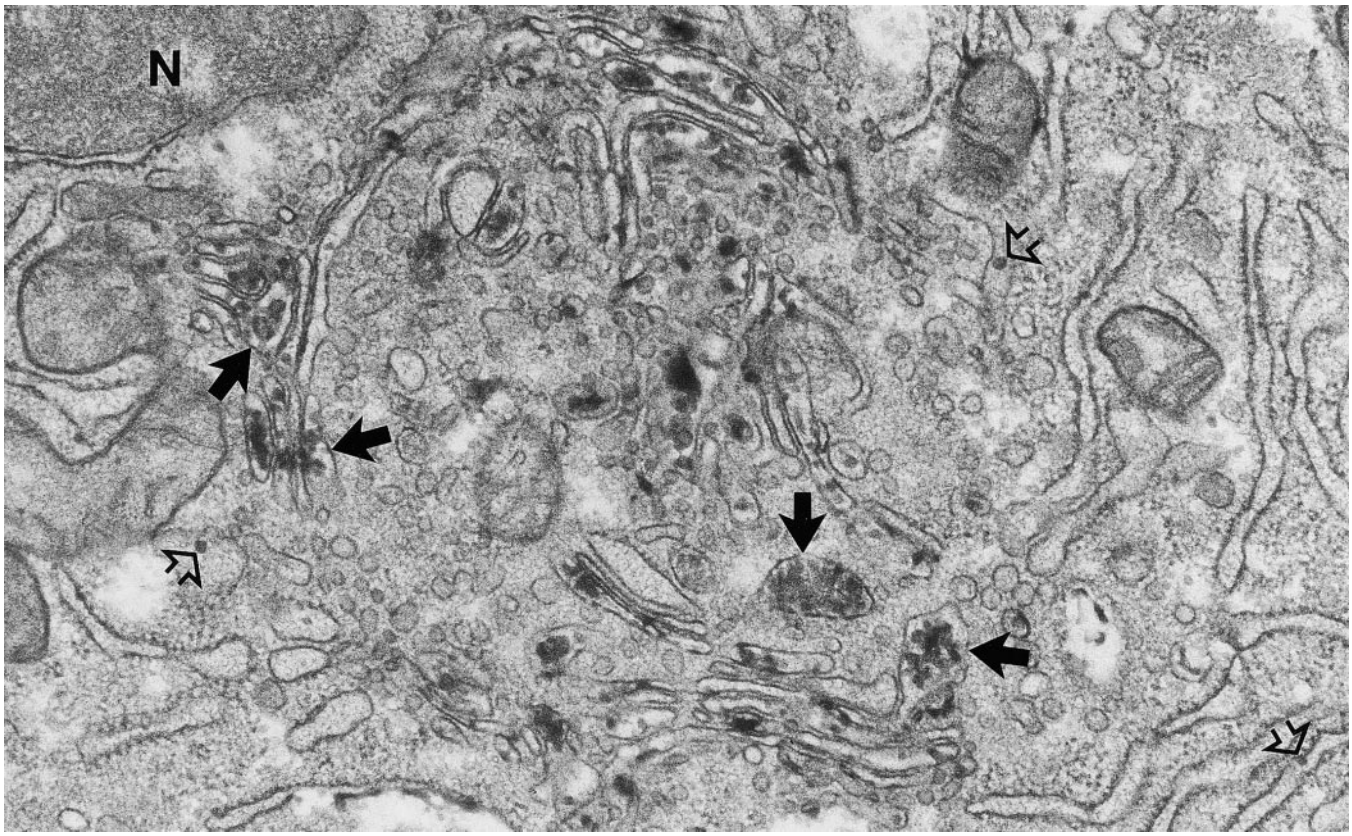

Fig. 8. Electron micrograph of E13.5 yolk sac visceral endoderm showing an abundance of smaller nascent VLDL particle (mostly 250–400 Å in diameter) in both RER cisternae (open arrows) and in incompletely filled forming secretory vesicles (solid arrows) of the Golgi apparatus. Original magnification, ×36,000.

At E18.5, expression of both MTP and apoB was higher in yolk sac samples obtained near the placenta than in regions farther from the placenta. The yolk sac nearer the placenta is thicker and more villous than more distant regions (29), and perhaps it participates more actively in lipid transport at these late developmental time points. Consistent with this topographic structural and functional difference, ultrastructural analyses demonstrated an apparent deficiency of lipoprotein particles in endodermal cells distant from the placenta. In contrast to the high level of apoB expression in rat placenta at E21 (30), we found only trace amounts of apoB expression in E18.5 mouse placenta.

In contrast to MTP, α-TTP expression was not detected in the yolk sac, except when assessed by RT-PCR. α-TTP mRNA and protein, however, were detectable at low levels in the fetal liver. The lack of significant expression in the yolk sac and the presence of α-TTP in the fetal liver are somewhat reminiscent of the situation in the adult intestine and liver: the adult intestine does not express α-TTP (15) and can absorb all stereoisomers of vitamin E, whereas the adult liver expresses α-TTP, and this expression contributes to the selective enrichment of hepatically derived lipoproteins in the α-tocopherol form of vitamin E. In the embryo, the yolk sac may be more intestine-like with regard to vitamin E absorption, and the embryonic liver may contribute to selective enrichment of circulating lipoproteins in the embryo with α-tocopherol. Such a function could be important because vitamin E is critical for normal rodent development (7, 9). A knockout of the α-TTP gene in mice will help to address this possibility.

Our ultrastructural observations are consistent with the proposed role of the yolk sac during the E7–10 period. Previously, we characterized the ultrastructural morphology of E9.5 yolk sac visceral endoderm using electron microscopy, demonstrating for the first time that this tissue assembles and secretes VLDL-sized particles at this early time point (10). In the current study, we observed evidence of lipoprotein assembly of large VLDL-sized lipoproteins in endoderm cells as early as E7.5 and even more abundant VLDL particles at both E8.5 and E9.5. The VLDL size of these lipoproteins presumably results from the incorporation of large amounts of triglycerides during their assembly, and, indeed, MTP activity was present at significant levels at these time points. The ultrastructural characteristics of nascent VLDL in the RER of yolk sac endodermal cells differed distinctly in one important respect from that in adult hepatocytes where no VLDL-sized particles are found in the RER cisternae. In the latter case, VLDL are found only at the smooth-surfaced terminal end of the RER and in the smooth ER proper (31). By the middle stages of development (e.g., E13.5), lipoprotein production appeared to be abundant, as indicated by the large numbers of particles, but the diameters of the lipo-

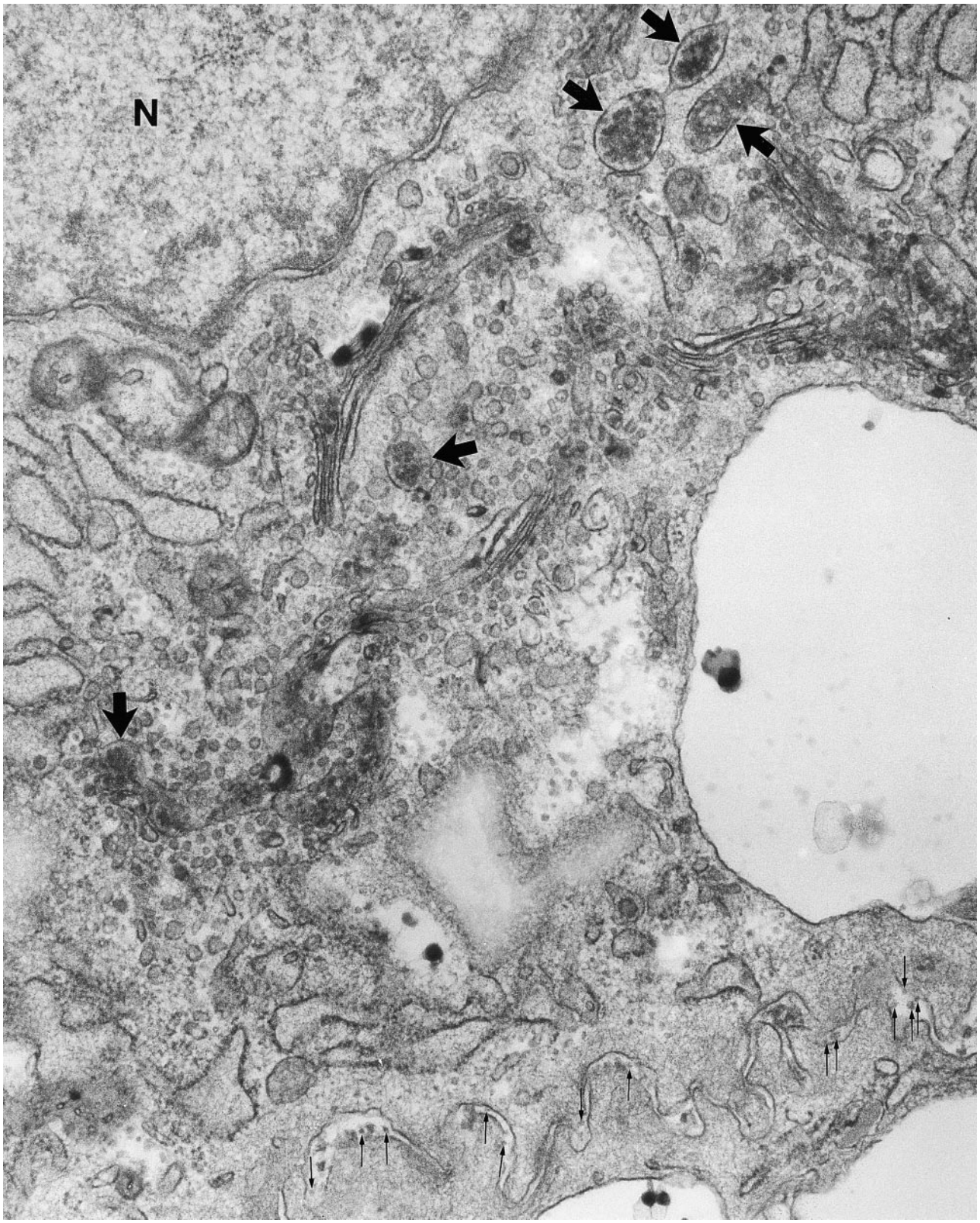

Fig. 9. Electron micrograph of yolk sac visceral endoderm at E18.5–19 showing mostly smaller (250–400 Å in diameter) lipid-staining putative apoB-containing lipoproteins clustered within forming secretory vesicles (large arrows) of the Golgi apparatus and in the basal extracellular spaces (small arrows) at the junction of the yolk sac visceral and parietal endoderm. Because of their very small size, these lipoproteins are barely resolved, consistent with observations in late gestational rat yolk sac (28), in which apoB-100 was secreted mostly in particles of LDL size. Original magnification, ×36,000.

proteins were significantly reduced. This finding seemed paradoxical, as this stage corresponded to the time point when the largest amounts of MTP activity were observed in the yolk sac. However, the ratio of apoB to MTP mRNA expression was higher at E13.5 than at the earlier time points, which does correlate with the apparent finding of less lipid per particle (i.e., smaller particle size). The morphologic data at E13.5 would be consistent with a reduction in the transport of bulk triglycerides, but perhaps a continued production of smaller, more cholesterol-rich VLDL. Precedent for this finding comes from experiments performed in the developing chick, where the yolk sac endoderm secretes large amounts of apoB-containing cholesteryl ester-rich VLDL for transport to the developing embryo (32).

In a series of studies, Franke et al. (33) and Plonné et al. (34, 35) have characterized the ultrastructure of rat yolk sac endoderm lipoprotein secretion and the biochemical properties of lipoproteins secreted by late-term E22 yolk sacs. These investigators observed that, in in vitro yolk sac incubations, lipoprotein production was lower on the last day (E22) of gestation than on E18 and was much lower than on E16 (35). Although these investigators observed that primarily LDL-sized particles were produced by rat yolk sac endoderm, their observations were made at late gestational time points (35). Thus, their observations in rats are consistent with our observations of smaller diameter particles in mice at later developmental time points. We suspect that larger, VLDL-sized particles are secreted by yolk sac endoderm in rat embryos at earlier time points.

The vital requirement of lipoprotein production by yolk sac endoderm in developing mouse embryos prompts the question of which essential nutrients are provided via lipoproteins. Possibilities include many hydrophobic substances normally transported on lipoproteins, such as triglycerides (for fuel), cholesterol or cholesteryl esters, essential fatty acids (for membranes and neurologic development), or fat-soluble vitamins (e.g., vitamin E or vitamin A). Because of the similarities in phenotypes of mouse embryos lacking apoB and rat models of vitamin E deficiency (7–9), the lethality observed in apoB-deficient embryos may relate, at least in part, to vitamin E deficiency. However, the lack of apoB-containing lipoproteins may result in a more global nutritive defect (i.e., deficiencies of other lipids in addition to vitamin E). Evidence supporting this possibility is provided by recent studies addressing the role of cholesterol in development (reviewed in ref. 36), which have delineated a role for cholesterol in modifying an important developmental signaling molecule, the hedgehog protein (37).

In this study, we have further characterized the mouse yolk sac endoderm as a lipoprotein-secreting organ of physiologic relevance in rodents. Although apoB-containing lipoproteins are essential for the normal development of mouse and perhaps rat embryos, adult human subjects survive with a near-total absence of apoB-containing lipoproteins (38). Human subjects deficient in either MTP or α-TTP have also been described (12, 13, 21, 22). For deficiencies of each of these gene products in humans, developmental abnormalities have not been recognized, and the primary neurological problems that result relate to vitamin E deficiency in adults. Thus, although direct evidence is lacking, apoB-containing lipoprotein formation by the yolk sac may not be critical for humans. This could be due to different embryonic growth rates, nutrient requirements, or maternal–fetal transport methods. The placental circulation is established very early during human gestation, and the transport mechanisms for lipids in the placenta and lipid requirements may be quite different from those in the mouse yolk sac.

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