

# Tissue-specific Sorting of the Human LDL Receptor in Polarized Epithelia of Transgenic Mice

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**Abstract.** The distribution of human low density lipoprotein (LDL) receptors was studied by immunofluorescence and immunoelectron microscopy in epithelial cells of transgenic mice that express high levels of receptors under control of the metallothionein-I promoter. In hepatocytes and intestinal epithelial cells, the receptors were confined to the basal and basolateral surfaces, respectively. Very few LDL receptors were present in coated pits or intracellular vesicles. In striking contrast, in the epithelium of the renal tubule the receptors were present on the apical (luminal) surface where they appeared to be concentrated at the base of microvilli and were abundant in vesicles of the

endocytic recycling pathway. Intravenously administered LDL colloidal gold conjugates bound to the receptors on hepatocyte microvilli and were slowly internalized, apparently through slow migration into coated pits. We conclude that (a) sorting of LDL receptors to the surface of different epithelial cells varies with each tissue; and (b) in addition to a signal for clustering in coated pits, the LDL receptor may contain a signal for retention in noncoated membrane that is manifest in hepatocytes and intestinal epithelial cells, but not in renal epithelial cells or cultured human fibroblasts.

THE low density lipoprotein (LDL)<sup>1</sup> receptor binds cholesterol carrying lipoproteins and carries them into cells via endocytosis in coated pits (9). After discharging its ligand in the endosome, the receptor returns to the surface. The steady-state distribution of LDL receptors has been studied by immunoelectron microscopy in human skin fibroblasts that were induced to produce large amounts of receptors (1). These fibroblasts have a smooth surface that is relatively free of microvilli. In the steady state ~70% of the cell surface LDL receptors are located in coated pits, and the rest are found in small clusters that are randomly distributed on the membrane. Receptors are also present in endocytic vesicles, multivesicular bodies, and small recycling vesicles (22, 23).

Indirect studies suggest that the steady-state distribution of receptors may be different on epithelial cells. Two types of epithelial cells have been studied by electron microscopy with the use of <sup>125</sup>I-LDL and colloidal gold-LDL complexes. In livers of rats with high level expression of LDL receptors induced by 17 $\alpha$ -ethinyl estradiol, bound LDL is diffusely distributed on the microvilli of the sinusoidal (basal) surface (5, 11, 12, 13). A relatively small amount of LDL is visualized in coated pits. A similar finding was reported in cultured ovarian granulosa cells (21). In both liver (11, 12) and granulosa cells (21), time course studies revealed that LDL-gold complexes are internalized through slow migration from the microvillar surface into coated pits.

The previous studies of hepatocytes used LDL-gold to mark cell surface LDL receptors, a method that cannot be used to study the localization of receptors in membrane compartments of fixed cells. In the past, it has not been possible to use immunoelectron microscopy for these measurements since the number of hepatic LDL receptors is less than the number on induced fibroblasts and is below the limits for reliable quantitation.

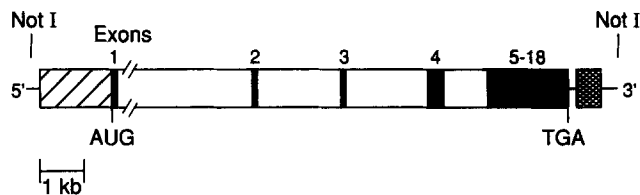
To circumvent this problem, in the current paper we have studied the distribution of LDL receptors in epithelial cells from organs of mice that express high levels of human LDL receptors encoded by a transgene (14). In epithelial cells of liver and small intestine, the vast bulk of receptors was distributed diffusely on the basal and basolateral surfaces, respectively, and only a small number was visualized in endocytic vesicles. In sharp contrast, in the epithelium of the renal tubule the expressed LDL receptors were found on the apical (luminal) surface where they appeared to be concentrated at the base of the microvilli. They were also abundant in endocytic vesicles. The data suggest that different polarized epithelial cells may sort LDL receptors differently and that a specific signal may be required to retain LDL receptors on the surface of liver and intestinal epithelial cells.

## Materials and Methods

### Materials

Chemicals used for immunofluorescence and electron microscopy were ob-

1. *Abbreviation used in this paper:* LDL, low density lipoprotein.



**Figure 1.** LDL receptor transgene 2. The promoter and transcription initiation sites derived from the mouse metallothionein-I gene are indicated by a striped box. LDL receptor exons are denoted by filled-in areas and introns by open areas. Vertical slashes in intron 1 indicate a region where a portion of this intron was deleted. The exons are numbered above the diagram. Thin lines represent poly-linker sequences. Sequences from the 3' end of the human growth hormone gene containing signals for transcription termination and polyadenylation are indicated by a stippled box.

tained from previously reported sources (15, 22). We obtained rabbit anti-mouse IgG and goat anti-rabbit IgG labeled with FITC from Zymed Laboratories (San Francisco, CA); sheep anti-rabbit IgG from Organon Teknika-Cappel (Malvern, PA); monoclonal mouse antidiuretic hormone IgG from Oxford Biomedical (Oxford, MI); and goat anti-rabbit IgG conjugated to gold (10 nm) from Energy Sciences (Woburn, MA). A polyclonal rabbit IgG directed against the LDL receptor was prepared by immunizing a New Zealand white rabbit with purified bovine LDL receptor as previously described (25). Rabbit anti-human LDL antiserum was purchased from Calbiochem-Behring Corp. (San Diego, CA), and the IgG fraction was purified on a protein A Sepharose column. A nonimmune rabbit IgG was prepared as reported earlier (25).

### Preparation of LDL-Gold Conjugate

Colloidal gold, 15 nm in diameter, was prepared as previously described (23). The LDL-gold conjugate was made as follows: human plasma LDL (d 1.019-1.063 g/ml) (8) was loaded onto a PD-10 column (equilibrated in distilled water), eluted with distilled water, and adjusted to a protein concentration of 1 mg/ml. Multiple 10-ml aliquots of colloidal gold (adjusted to pH 5.5 with 1% [wt/vol] potassium carbonate) were each incubated with 100-120- $\mu$ g LDL protein, pooled together in the presence of BSA at a final concentration of 0.01% (wt/vol), and centrifuged in 30-ml polysulfone tubes in a Ti 50 rotor (Beckman Instruments, Palo Alto, CA) at 12,000 rpm at 4°C for 40 min. Virtually all of the conjugate from each 30-ml of starting material was recovered in a loose pellet of  $\sim$ 0.25 ml. Several loose pellets were pooled, dialyzed against PBS, gel filtered over a PD-10 column equilibrated with PBS, and stored at 4°C. Before injection into mice, the conjugate was warmed to 37°C, and BSA was added at a final concentration of 0.2% (wt/vol). Estimation of the protein concentration of LDL in the LDL-gold conjugate was based on a recovery of 85% as determined by parallel incubations in which  $^{125}$ I-LDL of known specific radioactivity was conjugated to gold.

### Construction of Metallothionein Promoter-LDL Receptor Minigene Plasmid

An expression plasmid containing the mouse metallothionein-I promoter fused to a hybrid human LDL receptor gene (designated transgene 2) was constructed by standard methods of genetic engineering (19), involving a total of nine intermediate plasmids. The final insert (Fig. 1) was harbored in the pTZ18R vector (Pharmacia Fine Chemicals, Piscataway, NJ) and contained in the following linear order, 5'-3': 1.7 kb of DNA corresponding to the 5'-flanking region of the mouse metallothionein-I gene (14); exon 1 of the LDL receptor gene; a truncated form of intron 1 of the LDL receptor gene from which sequences between an Rsa I site 77 nucleotides 3' of exon

1 and an Eco RI site located 3.1 kb 5' of exon 2 (26) had been deleted; exons and introns 2-4 of the LDL receptor gene; a cDNA fragment corresponding to exons 5-18 of the LDL receptor (27); and sequences from the 3' end of the human growth hormone gene containing signals for transcription termination and polyadenylation (14). The length of the metallothionein promoter-LDL receptor minigene is  $\sim$ 14.7 kb, and the expected size of the correctly processed mRNA is 2.9 kb. Not I sites at the 5' and 3' ends of the minigene were used to excise the DNA from the plasmid vector prior to microinjection into fertilized mouse eggs.

### Transgenic Mice

A total of 405 eggs were microinjected with transgene 2 and transferred into pseudopregnant females (3). Among the 58 offspring, 25 (43%) contained the transgene as determined by dot hybridization of DNA obtained from tail homogenates. These mice were subjected to a partial hepatectomy at  $\sim$ 5-6 wk of age. 15 of 24 founder mice produced mRNA derived from the transgene in the liver as determined by solution hybridization (18). Of these 15 mice, 4 showed a chronic absence ( $<$ 1 mg/dl) of apoprotein B-100 in plasma as measured by rocket immunoelectrophoresis (14). These four mice were bred to C57Bl/6J  $\times$  SJL F1 mice, and lines of transgenic mice were established. Mice from one of these 4 lines expressing transgene 2 (line 192-2) were used in this study. The line of mice carrying transgene 1 (line 93-4) (14) was bred to homozygosity with respect to the transgene and has been maintained in this state. Where indicated, mice (20-25 g body weight) were treated using one of two methods for induction of the metallothionein promoter with zinc: either ZnSO<sub>4</sub> (25 mM) in the drinking water for 7 d before sacrifice, or 5 mg/kg of ZnSO<sub>4</sub> in 0.1 ml injected intraperitoneally 12 and 6 h before sacrifice.

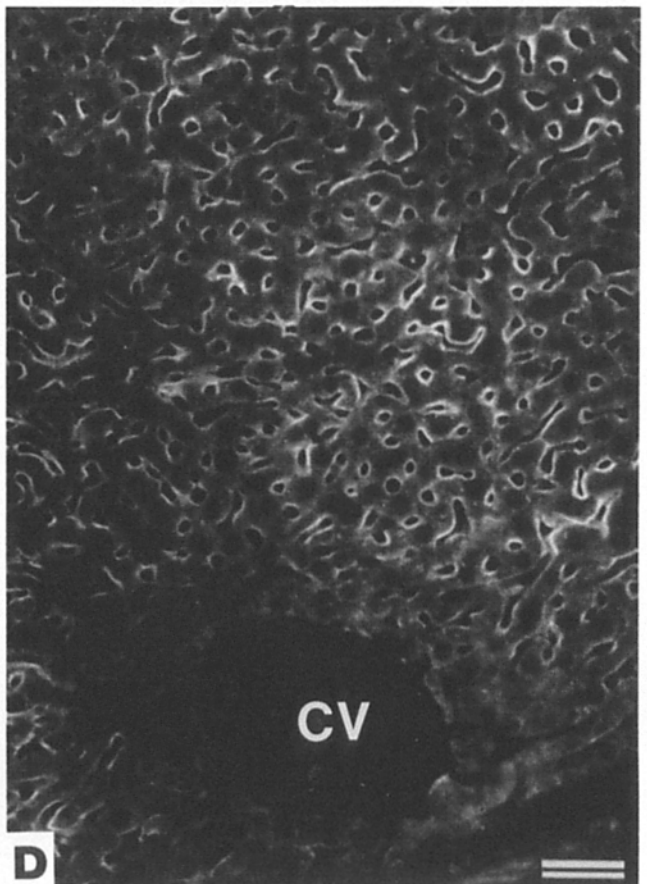
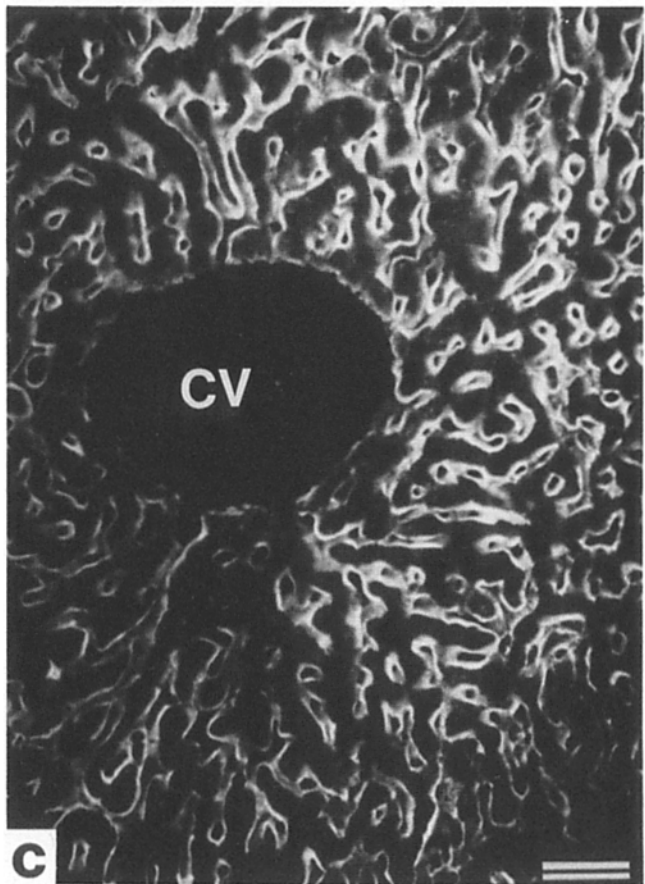
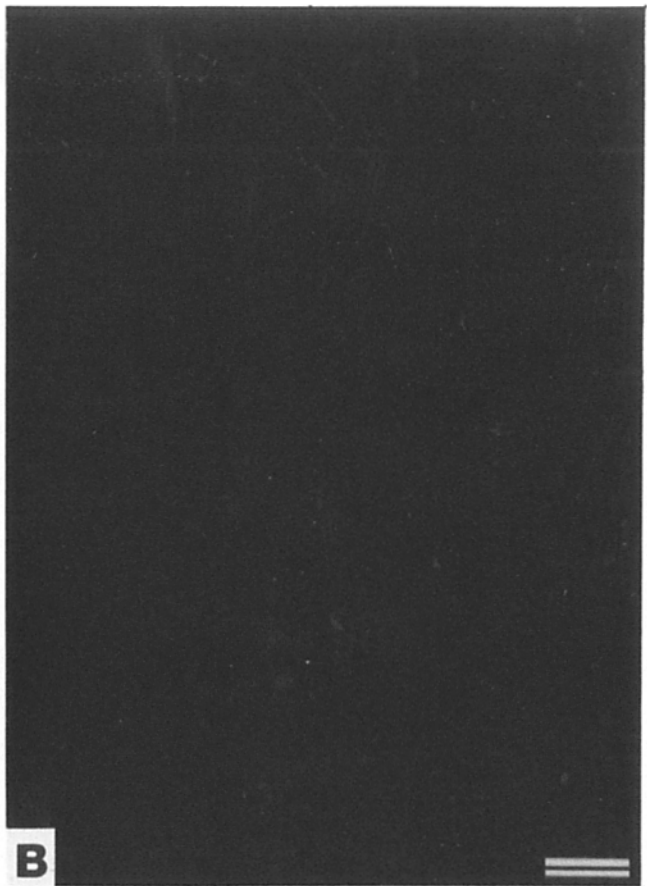
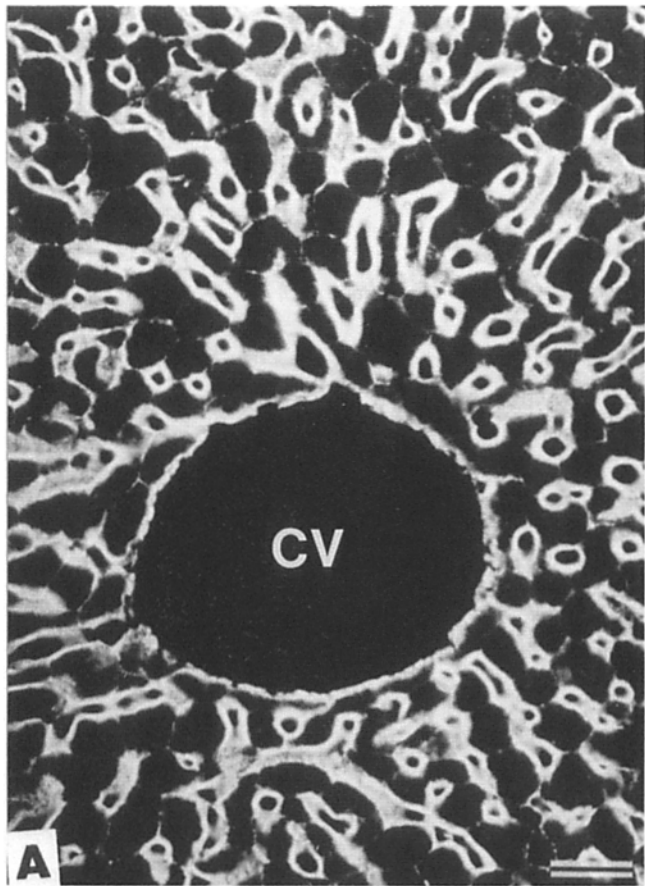
### Injection of Mice with LDL and LDL-Gold

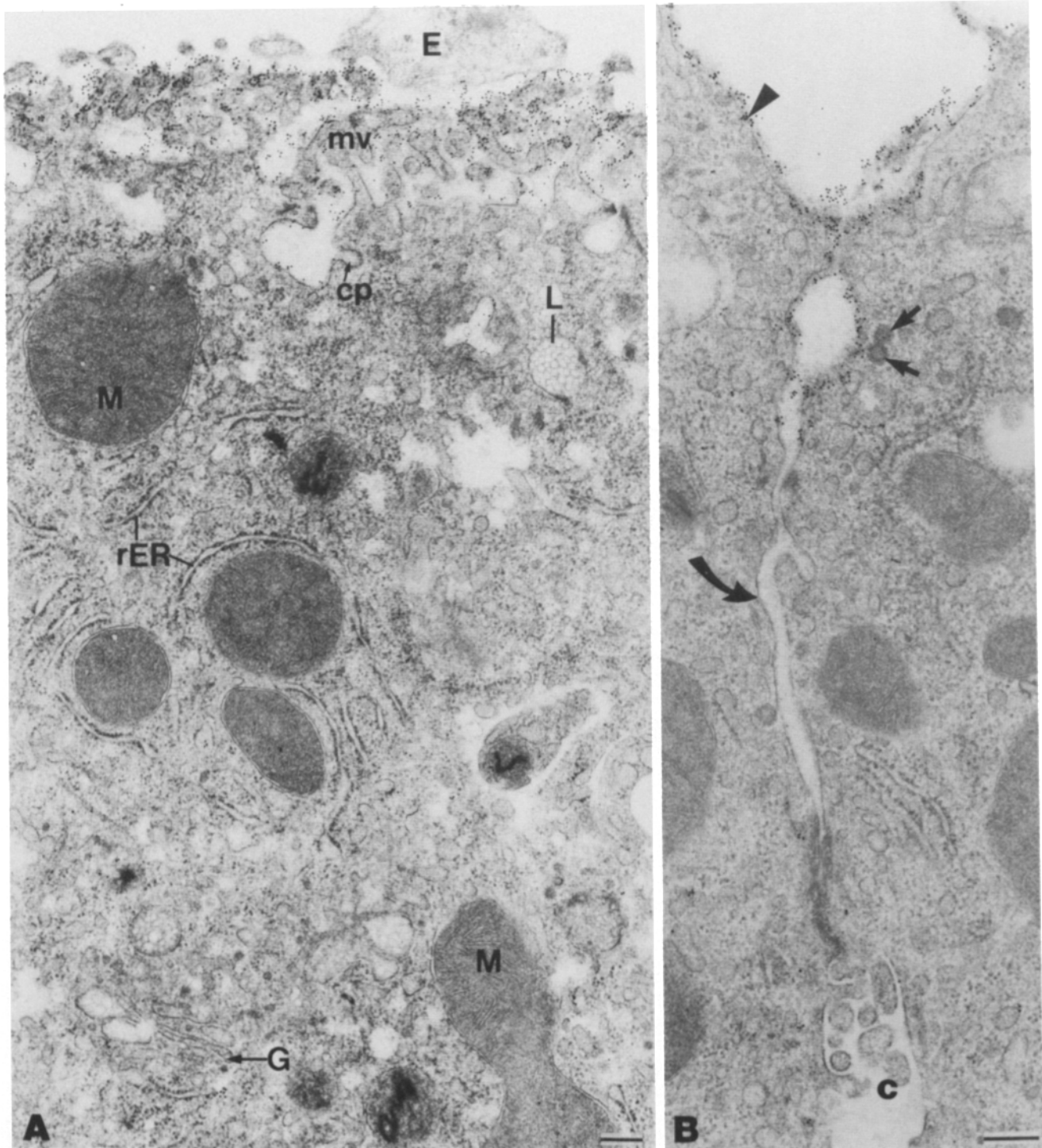
Mice were anesthetized with sodium pentobarbital (90 mg/kg body weight) and injected intravenously (0.15-0.2 ml/injection) via the external jugular vein with either human LDL (one injection, 0.5 mg protein) or LDL-gold (four injections, 0.3 mg protein for each injection at 15-min intervals) as indicated in the figure legends. Body temperature of the animals was maintained by keeping them on a warming tray throughout the experiment. The lipoproteins were injected in PBS or 0.15 M sodium chloride containing 0.2% (wt/vol) BSA.

### Immunofluorescence Microscopy

Mice were anesthetized as described above and were perfused through the ascending aorta with 40 ml of Hank's balanced salt solution for 5 min to remove blood. Mice were then fixed by the perfusion of 60 ml of 3% (wt/vol) paraformaldehyde in 100 mM sodium phosphate buffer at pH 8.0 containing 3 mM trinitrophenol, 4 mM KCl, and 2 mM MgCl<sub>2</sub> for 5 min. Samples of liver, kidney, and jejunum were removed and fixed in a fixative containing 60% (vol/vol) methanol, 10% (vol/vol) glacial acetic acid, 30% (vol/vol) inhibitol (1,1,1-trichloroethane) for 24 h. Further processing of tissues, paraffin embedding, sectioning, and indirect immunofluorescence was done as previously described (15). Briefly, deparaffinized, hydrated tissue sections were soaked in buffer A (20 mM Tris-chloride at pH 8.5, 200 mM NaCl, 0.1% [wt/vol] BSA, and 0.02% [wt/vol] sodium azide) for 30 min after which the sections were incubated sequentially with antibodies as follows: overnight incubation with 50  $\mu$ g/ml of rabbit anti-human LDL IgG, anti-LDL receptor IgG, or a nonimmune IgG, followed by a 2-h incubation with 25  $\mu$ g/ml affinity purified, goat anti-rabbit IgG conjugated to FITC. After each incubation, sections were rinsed with buffer A three times for 5 min. Finally, sections were rinsed in distilled water for 30 s before mounting in 1,4-diazobicyclo(2.2.2)octane. Sections were observed and photographed using a Zeiss photomicroscope III with appropriate filter package.

**Figure 2.** Immunofluorescence localization of the human LDL receptor (A-C) or injected human LDL (D) in livers of zinc-treated (A) or untreated (C and D) transgenic mice and in a zinc-treated normal mouse (B). Mice expressing transgene 2 (A) or normal mice (B) were injected with zinc intraperitoneally before the experiment. Liver samples were perfusion fixed and embedded in paraffin for immunostaining as described in Materials and Methods. A transgenic animal that was not injected with zinc (C and D), received an intravenous bolus of human LDL (0.5 mg protein) 15 min before the liver was perfused, fixed, and processed. CV, central vein. Bar, 10  $\mu$ m.



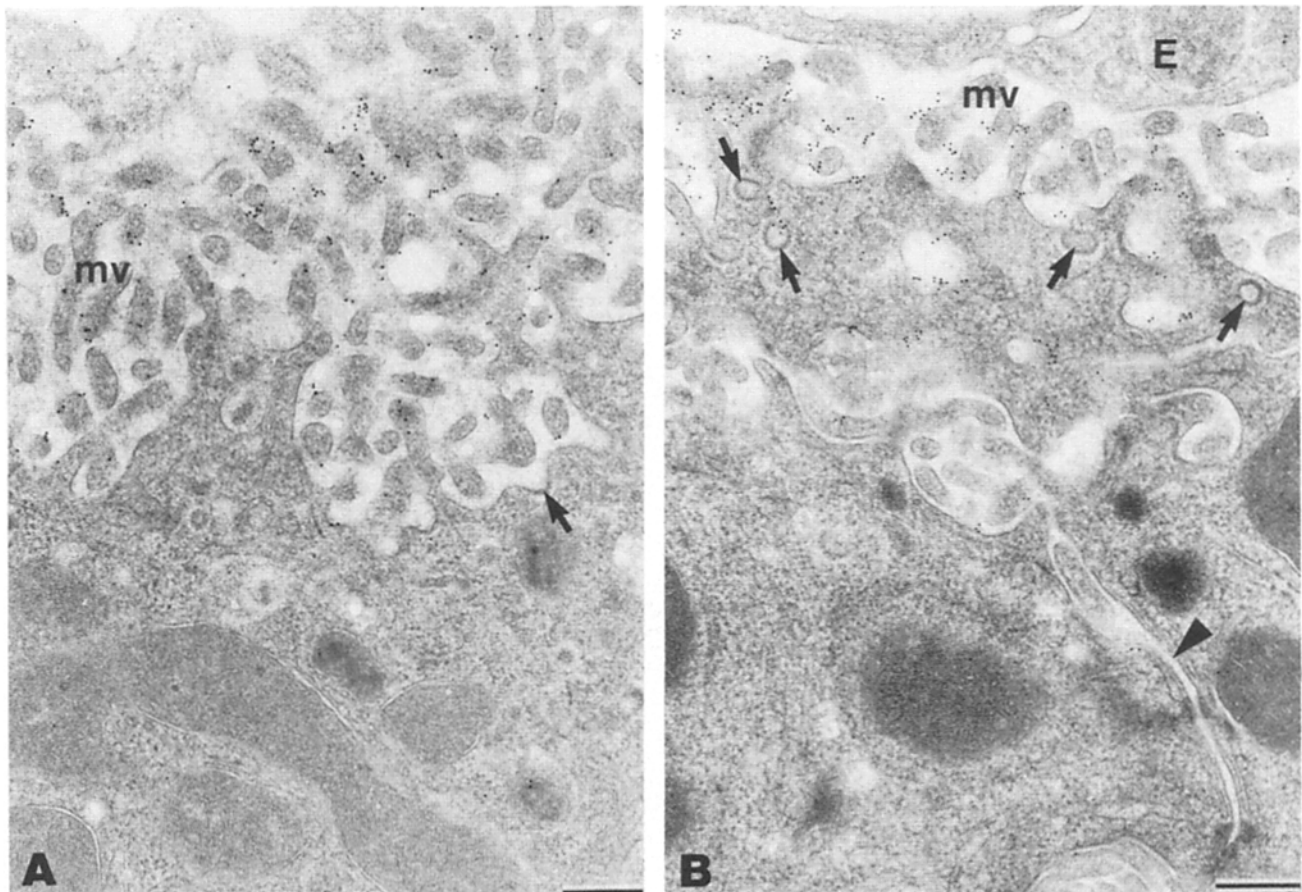


**Figure 3.** Immunogold localization of the human LDL receptor in hepatocytes from zinc-treated transgenic mice. Mice expressing transgene 2 were treated with zinc in drinking water as described in Materials and Methods. Tissue samples were prepared for immunogold labeling as described in Materials and Methods. (A) Overview of a hepatocyte. *E*, endothelial cell; *mv*, microvilli; *cp*, coated pit; *L*, lipoprotein-rich vesicles; *M*, mitochondria; *rER*, rough endoplasmic reticulum; *G*, Golgi apparatus. (B) High magnification of basal surface (arrowhead), lateral surface (curved arrow), and canalicular surface (c) of hepatocyte. (Straight arrows) coated vesicles. Bars, 0.3  $\mu\text{m}$ .

### Immunoelectron Microscopy

Tissue samples from perfusion-fixed mice were dissected out and kept overnight in the perfusion fixative. Vibratome sections (60–80  $\mu\text{m}$  thick) were prepared and washed in 100 mM sodium phosphate buffer, pH 7.8, containing 50 mM ammonium chloride for 30 min and then transferred to buffer B (buffer A containing 0.05% [wt/vol] saponin) for 1 h. All the primary anti-

bodies, rabbit anti-human LDL IgG, anti-LDL receptor IgG, and the nonimmune IgG, were diluted in buffer B at a final concentration of 50  $\mu\text{g/ml}$ . Groups of 6–8 sections were incubated overnight with each of the antibodies. This was followed by a 3-h incubation with 20  $\mu\text{g/ml}$  of sheep anti-rabbit IgG conjugated with dinitrophenol in buffer B (22). Sections were washed after each incubation three times for 5 min each in buffer B. After a final wash, sections were rinsed twice in 100 mM sodium phosphate



**Figure 4.** Immunogold localization of the human LDL receptor (*A*) and injected human LDL (*B*) on the basal surface of hepatocytes of noninduced transgenic mice. Liver samples from the same animal described in Fig. 2 (*C* and *D*) were processed to localize either LDL receptors (*A*) or LDL (*B*) by immunogold labeling. *E*, endothelial cell; *mv*, microvilli; *arrows*, coated pits or vesicles; *arrowhead*, lateral surface. Bars, 0.4  $\mu\text{m}$ .

buffer (pH 7.8), and fixed with 1.33% (vol/vol) glutaraldehyde for 2 h in the same buffer. Tissue sections were postfixated with 1% (wt/vol) osmium tetroxide in phosphate buffer, dehydrated, embedded in Epon, sectioned, and processed to localize DNP groups by immunogold labeling as previously described (15).

#### Immunoblot Analysis

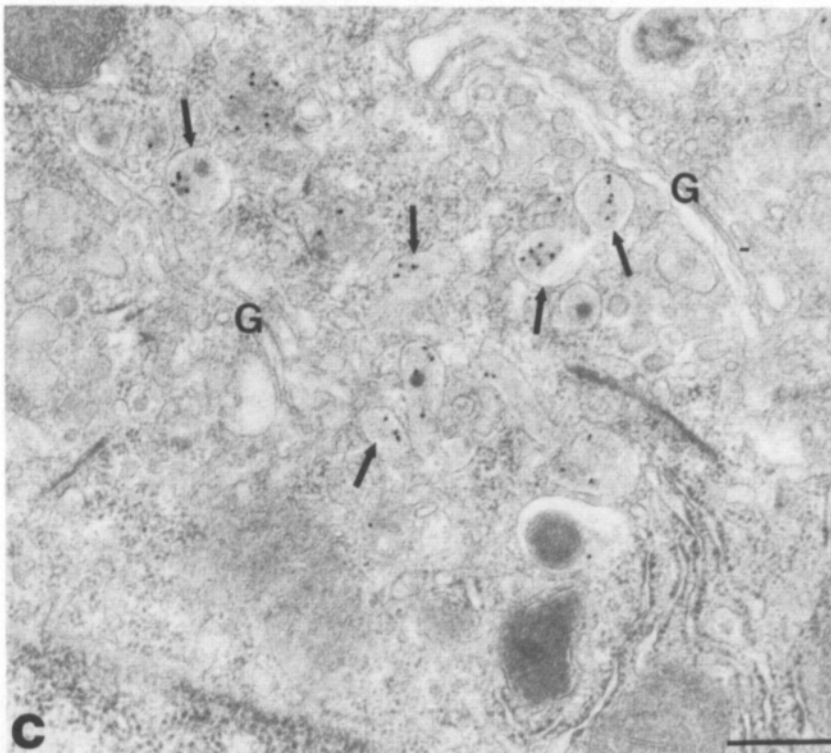
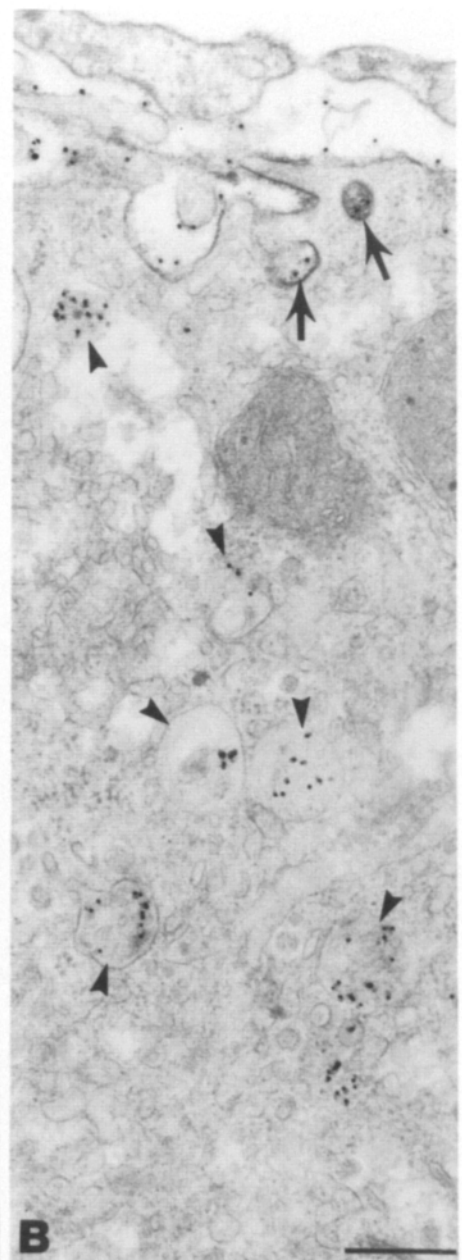
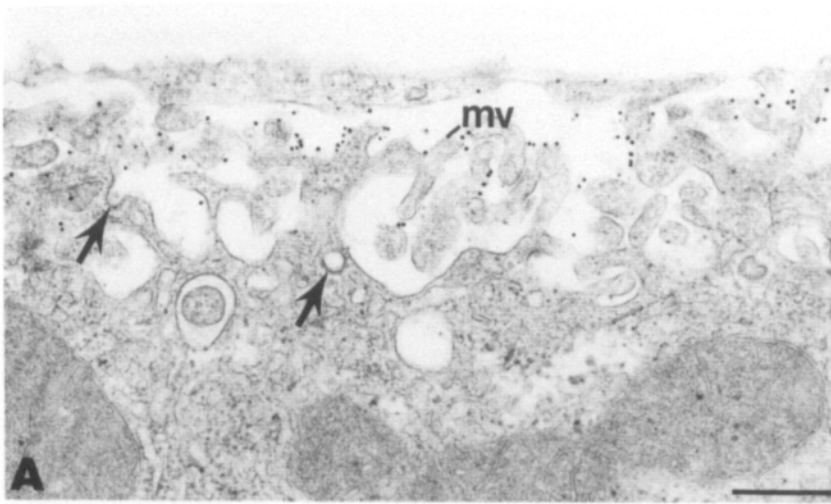
Mouse tissues (0.5–1.1 g) were homogenized in a polytron homogenizer in 5 vol (wt/vol) of buffer containing 20 mM Tris-chloride at pH 8.0, 1 mM  $\text{CaCl}_2$ , 150 mM NaCl, 1 mM PMSF, 1 mM 1,10-phenanthroline, 0.1 mM

**Table I. Distribution of LDL and LDL Receptors in Normal and Transgenic Mouse Liver**

Mouse	Induction of receptors with $\text{ZnSO}_4$	Intravenous injection of LDL	IgG for immunogold labeling	Number of coated pits counted	Total $\mu\text{m}$ of coated membrane	Gold particles/ $\mu\text{m}$ of coated membrane ( <i>a</i> )	Gold particles/ $\mu\text{m}$ noncoated membrane ( <i>b</i> )	Ratio ( <i>b/a</i> )
<b>Experiment A</b>								
Normal	Yes	No	Anti-LDL receptor	106	42	0.14 (6)*	0.12 (15)*	—
Transgenic	Yes	No	Nonimmune	339	136	0.12 (16)	0.30 (46)	—
Transgenic	Yes	No	Anti-LDL receptor	378	151	0.48 (73)	41.5 (4,860)	86
<b>Experiment B</b>								
Transgenic	No	No	Anti-LDL	163	65	0.077 (5)	0.16 (17)	—
Transgenic	No	No	Anti-LDL receptor	421	168	0.39 (66)	12.3 (1,591)	32
Transgenic	No	Yes	Anti-LDL	411	164	0.82 (134)	12.0 (1,503)	15
Transgenic	No	Yes	Anti-LDL receptor	138	55	0.73 (40)	12.9 (1,610)	18

Treatment of mice, preparation of liver, and immunogold labeling were performed as indicated in the legends to Figs. 3 and 4. Random photographs were taken at a magnification of 9,000, and negatives were printed at a final magnification of 24,000. At least 50 different photographs were taken for each treatment. Coated pits/vesicles located  $<0.5 \mu\text{m}$  from the hepatocyte basal surface membrane were identified on the basis of morphology. The linear membrane present in each compartment was measured with a Map Measuring device (Swirl Handle Type, Keuffel and Esser, Switzerland), and the gold particles over each portion of membrane were tabulated.

\* The total number of gold particles found in coated pits are shown in parentheses.



leupeptin, 1  $\mu\text{g/ml}$  pepstatin, and 0.5  $\mu\text{g/ml}$  aprotinin. The homogenate was centrifuged at 470  $g$  for 10 min at 4°C in a rotor (SA 600; Sorvall Co., Dupont, Wilmington, DE), and the supernatant was spun at 100,000  $g$  in a rotor (TFT 65.13; Sorvall Co.) for 1 h. The pellet was resuspended with a 21-G needle in 1 ml of 125 mM Tris-maleate at pH 6.0, 1 mM  $\text{CaCl}_2$ , 160 mM NaCl, and 1% (vol/vol) Triton X-100 containing all of the above protease inhibitors. The suspension was centrifuged at 100,000  $g$  for 1 h at 4°C in a rotor (TFT 65.13; Sorvall Co.). The supernatant was subjected to 7% SDS-PAGE under nonreducing conditions and transferred to nitrocellulose. After transfer the filters were incubated in 50 mM Tris-chloride at pH 8, 2 mM  $\text{CaCl}_2$ , 80 mM NaCl, 5% (wt/vol) nonfat dry milk, 0.2% (vol/vol) NP-40, and 0.01% (vol/vol) Antifoam A emulsion for 1 h at room temperature. Filters were then incubated with 5  $\mu\text{g/ml}$  of polyclonal rabbit anti-LDL receptor IgG in the above buffer for 1 h at room temperature. After two 15-min washes with the above buffer, the filters were incubated with  $^{125}\text{I}$ -labeled goat anti-rabbit IgG (5  $\times 10^6$  cpm/ml;  $\sim 7,000$  cpm/ng) for 1 h at room temperature. After two 15-min washes, the filters were dried and subjected to autoradiography.

## Results

We examined two lines of transgenic mice, each containing a different LDL receptor transgene. One line expressing LDL receptor transgene 1 was previously described by Hofmann et al. (14). LDL receptor transgene 1 contains the mouse metallothionein-I promoter fused to the coding region of the cDNA for the human LDL receptor. Marked overexpression is achieved through administration of cadmium, which induces the metallothionein promoter (14). During the early stages of the current study, we found that this transgene contained a single base insertion at nucleotide position 2,564. The frameshift resulted in a change in the COOH-terminal five amino acids of the LDL receptor (glu-asp-asp-val-ala-stop) to gly-gly-stop. To be certain that this COOH-terminal mutation did not alter the distribution of the receptor, we made a second group of transgenic mice that expressed the correct receptor sequence, designated transgene 2 (Fig. 1). Transgene 2 is a minigene of the LDL receptor that contains the promoter and transcription initiation site derived from the mouse metallothionein-I gene, a portion of the LDL receptor gene (exons 1–4 and their corresponding introns), and the coding region of the LDL receptor cDNA containing exons 5–18. As will be described below, we found no differences in the function or in the cellular distribution of the human receptor encoded by transgenes 1 or 2.

### Liver

Previous studies in mice expressing LDL receptor transgene 1 showed that cadmium treatment stimulated expression of the human receptor in liver, and this led to the elimination of LDL from plasma (14). After cadmium treatment, hepatocytes from these animals internalized large amounts of intravenously injected fluorescently labeled human LDL (14). Fig. 2 shows the localization of human LDL receptors by indirect immunofluorescence in the hepatocytes of mice that expressed transgene 2 and were injected intraperitoneally

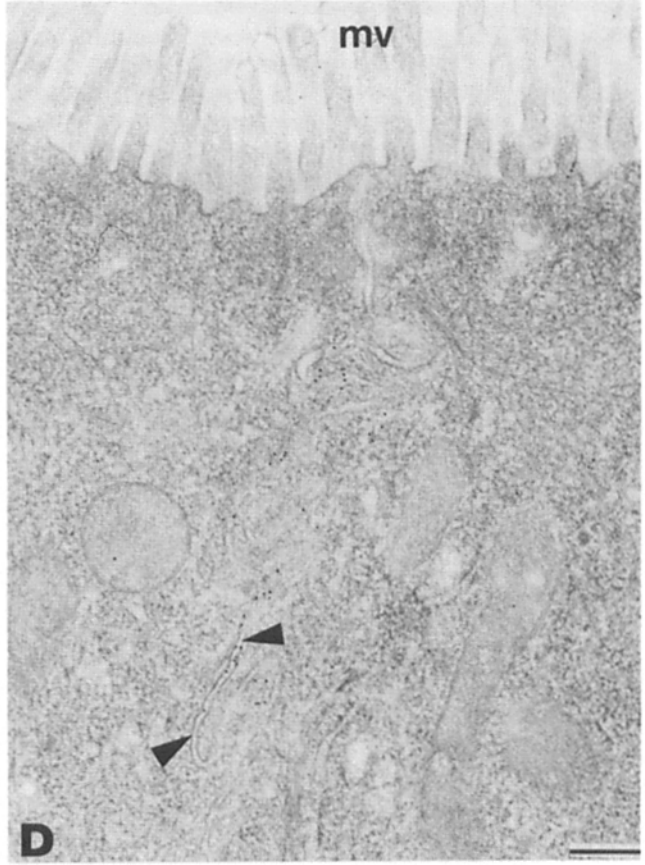
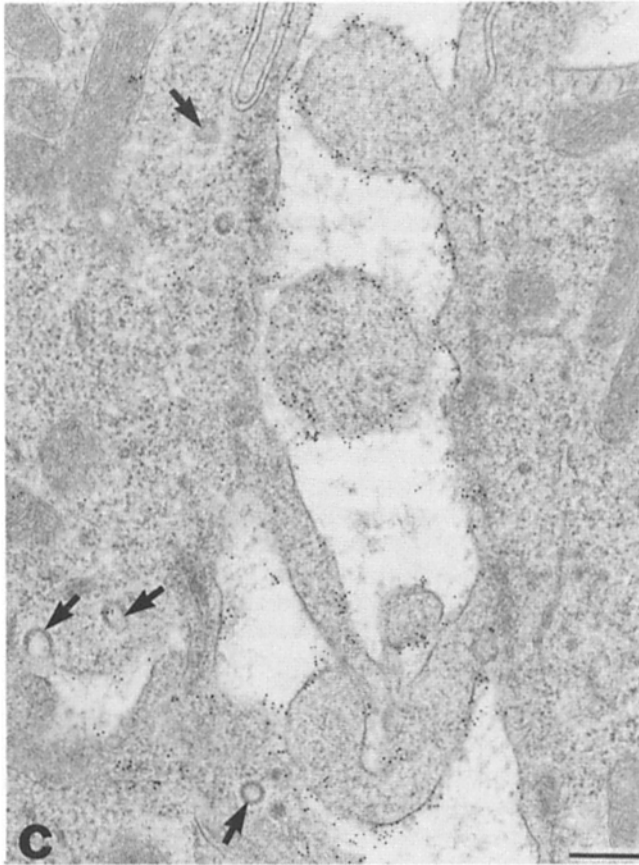
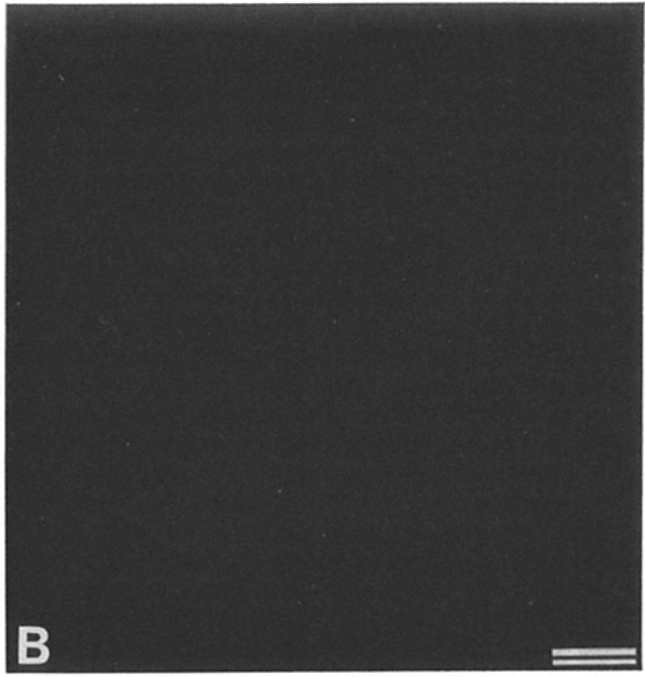
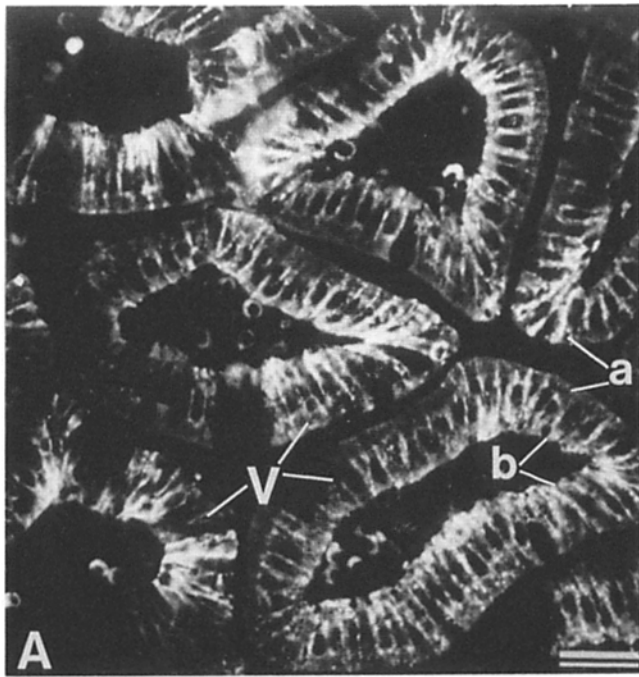
with  $\text{ZnSO}_4$  to induce high levels of LDL receptors. Abundant immunostaining of receptors was found at the sinusoidal surface (Fig. 2 A). The intensity of the fluorescence signal was uniform among all sinusoidal surfaces, including the surface of hepatocytes near the central vein. There was no indication of antibody binding within the cell. Receptors were not present on the surface of endothelial or Kupffer cells, and there was little or no activity in the portal triad. Even though the antireceptor IgG reacts with the mouse LDL receptor, the hepatocytes of normal animals treated intraperitoneally with zinc did not stain, presumably because the number of receptors is relatively small (Fig. 2 B). In addition, a nonimmune IgG did not stain hepatocytes of zinc-treated transgenic animals (data not shown).

Founder mice expressing transgene 2 and mice from line 192-2 used in this study had undetectable levels of plasma apo B-100 even in the absence of zinc induction (data not shown), suggesting that the basal level of transgene expression was sufficient to clear mouse LDL from the plasma. Fig. 2 (C and D) compare the distribution of the LDL receptor with the distribution of receptor bound human LDL in a noninduced transgenic animal that had received an intravenous injection of human LDL 15 min before removing the liver. The receptor and the LDL were both localized to the basal surface of the hepatocytes. Little if any intact immunoreactive LDL was detected within the cell.

The immunofluorescence images indicated that the LDL receptor in transgenic animals was preferentially located on the basal surface (sinusoidal surface) of the hepatocytes. Electron microscopic images of the receptor distribution using indirect immunogold labeling confirmed this observation (Fig. 3). Numerous gold particles were distributed over the basal surface of the hepatocytes of zinc-treated, transgenic animals (Fig. 3 A). The amount of labeling diminished as the basal surface membrane merged with the lateral surface membrane (*curved arrow*, Fig. 3 B), and there was virtually no label on the canalicular (apical) membrane (*c*, Fig. 3 B). At the basal surface, immunogold labeling was found on the microvilli and the intermicrovillus membrane (Fig. 3, A and B). The density of labeling over clathrin-coated pits (*cp*, Fig. 3 A; *straight arrows*, Fig. 3 B) was reduced compared with the other portions of membrane (*mv*, Fig. 3 A; *arrowhead*, Fig. 3 B). Labeling was not present over endothelial cells or Kupffer cells of transgenic animals (E, Fig. 3 A). No label was present on the surface of hepatocytes from zinc-treated normal animals (data not shown).

In noninduced transgenic mice hepatocytes express fewer human LDL receptors than they do after zinc induction. To determine whether the receptor distribution was different at this lower level of expression, we examined noninduced livers by immunoelectron microscopy (Fig. 4 A). The distribution of receptors in the noninduced transgenic hepatocytes was similar to that in the zinc-treated animals. There was

**Figure 5.** Binding and internalization of intravenously administered LDL-gold in hepatocytes and sinusoidal cells from transgenic (A–C) and nontransgenic (D) livers. Normal mice (D) or mice expressing transgene 2 that had been injected intraperitoneally with zinc (A–C) were injected intravenously with human LDL-gold as described in Materials and Methods. At the end of 1 h, the livers were perfusion fixed and processed for electron microscopy. (A) *mv*, microvilli; *arrows*, coated pits. (B) *arrows*, coated pits; *arrowheads*, endocytic compartments. (C) *G*, Golgi complex; *arrows*, Golgi-associated vesicles containing LDL-gold. (D) *L*, lysosome; *mv*, microvilli; *arrows*, coated pits. Bars, 0.5  $\mu\text{m}$ .





very little labeling in coated pits as compared with the remainder of the cell surface. Even in cells expressing only a few receptors, the receptors were preferentially located on the apex of the microvilli (*mv*, Fig. 4 *A*). In the same experiment we used an anti-LDL antibody to determine the cellular distribution of injected human LDL (Fig. 4 *B*). This distribution was similar to the distribution of receptors.

These qualitative observations were confirmed by quantitative analysis (Table I). In experiment A, the anti-LDL receptor IgG showed little labeling of hepatocytes from nontransgenic mice treated with zinc. When liver sections from zinc-treated transgenic mice were incubated with anti-LDL receptor IgG, the density of labeling with gold particles was 86 times greater over noncoated membranes (41.5 particles/ $\mu\text{m}$ ) than over coated membranes (0.48 particles/ $\mu\text{m}$ ). In experiment B, we analyzed the distribution of LDL and LDL receptors in noninduced transgenic mice that express fewer LDL receptors. In these animals, the density of labeling over noncoated membranes was reduced when compared with the noninduced animals (12.3 particles/ $\mu\text{m}$  versus 41.5), but the density of labeling in coated pits was nearly the same (0.39 versus 0.48). Thus, in the noninduced animals, the ratio of noncoated membrane labeling to coated membrane labeling was lower than that in the zinc-treated animal (32 versus 86), but there was still a marked preference for noncoated membranes. When the noninduced animals were injected with LDL, there was a slight increase in the labeling of coated pits with the anti-receptor IgG (0.73 particles/ $\mu\text{m}$  versus 0.39). Nevertheless, the vast bulk of receptors remained outside of coated pits. An antibody against LDL gave a similar distribution to that seen with the anti-receptor antibody.

There was little immunogold labeling of LDL receptors in intracellular, vacuolar compartments of the zinc-treated transgenic liver (Fig. 3). Very few gold particles were found in morphologically identifiable endosomes, multivesicular bodies, lipoprotein-rich vacuoles (*L*, Fig. 3 *A*), lysosomes or Golgi apparatus (*G*, Fig. 3 *A*). Likewise, when we used immunogold labeling to analyze the distribution of LDL in nontreated transgenic mice that had been perfused with human LDL (Fig. 4 *B*), there was little immunodetectable LDL in endocytic compartments.

To determine whether receptor-bound LDL was able to enter the hepatocyte, we injected LDL-gold intravenously into normal and transgenic mice that were treated intraperitoneally with zinc. Each animal received four injections of LDL-gold at 15 min intervals, after which the livers were fixed and processed for electron microscopy. In the transgenic mice most of the hepatocytes had numerous gold particles distributed over the basal surface (Fig. 5 *A*) with the highest concentration on the microvilli. Occasionally, one or two particles were found in coated pits, but the majority of the labeling mirrored the receptor distribution detected by

immunogold (Fig. 3). Normal mice had a negligible amount of gold labeling on the hepatocyte surface (Fig. 5 *D*). Although most of the gold on the cell surface was on microvilli, transgenic hepatocytes did internalize LDL-gold. Gold particles were found in endosomes (*arrowheads*, Fig. 5 *B*) at different stages of maturation and in lysosome-like vesicles near the apical cell surface. In addition, vesicles associated with the *trans*-Golgi apparatus often contained gold particles (Fig. 5 *C*). None of these compartments were labeled in normal mice injected with LDL-gold.

Occasionally, we observed a hepatocyte that contained gold particles throughout the endocytic pathway. In these cells, gold was found in coated pits (*arrows*, Fig. 5 *B*), endosomes, and other endocytic compartments. Such cells appeared to be taking up LDL-gold at discrete regions of the surface while other areas of the surface of the same cell were quiescent.

The endothelial and Kupffer cells lining the sinusoid also internalized LDL-gold (Fig. 5 *D*). This uptake was equal in the normal and transgenic animals, and therefore it was not mediated by the human LDL receptor. We saw little gold on the surface of Kupffer cells, but large numbers of particles were found in lysosomes.

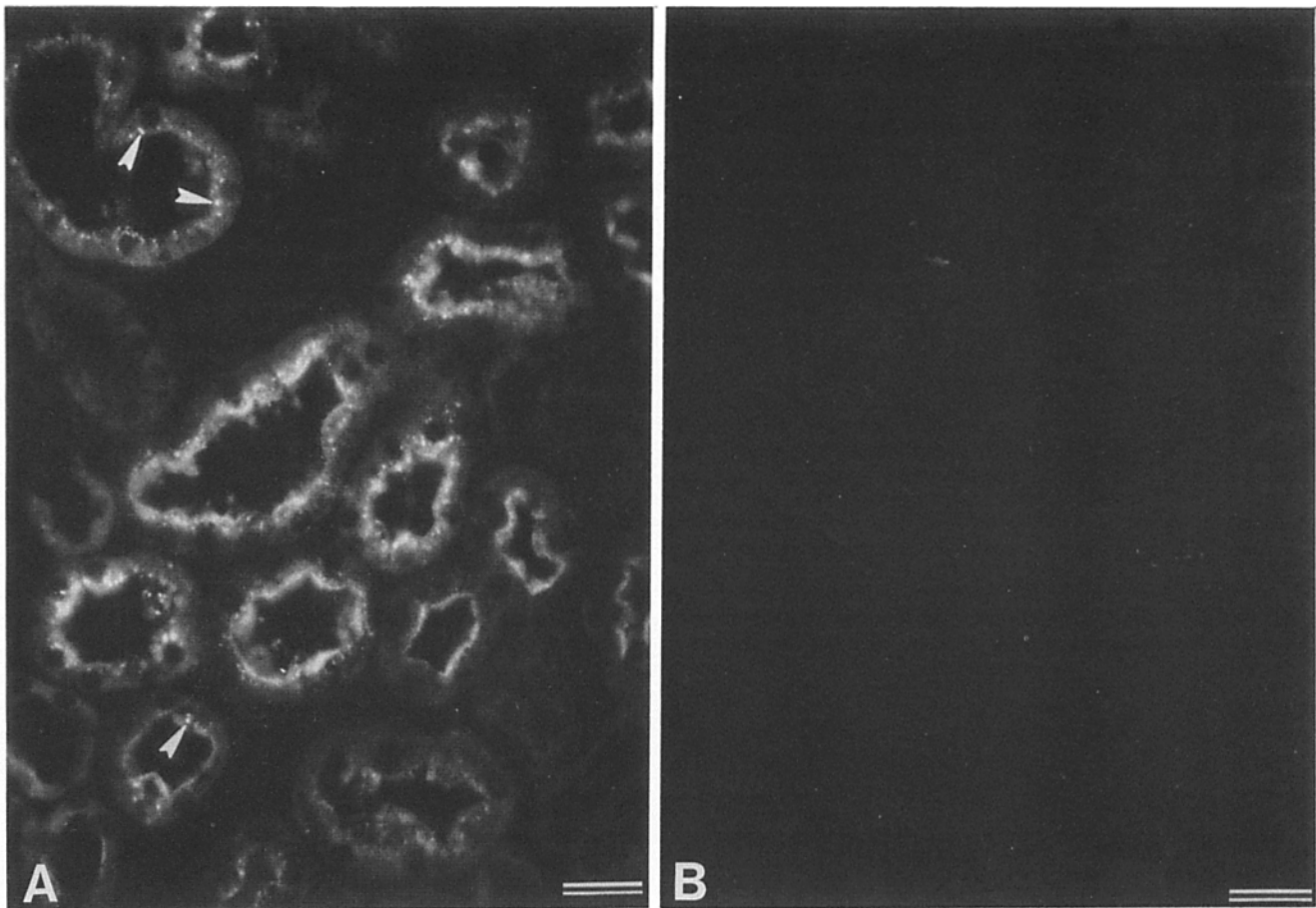
### Intestinal Absorptive Cells

The intestinal absorptive cells from jejunum in zinc-treated transgenic animals expressed relatively high levels of LDL receptors (Fig. 6). Indirect immunofluorescence showed that receptors were confined to the basal (*b*, Fig. 6 *A*) and lateral surfaces of the cell; there was almost no staining of the apical surface (*a*, Fig. 6 *A*). Nonimmune IgG did not stain the same tissue sample (Fig. 6 *B*). These findings are similar to those reported by Fong et al. (7) who studied by light microscopy the immunoperoxidase distribution of LDL receptors in the jejunum of rats. By electron microscopy, immunogold labeling was uniform over the convoluted, lateral cell surface and the basal surface (Fig. 6, *C*, *D*, and *E*). Coated pits appeared to have the same density of gold particles as the noncoated membrane (Fig. 6 *C*). Sparse gold labeling of intracellular vacuoles was observed, and the apical microvillar surface of the cells was devoid of label (*mv*, Fig. 6 *D*).

### Kidney

The cells lining the convoluted tubules of the kidney expressed the transgene 2-encoded human LDL receptor. Both proximal and distal segments of the loop of Henley were positive by immunofluorescence (Fig. 7 *A*). In contrast to the distribution in liver and intestine, in the renal epithelium LDL receptors were preferentially located at the apical surface. The immunofluorescence pattern suggested that receptor was concentrated at the base of the microvilli. Moreover,

**Figure 6.** Immunofluorescence (*A* and *B*) and immunogold (*C*–*E*) localization of the human LDL receptor in intestinal absorptive cells (jejunum) of transgenic mice. Mice expressing transgene 2 were treated with zinc either orally (*A*–*C*, and *E*) or intraperitoneally (*D*). (*A*) Cross section of intestinal villi (*V*) incubated with anti-LDL receptor IgG; *a*, apical cell surface; *b*, basal surface. (*B*) Cross section of intestinal villi incubated with nonimmune IgG. (*C*) Lateral surface near the base of two absorptive cells; *arrows*, coated vesicles. (*D*) Apical and lateral surfaces of two absorptive cells; *mv*, microvilli; *arrowheads*, lateral plasma membrane. (*E*) High magnification view of lateral surface near the middle of two cells. Bars, (*A* and *B*) 10  $\mu\text{m}$ ; (*C*, *D*, and *E*) 0.4  $\mu\text{m}$ .



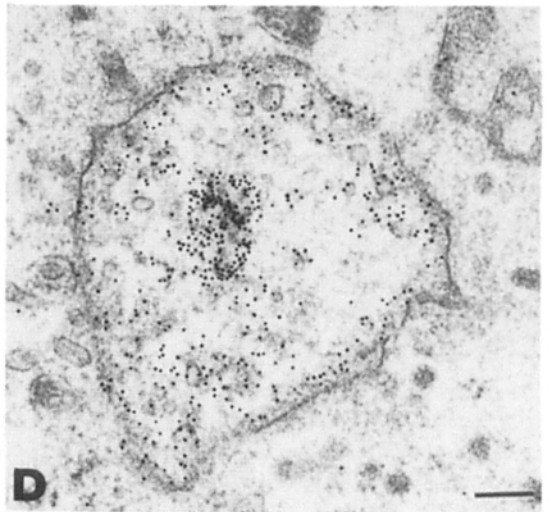
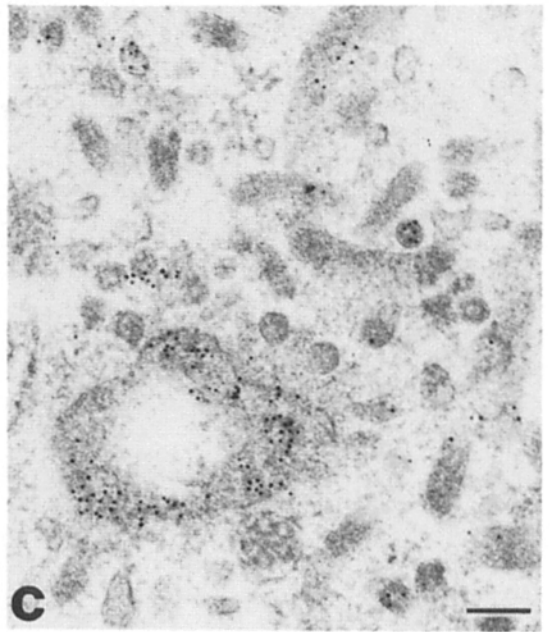
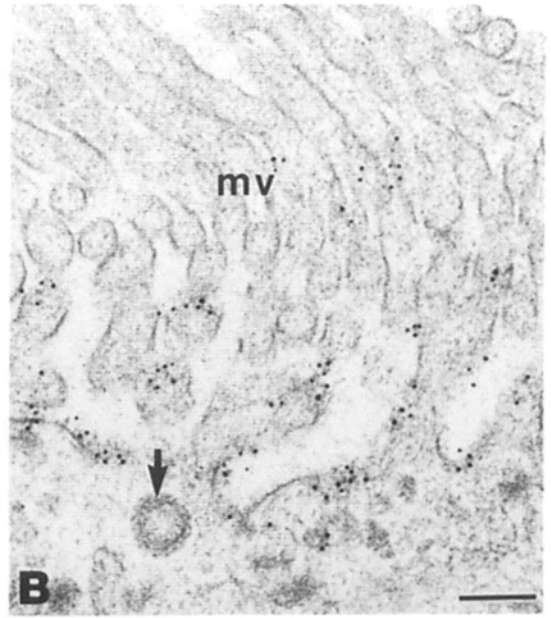
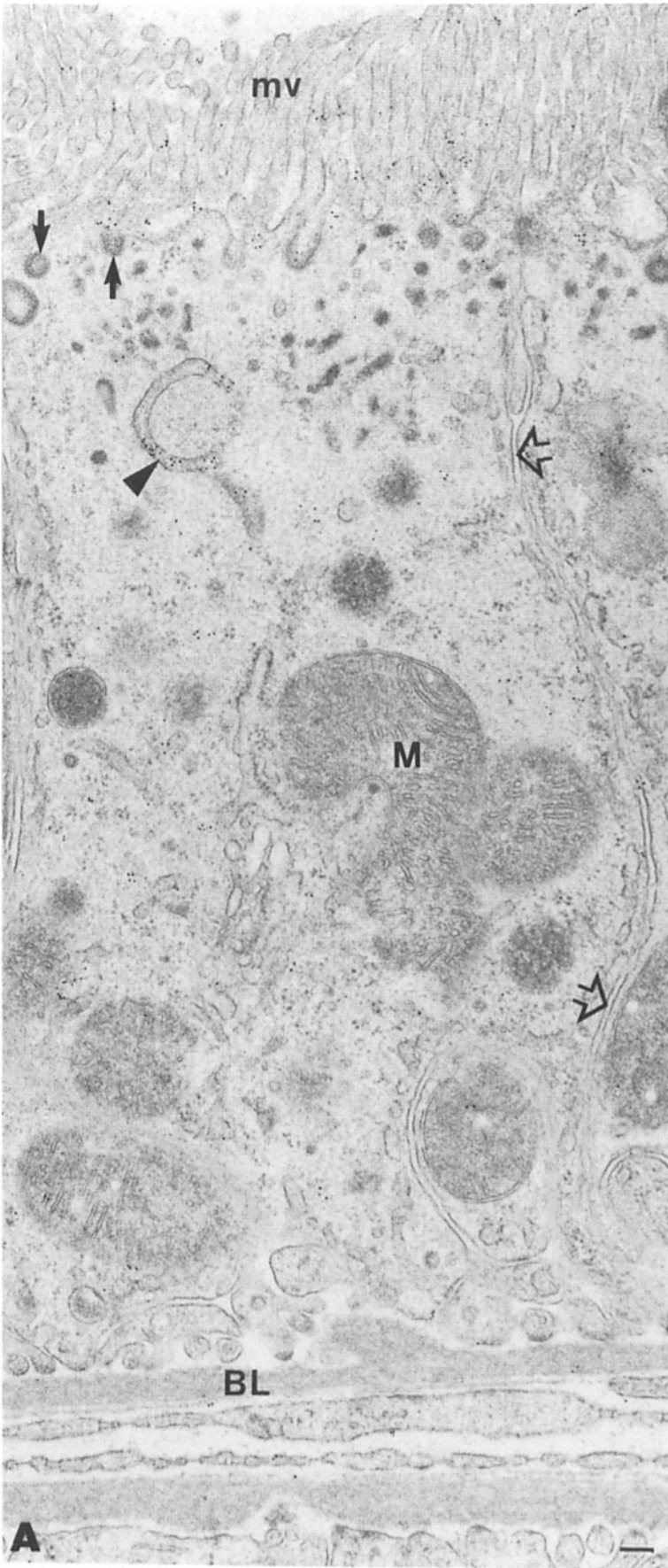
**Figure 7.** Immunofluorescence localization of the human LDL receptor in the kidney of transgenic (*A*) and normal mice (*B*). Animals expressing transgene 2 were treated orally with zinc before fixation and processed for immunofluorescence using anti-LDL receptor IgG. Arrows, vesicles in apical cytoplasm. Bar, 10  $\mu\text{m}$ .

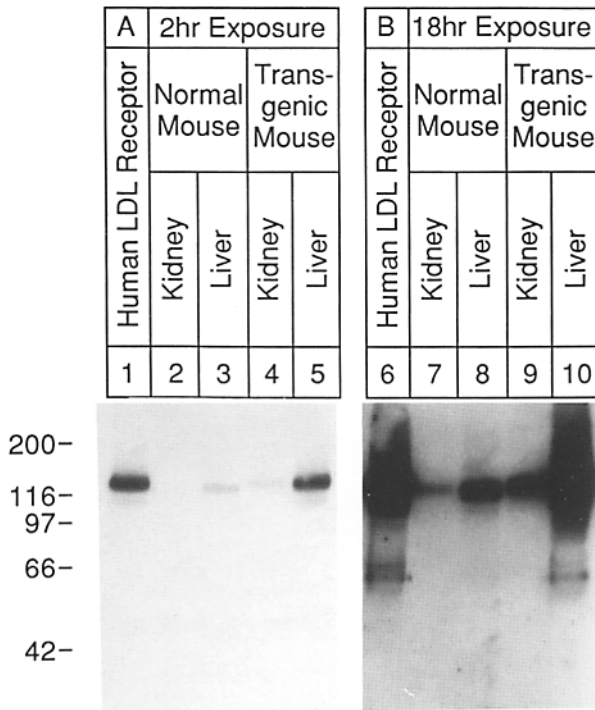
many of the cells (*arrowheads*, Fig. 7 *A*) had prominent vacuolar staining in the apical cytoplasm. The same tissue incubated with a nonimmune antibody did not show any staining (data not shown). No staining with the anti-LDL receptor antibody was seen in normal mouse kidneys (Fig. 7 *B*).

Fig. 8 *A* shows a cross section of an entire kidney epithelial cell that was prepared for immunogold labeling of the LDL receptor. The apical surface contained most of the gold particles with the highest concentration at the base of the microvilli (Fig. 8 *B*). The apical cytoplasm showed numerous labeled vesicles (Fig. 8, *A*, *C*, and *D*). Some vesicles had the morphology of multivesicular bodies (Fig. 8 *D*), whereas others were tubular (Fig. 8 *A*) or vesicular-tubular (Fig. 8 *C*). These morphologic features are characteristic of endosome and receptor recycling compartments (20, 22, 23). A similar distribution of LDL receptors was observed in a zinc-treated animal that expressed transgene 1 (data not shown).

The finding of immunostainable LDL receptors only on the luminal surface of the renal tubule raised the possibility that the antibody was detecting fragments of the receptor that had been shed from another tissue and had entered the glomerular filtrate. To rule out this possibility, we prepared homogenates from liver and kidney of a transgenic mouse, subjected them to SDS gel electrophoresis and immunoblot analysis, and stained the proteins with an anti-LDL receptor antibody, which was visualized by autoradiography (Fig. 9). After a 2-h exposure, there was faint immunostaining of the LDL receptor in normal mouse liver (Fig. 9, lane 3) and an intense band in liver membranes from the transgenic mouse (Fig. 9, lane 5). After a prolonged 18-h exposure the LDL receptor in transgenic mouse kidney was visualized as an intense band (Fig. 9, lane 9), which was much more intense than the band in normal mouse kidney (Fig. 9, lane 7). The transgene-encoded human receptor from liver and kidney migrated slightly slower than the mouse receptor, and the

**Figure 8.** Immunogold localization of the human LDL receptor in kidney epithelial cells of transgenic mice. (*A*) Low magnification view of epithelial cell; *BL*, basal lamina; *M*, mitochondria; *arrows*, coated pit or vesicle; *arrowhead*, tubular endosome; *open arrows*, lateral plasma membrane. (*B*) High magnification view of apical surface of epithelial cell surface; *mv*, microvilli; *arrow*, coated vesicle. (*C* and *D*) High magnification views of endosomal compartments. Animals expressing transgene 2 were treated with zinc either orally (*C*) or intraperitoneally (*A*, *B*, and *D*) before processing for immunogold localization. Bars, 0.2  $\mu\text{m}$ .





**Figure 9.** Immunoblot analysis of human LDL receptors from kidney and liver of transgenic mice. Normal mice and mice expressing transgene 2 were treated with zinc intraperitoneally. Aliquots of the 100,000 *g* membrane fraction from liver and kidney (lanes 2 and 4, 80  $\mu$ g protein; lanes 3 and 5, 180  $\mu$ g protein) were prepared as described in Materials and Methods, subjected to SDS-PAGE, and transferred to nitrocellulose filters. The filters were immunoblotted with a polyclonal rabbit anti-bovine LDL receptor antibody, probed with a  $^{125}$ I-labeled goat anti-rabbit IgG, and exposed to XAR-5 film with an intensifying screen at  $-70^{\circ}\text{C}$  for either 2 h (A) or 18 h (B). Lane 1 contained 200  $\mu$ g protein of a detergent-solubilized whole cell extract of CHO cells transfected with a plasmid encoding the human LDL receptor cDNA (6).

mobility of the human receptor in kidney was identical to that in liver. There was no evidence for an immunoreactive receptor fragment in the transgenic mouse kidney.

## Discussion

The current immunocytochemical studies in transgenic mice have revealed differential sorting of the LDL receptor in different epithelial cells. In hepatocytes and intestinal epithelium, the human LDL receptors were distributed diffusely on the basal and basolateral surfaces, respectively, and were observed only rarely in coated pits and endosomes. In contrast, in renal tubular epithelium, the LDL receptors were preferentially located at the base of microvilli on the apical (luminal) surface and appeared frequently in endosomes, suggesting that they were actively engaged in endocytosis.

The diffuse distribution of LDL receptors on microvilli of liver cells stands in marked contrast to their distribution in cultured fibroblasts (1) and fibroblast-like Chinese hamster cells (6) where the bulk of receptors are located in coated pits and in membranes of the endocytic recycling pathway. The current findings are in agreement, however, with studies that have shown a diffuse, microvillar distribution of receptor-

bound LDL-gold complexes in perfused livers from estradiol-treated rats (11, 12) and in cultured rat ovarian granulosa cells (21). In both of these studies, the LDL-gold complexes appeared to move slowly to the base of the microvilli and were then internalized by coated pits. These findings imply that the transgene-encoded receptors must also move to coated pits as they deliver LDL into the cell. In prior experiments, we demonstrated uptake of intravenously administered fluorescently labeled LDL into hepatocytes (14), and, in the current experiments, we observed abundant internalization of gold-labeled LDL in the transgenic mouse livers. This uptake was far greater than was observed in the livers of nontransgenic animals, indicating that it was mediated by the human LDL receptor. Moreover, animals that received LDL displayed a slight increase (twofold) in the density of LDL receptors over coated pits (Table I), raising the possibility that LDL binding might accelerate this movement.

Coated pit-mediated internalization of hepatic LDL receptors is also suggested by the metabolic abnormality in three human subjects with the internalization-defective form of familial hypercholesterolemia (6, 17). These individuals have mutations in the cytoplasmic domain of the LDL receptor that prevent the receptor from moving into coated pits and from carrying bound LDL into the cell. Despite the normal ability of these receptors to bind LDL, individuals with these mutations have plasma LDL levels that are as high as those found in individuals with absent receptors (10). Since the plasma LDL level is controlled largely by LDL receptors in the liver (4), the data indicate that internalization defective receptors do not function in liver and imply that normal LDL receptors function in liver cells by moving into coated pits.

A major question raised by our studies is whether the diffuse distribution of LDL receptors in hepatocytes is a consequence of the saturation of coated pit binding sites, owing to the high level of receptor expression. Two observations suggest that this is not the case. First, lowering receptor concentration by omitting treatment with zinc did not change the diffuse distribution. Second, despite the high receptor density on the uncoated membrane of zinc-treated animals (41.5/ $\mu\text{m}$ ), the density of receptors in coated pits was only 0.48/ $\mu\text{m}$  (Table I). In human fibroblasts, receptor density in coated pits is 20/ $\mu\text{m}$  (22), suggesting that the liver coated pits are not saturated with LDL receptors. We cannot rule out the possibility that in hepatocytes coated pits are saturated with other receptors such as the asialoglycoprotein receptor. This would imply that the other receptors bind to coated pits with higher affinity than do LDL receptors. At present, there is no evidence to support this conclusion. We prefer the hypothesis that the movement of LDL receptors from microvilli to coated pits is a regulated event and that liver cells have the capacity to retain LDL receptors for a prolonged time on the microvillar surface, admitting them to coated pits only slowly.

In contrast to the results in liver, the appearance of LDL receptors on the apical surface of renal tubular epithelial cells was unexpected. Biochemical assays do not detect large numbers of LDL receptors in kidney membrane preparations (16). Moreover, we did not detect any endogenous LDL receptor either by immunogold or immunofluorescence labeling techniques. Therefore, we are unable to relate our findings in the kidney of the transgenic mouse to the distribution of the endogenous receptor in this tissue. Nevertheless,

the observation that the transgenic receptor is sorted to the apical surface, in contrast to its sorting to the basal surface in liver and basolateral surface in intestine, raises the possibility that renal tubular epithelial cells may have a mechanism for apical sorting that differs from the mechanism in other epithelia.

Our current understanding of protein sorting in polarized epithelial cells is largely derived from studies of MDCK cells in vitro (24) and hepatocytes in vivo (2). Whereas in rat hepatocytes proteins destined for the apical surface are first directed to the basal surface (2), in the MDCK cells apical proteins are delivered directly to the apical surface (24). Further studies with LDL receptors containing various mutations in their cytoplasmic or extracellular domains should help to clarify the mechanism for this interesting tissue-specific sorting.

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