Fate of DNA Targeted to the Liver by Asialoglycoprotein Receptor-mediated Endocytosis in Vivo
PROLONGED PERSISTENCE IN CYTOPLASMIC VESICLES AFTER PARTIAL HEPATECTOMY

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After intravenous injection, DNA complexed with asialoglycoprotein-polylysine conjugates is endocy-
tosed by hepatocytes via asialoglycoprotein receptors and is expressed transiently. Long term persistence and expression occurs when partial hepatectomy is performed after gene delivery. To determine the intracellular location of the persisting DNA, we transferred a plasmid expressing bacterial chloramphenicol acetyltransferase into the liver of rats in vivo by asialoglycoprotein receptor-mediated endocytosis. The internalized DNA was measured by Southern blot. Twenty min after administration, 80–85% of the plasmid appeared in the liver, 80% of which was within hepatic cells (12,000–18,000 copies/hepatocyte). In sham-operated control rats, the transgene concentration decreased to 8–12 and 2–4% of the initial levels in 4 and 24 h, respectively, and became undetectable at 7 days. In rats subjected to 66% hepatectomy 20 min after DNA administration, 20, 9, and 7% of the plasmid in the residual liver persisted at 4 h, 24 h, and 7 days, respectively. Liver homogenates were fractionated by differential centrifugation and Percoll gradient centrifugation. In 66% hepatectomized rats, the plasmid persisted in an undegraded, transfaction-competent form in plasma membrane/endosome-enriched fractions throughout the duration of the experiment (7 days), indicating that cytoplasmic vesicles are the main site of persistence of the endocytosed DNA.

Endocytosis, mediated by asialoglycoprotein (ASGP) receptor has been utilized for delivering DNA to the liver in vivo (1–5). Following intravenous administration of bacterial (1, 2) or mammalian (3–5) genes complexed with a ASGP-polylysine conjugate, the DNA is rapidly cleared from circulation, is internalized by the liver and expressed transiently (1, 4). However, prolonged expression of genes delivered to the liver in this manner has been shown to occur in two rodent models, Sprague-Dawley rats expressing bacterial chloramphenicol acetyltransferase (CAT) (2) and analbuminemic rats (NAR strain) expressing human serum albumin (3), when partial hepatectomy is performed shortly after internalization of the gene. The intracellular site of the persisting endocytosed DNA and the mechanism by which partial hepatectomy results in the inhibition of lysosomal degradation is unknown. Elucidation of this phenomenon is important in the understanding of the pathway by which macromolecules internalized by receptor-mediated endocytosis are translocated and degraded in the lysosome. This information may also lead to the development of rational pharmacological strategies for achieving prolonged expression of therapeutic genes targeted to the liver in vivo.

The focus of the present study was to determine the time course of degradation of the internalized DNA and the intracellular location of the persisting DNA upon targeted delivery and partial hepatectomy. Our findings indicate that, normally, the endocytosed DNA is degraded rapidly. When partial hepatectomy is performed after endocytosis, the DNA persists in a low density cytoplasmic vesicular compartment. Events that result in the persistence of the bulk of the endocytosed DNA apparently precede the wave of DNA synthesis and mitosis that occur as a result of partial hepatectomy.

EXPERIMENTAL PROCEDURES

Animals
Male Sprague-Dawley rats (150–200 g) were obtained from our colony at the Albert Einstein College of Medicine. The rats were maintained on standard laboratory rat chow in a 12-h light/dark cycle.

The Plasmid, p(9–12)AlbCAT
The plasmid containing the gene for bacterial chloramphenicol acetyltransferase (CAT) driven by an albumin promoter/enhancer was provided by James M. Wilson, University of Michigan, Ann Arbor. This plasmid, which was previously shown to express CAT activity after targeted delivery to the liver, in vivo (3), was used for determination of the intracellular fate of endocytosed DNA in this study.

Synthesis of the ASGP-Polylysine Conjugate and Formation of the DNA-Carrier Protein Adduct
Asialoorosomucoid was produced by treating orosomucoid (Sigma) with neuraminidase and covalently linked with polylysine (Sigma,
average molecular weight 59,000) as previously described (3, 5). To form targetable carrier DNA complexes, plasmid DNA, 0.6 mg in 1 ml of 2 M NaCl was added to the asialoorosomucoid-polysyaline conjugate (containing 0.15 mg of asialoorosomucoid) in 9.6 ml of 2 M NaCl at 25 °C. The mixture was placed in a dialysis tubing (1.0 cm flat width) with an exclusion limit of 12,000 to 14,000 dalton and dialyzed against 1.0 M NaCl for 3 h at 4 °C for 3 h at 4 °C. After dialysis, the following concentrations of NaCl: 1.5 M, 1.0 M, 0.5 M, 0.25 M, and 0.15 M. After the final dialysis, the complex was filtered through 0.45-

Administration of the DNA-Carrier Complex and Partial Hepatectomy

Rats were anesthetized with ether, and the right external jugular vein was exposed. The soluble complex containing 22 pmol of the DNA in a volume of 0.5 ml was infused into the right external jugular vein, the vein was ligated, and the incision was closed. In one group, 66% hepatectomy was performed through a midline incision (6) under ether anesthesia, 20 min after infusion of the DNA-carrier polysyaline adduct. In preliminary studies, it was determined that 20 min was required for maximum internalization of the injected DNA in the liver. The removed lobes of the liver were subjected to DNA analysis as described below. Rats in both control and partial hepatectomy group were killed at 20 min, 1 h, 4 h, 24 h, and 7 days after infusion of the DNA-carrier complex, and the livers were removed and subjected to DNA analysis.

Collagenase Perfusion and Separation of Parenchymal and Non-parenchymal Cell Fractions

Rats were anesthetized with diethyl ether, the peritoneal cavity was opened, and the portal vein was cannulated. The liver was perfused in situ as described by Berry and Friend (7) and modified by Seglen (8). Hepatocytes and non-parenchymal cells were separated by a modification of a previously reported protocol (9) as follows. Cell were diluted in the medium RPMI (GIBCO) to 2 × 10 ^{6} cells/ml and layered on a preformed Percoll gradient (1.03–1.10 g/ml) and centrifuged at 750 × g for 30 min in a swinging rotor. Buoyant density of fractions was determined using density marker beads (Pharmacia LKB Biotechnology Inc.) in a balancing tube. Fractions containing non-parenchymal cells (buoyant density 1.04–1.06 g/ml) were separated from fractions containing hepatocytes (buoyant density 1.07–1.09 g/ml) (9). Cell number in the fractions was determined in a Coulter counter. For characterization of the cell fractions, the cells were centrifugally attached to glass slides and immunocytochemistry was performed using an anti-rat albumin rabbit antiserum as previously described (10). DNA from each fraction was extracted and subjected to Southern blot analysis as described below.

Separation of Subcellular Fractions

All procedures were performed at 4 °C. Liver samples were minced and homogenized in 4 ml of 0.2 M Tris acetate, pH 8.0, containing 0.25 M sucrose and 150 mM NaCl. For liver tissue, a glass homogenizer provided with a motor-driven Teflon pestle. The homogenate was centrifuged at 750 × g for 10 min, and the supernatant was separated. The pellet was washed once with the homogenization buffer, and the wash was pooled with the supernatant.

Subfractionation of the 750 × g Pellet—To separate nuclear from cosedimenting intracellular membranes, the 750 × g pellet was resuspended to the original homogenate volume in the homogenization buffer and layered over a cushion of 0.2 M sucrose, 0.2 M disodium EDTA and centrifuged at 105,000 × g for 2 h. The membrane fraction at the interface of 2.0 M sucrose and 0.25 M sucrose, and the nuclear fraction at the bottom of the tube were collected.

Subfractionation of the 7500 × g Supernatant—Percoll (Pharmacia, Uppsala, Sweden) was added to the supernatant to a final concentration of 25% (11), and the mixture was centrifuged at 105,000 × g for 2 h in a swinging rotor. Samples were collected in 10 equal fractions, designated fractions 1–10, in increasing order of buoyant density.

Assay of Marker Enzymes and Protein in Subcellular Fractions

Glucose-6-phosphatase activity was determined according to Swan son (12). β-Glucuronidase activity was assayed by the method of Levy and Marsh (13), and 5’-nucleotidase activity was measured with adenosine monophosphoric acid as substrate according to Touster et al. (14). Protein was determined according to Lowry et al. (15).
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**FIG. 1. Southern blot of DNA extracted from the parenchymal and non-parenchymal liver cells.** The plasmid pAlb(9-12)CAT was targeted to the liver in vivo by ASGP receptor-mediated endocytosis as described in the text. Twenty min after DNA administration, liver cells were isolated by in situ collagenase perfusion and the parenchymal and non-parenchymal fractions were separated by Percoll gradient centrifugation. Total DNA was extracted from parenchymal and non-parenchymal cells (10⁶ cells from each fraction) and the input plasmid (40 ng) were resolved by agarose gel electrophoresis without digestion (lanes 1-3) or after digestion (lanes 4-6) with XbaI (which has a single restriction site on the plasmid). *Lanes 1 and 4,* input plasmid; *lanes 2 and 5,* DNA from parenchymal cells; *lanes 3 and 6,* DNA from the non-parenchymal cells.

**FIG. 2. Distribution of marker enzymes in subcellular fractions.** Subcellular fractions of liver homogenate were prepared by differential centrifugation and Percoll gradient centrifugation as described in the text. The purified nuclear fraction (N) was separated from the low speed membrane fraction (LM) by centrifugation of the 750 × g pellet through a cushion of 2 M sucrose. The 750 × g supernatant was separated into the cytosolic fraction (C) and a membrane fraction, which was further subfractionated by centrifugation in a Percoll gradient (P1 through P10 in ascending order of buoyant density). Enzyme activities are shown as percent of total recovery in all fractions. Means of four experiments and standard errors of mean.

**Assay of CAT Activity**

CAT activity in the liver homogenates was assayed in triplicate using [14C]chloramphenicol as a substrate and thin layer chromatographic analysis as previously described (20). The assay was performed in triplicate.

**RESULTS**

**Distribution of the Internalized DNA between the Parenchymal and Non-parenchymal Cell Fractions—Immunocytochemistry of the hepatocyte fractions obtained from the Percoll gradient showed that all cells in this fraction were positive for albumin and had morphological appearance of hepatocytes. Only 2–5% of the cells in the non-parenchymal fraction were positive for albumin.** Fig. 1 shows a Southern blot of DNA extracted from the two cell populations. Densitometric analysis of the Southern blots showed that approximately 80% of the internalized DNA was present in the parenchymal cell fraction, representing approximately 12,000 copies of the plasmid/hepatocyte at 20 min after the injection.

**Distribution of Marker Enzymes in Subcellular Fractions—** Recovery of marker enzyme activities are shown in Fig. 2. The endoplasmic reticulum marker glucose-6-phosphatase...
and the lysosomal marker β-glucuronidase were recovered mainly in the 750 × g supernatant fraction. Thirty-three percent of the 5′-nucleotidase activity (a marker for plasma membranes) was recovered in the 750 × g pellet; the remainder was in the supernatant. When the 750 × g pellet was subfractionated, 5′-nucleotidase activity was recovered in the membrane fraction which was 2-fold enriched in this enzyme activity over the homogenate. Specific enzyme activities for glucose-6-phosphatase (a marker for the endoplasmic reticulum) and β-glucuronidase (a lysosomal enzyme) were reduced seven times and 10 times, respectively, in the membrane fraction of the 750 × g pellet, compared to the homogenate. Fraction 4 of the percoll gradient was enriched 11-fold over the homogenate in 5′-nucleotidase activity and depleted 22 times and 25 times in β-glucuronidase and glucose-6-phosphatase, respectively, indicating that this fraction is enriched in plasma membranes. Fraction 10 of the Percoll gradient, the highest density fraction, was enriched 4-fold in β-glucuronidase activity and depleted 10 times in 5′-nucleotidase activity.

Electron Micrography—Fig. 3 shows electron micrograph of selected subcellular fractions. The membrane fraction of the 750 × g pellet showed open sheets of membrane and some comparative large vesicles with minor contamination by other organelles (Fig. 3, panel a). The plasma membrane/endosome-enriched Percoll gradient fraction. Fraction 4 (Fig. 3, panel b) showed small closed spherical membrane vesicles with little contamination from lysosomes, mitochondria, or rough endoplasmic reticulum. Fraction 10 of the Percoll gradient (Fig. 3, panel c) consisted of primarily mitochondria and lysosomes.

Localization of the Internalized Plasmid in Subcellular Fractions—Amounts of the internalized plasmid DNA present in the subcellular fractions was determined by Southern blot (Fig. 4) and quantified by densitometry (Fig. 5). Twenty min after the injection of the DNA-carrier complex (Fig. 4, panel a and Fig. 5), the internalized plasmid was present in the membrane fraction of the 750 × g pellet and bimodally distributed in the Percoll gradient fractions of the 750 × g supernatant in the plasma membrane/endosome-enriched fractions (Fractions 4 and 5) and the lysosome-enriched fractions (Fractions 8–10). The internalized DNA was not detectable in the nuclear fraction of the 750 × g pellet. A major portion of the DNA present in the 750 × g pellet membrane fraction and in the lysosome-enriched fraction was already degraded at 20 min after administration of the plasmid as indicated by smears in the corresponding lanes. In sham-operated rats, at 4 h (Fig. 5, panel a), there was a decrease in the amount of plasmid DNA in all fractions, including the plasma membrane/endosome-enriched fractions, consistent with translocation of the endocytosed DNA to the lysosomes undergoing 66% hepatectomy following DNA administration.

Liver was harvested at various intervals after DNA administration. The 20-min sample (panel a) was collected at the time of partial hepatectomy; for other samples (panels b–d), rats were sacrificed at stated time points (4 h, 24 h, and 7 days, respectively). Subcellular fractions were prepared as in Fig. 1. The internalized DNA was extracted from each fraction, linearized by treatment with XbaI, and subjected to Southern blot analysis. DNA extracted from subcellular fractions of the liver homogenate was resolved by electrophoresis on 0.8% agarose gel (DNA extracted from subcellular fractions derived from 1 g of liver was applied per lane). Southern blot was performed using a 301-bp double-stranded DNA probe directed to the CAT coding region. Pi, input plasmid; N, isolated nuclei; LM, low speed (750 × g) pellet membranes; C, cytosol (105,000 × g/1 h supernatant of the 750 × g supernatant); 1–10, Percoll gradient fractions of the membrane pellet (105,000 × g/60 min pellet) of the 750 × g supernatant in increasing order of buoyant density. Six experiments were performed, and results of a representative experiment are shown.
and subsequent degradation. At 24 h after injection into the sham-operated rats, only traces of the plasmid were present in the lysosomal fractions, and at 7 days the internalized plasmids were not detectable in any fraction by Southern blot (Fig. 5, panel a). In the 66% hepatectomy group, 4 h after the injection of the plasmid (Fig. 4, panel b and Fig. 5, panel b), there was a marked decline in the amount of plasmid DNA in the membrane fraction of the 750 × g pellet and the lysosome-enriched fractions. In contrast, in the plasma membrane/endoosome-enriched fraction, the plasmid DNA was present in apparently intact form and at an undiminished concentration. In the 24-h specimens, the intact internalized plasmid DNA was detected predominantly in the plasma membrane/endoosome enriched fractions (Fig. 4, panel c and Fig. 5, panel b). Seven days after administration of the plasmid, the internalized DNA was detectable only in the plasma membrane/endoosome-enriched fractions (Fig. 4, panel d and Fig. 5, panel b).

Amplification of the Transgene in Nuclear and Cytoplasmic Fractions—Since the internalized DNA was not detectable in the purified nuclei by Southern blot analysis, to determine whether small amounts of the endocytosed DNA are translocated to nuclei, a 301-bp segment of the CAT coding region was amplified by PCR. The transferred DNA was detected in the nuclei and plasma membrane/endoosome-enriched fractions from both sham operated and partially hepatectomized rats at 20 min, 4 h, and 24 h after administration of the DNA (Fig. 6). In the 7-day specimens from the sham-operated rats, no amplification product was detected. However, amplification products were detected with DNA extracted from liver nuclei and plasma membrane/endoosome-enriched fractions from the 66% hepatectomized rats.

Effect of DNase Treatment—Four h after administration of the carrier-DNA complex, DNA present in the plasma membrane/endoosome-enriched fractions was resistant to DNase (Fig. 7), supporting its compartmentation within the membrane vesicles. DNA in the lysosomal fraction was also partially resistant to exogenously added DNase. In contrast, DNA in the membrane fraction of the 750 × g pellet was sensitive to DNase, suggesting that it was not segregated within vesicles. To determine whether the plasmid in plasma membrane/endoosome-enriched fraction was protected from DNase digestion by being enclosed within membrane vesicles, in some experiments, these fractions were treated with Triton X-100 prior to DNase digestion. In these experiments, the plasmid was completely digested by DNase I (not shown in Fig. 7), indicating that the plasmid itself had not undergone structural modifications making it DNase-resistant. As shown in Fig. 5, 7 days after DNA administration, the endocytosed plasmid was detectable only in plasma membrane/endoosome-enriched fractions of the liver homogenate in the 66% hepatectomized rats; the persistent DNA remained resistant to DNase treatment.

Transfection of Bacteria by Plasmids Extracted from Liver Fractions—Transfection efficiency of the input plasmid pAlbCAT was compared with that of plasmids extracted from plasma membrane/endoosome-enriched fractions 24 h and 7 days after targeted delivery of the plasmid to the liver (Table I). Transfection efficiency of the input plasmid (8.2 × 10⁶ to 3.1 × 10⁶ ampicillin-resistant colonies/μg of plasmid) of the DNA extracted from the plasma membrane/endoosome-enriched fractions (6.1 × 10⁶ to 3 × 10⁶ colonies/μg of DNA).

CAT Activity—No CAT activity was detectable in the liver samples at 20 min or 4 h after administration of the plasmid. At 24 h, CAT activity was detected at approximately 1.0 microunit/mg protein in both sham-operated and 66% hepatectomized rats. Seven days after DNA administration, CAT activity was undetectable in sham-operated rats but was de-
Prepared as in Fig. 4. The liver was harvested 20 min after delivery of the plasmid pAlb(9-12)CAT to the liver by ASGP receptor-mediated endocytosis as described under "Experimental Procedures." The liver was harvested and used for transfection of E. coli as described in the text. Each data point represents average of three transfections.

### Table I

<table>
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<tr>
<th>Experiment</th>
<th>Input plasmid</th>
<th>Plasmid extracted at 24 h</th>
<th>Plasmid extracted at 7 days</th>
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<td>1.8 x 10^6</td>
<td>2.1 x 10^6</td>
</tr>
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</tr>
<tr>
<td>3</td>
<td>3.1 x 10^6</td>
<td>2.6 x 10^6</td>
<td>3.0 x 10^6</td>
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The transfected DNA was not detected by Southern blot analysis in isolated nuclei at any time point. We considered the possibility that the transfected DNA could be randomly integrated with the chromosomal DNA, so that digestion with a restriction enzyme with a single recognition site of the plasmid may lead to a large variety of restriction fragment lengths, resulting in a smear, rather than recognizable bands. To exclude this possibility, the DNA extracted from isolated nuclei was digested with a restriction enzyme with two sites within the plasmid. This procedure, which should release the segment of any integrated transgene between the two restriction sites, also did not produce recognizable bands by Southern analysis. Therefore, the nuclear concentration of the transgene appears to be below the limits of detection by Southern blot analysis. However, PCR amplification showed the presence of the transgene in the isolated nuclei in both sham-operated controls and 66% hepatectomized rats at earlier time points (up to 24 h). Seven days after DNA administration, the transgene was detected only in the partially hepatectomized rats. Thus, there was a temporal correlation between the presence of the transgene in hepatic nuclei and its persistence in the plasma membrane/endosome-enriched vesicles. Therefore, the nuclear concentration of the transgene appears to be below the limits of detection by Southern blot analysis. However, PCR amplification showed the presence of the transgene in the isolated nuclei in both sham-operated controls and 66% hepatectomized rats at earlier time points (up to 24 h). Seven days after DNA administration, the transgene was detected only in the partially hepatectomized rats. Thus, there was a temporal correlation between the presence of the transgene in hepatic nuclei and its persistence in the plasma membrane/endosome-enriched vesicles.

### Discussion

**Targeted DNA Is Endocytosed Predominantly by Hepatocytes**

Macromolecules presenting to the liver may be internalized by hepatocytes or phagocytic cells present in the liver, such as Kupffer cells; the proportion of administered macromolecules internalized by hepatocytes depends on the particle size (21) and biochemical characteristics (22) of the molecule. Our cell isolation studies show that under the conditions of our experiments, 80% of the DNA-carrier complex internalized by the liver is endocytosed by hepatocytes. This exceeds the estimated proportion of parenchymal cells in the liver, approximately 65%, and points to specificity of the targeted delivery. Moreover, as the reporter gene is expressed from a hepatocyte-specific promoter-enhancer, its expression in the liver further substantiates internalization by hepatocytes.

**Events Leading to Transgene Persistence Precede Regenerative DNA Synthesis**

Previous studies have shown that DNA targeted to the liver by ASGP receptor-mediated endocytosis is retained in high copy numbers, for a long time (at least 14 weeks), when 66% hepatectomy is performed shortly after delivery of the DNA. These initial experiments were based on the hypothesis that the wave of DNA synthesis and cell division that occurs during the regenerative response to partial hepatectomy may result in the integration of the transgene with the chromosomal DNA. However, results of the present work indicate that in sham-operated control rats, a great majority of the endocytosed DNA is degraded in 4 h, whereas the wave of DNA synthesis occurs 16–24 h after partial hepatectomy. In rats undergoing 66% hepatectomy, there is a marked difference in the hepatic content of undegraded transgene at 4 h after DNA administration and all time points thereafter. The findings indicate that partial hepatectomy leads to the persistence of the bulk of the undegraded transgene via effects on hepatocytes that precede DNA synthesis and mitosis.

**The Bulk of the Persistent Transgene Exists within Cytoplasmic Vesicles**

Twenty min after its administration, the internalized DNA was located predominantly in three subcellular fractions, the membranous fraction of the low-speed pellet, the plasma membrane/endosome-enriched fraction, and the lysosomal fraction. As expected with DNA transfection, the endocytosed DNA in all fractions was markedly diminished in amount in 4 h in sham-operated rats, and was hardly detectable by Southern blot beyond 24 h. In contrast, in the 66% hepatectomy group, the transgene present in the plasma membrane/endosome-enriched fraction persisted with little attenuation for the duration of the experiment. However, the endocytosed DNA in the membranous fraction of the low-speed pellet and the lysosomal fraction progressively decreased and ultimately disappeared.

The transfected DNA was not detected by Southern blot analysis in isolated nuclei at any time point. We considered the possibility that the transfected DNA could be randomly integrated with the chromosomal DNA, so that digestion with a restriction enzyme with a single recognition site of the plasmid may lead to a large variety of restriction fragment lengths, resulting in a smear, rather than recognizable bands. To exclude this possibility, the DNA extracted from isolated nuclei was digested with a restriction enzyme with two sites within the plasmid. This procedure, which should release the segment of any integrated transgene between the two restriction sites, also did not produce recognizable bands by Southern analysis. Therefore, the nuclear concentration of the transgene appears to be below the limits of detection by Southern blot analysis. However, PCR amplification showed the presence of the transgene in the isolated nuclei in both sham-operated controls and 66% hepatectomized rats at earlier time points (up to 24 h). Seven days after DNA administration, the transgene was detected only in the partially hepatectomized rats. Thus, there was a temporal correlation between the presence of the transgene in hepatic nuclei and its persistence in the plasma membrane/endosome-enriched vesicles.

**Transgene Persisting within Cytoplasmic Vesicles Is DNase Protected**

Electron microscopy of the plasma membrane/endosome-enriched fraction showed numerous closed vesicles. Internalized DNA in this fraction was resistant to digestion by exogenously added DNase I. Disruption of the vesicles by detergent rendered the DNA susceptible to DNase. Together, these findings suggest that DNA in this fraction is located within a protected vesicular space, rather than being adherent to the surface of membranes. This may explain the persistence
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of the transgene within endosomes. DNA within the lysosomal fraction was also partially protected from exogenously added DNase I. Disappearance of the internalized DNA from lysosomal fractions at 24 h after DNA administration may result from its escape from lysosomes or degradation within lysosomal vesicles. The membranous fraction of the 750 g pellet was enriched in the plasma membrane marker 5'-nucleotidase, but showed predominantly open sheets of membrane. The internalized plasmid DNA present in this fraction was not protected from DNase, and therefore may have been simply adherent to the membranes. This component of the DNA rapidly declined and eventually disappeared, perhaps because of degradation by cellular DNase.

The Persistent Transgene Is Undegraded and Transfection-competent—When the extracted internalized DNA was subjected to Southern analysis without digestion with a restriction enzyme, supercoiled, nicked circular, and linear forms of the DNA were found to comigrate with the corresponding forms in the input plasmid. After digestion with a single cutter, the linearized endocytosed plasmid again comigrated with similarly linearized input plasmid. These findings suggest that DNA persisting within the liver in the 66% hepatectomized rats, beyond 24 h after intravenous administration, remains undegraded. Functional integrity of the persistent plasmid DNA was shown by the observation that plasmid extracted from the plasma membrane/endosome-enriched fractions of the liver 7 days after intravenous administration showed a transfection efficiency similar to that of the input plasmid. The concept of episomal persistence of endocytosed DNA is further supported by the findings of Wilson et al. (5) that after targeted delivery to the liver and subsequent partial hepatectomy, no non-methylated plasmids were detected, indicating that the transgene is stabilized but not replicated within the liver.

Possible Mechanisms of Transgene Persistence—Ligands that are internalized via ASGP receptor are initially located within endosomal vesicles. Translocation of these endosomal vesicles to lysosomes and subsequent fusion with lysosomes require attachment to microtubules and the action of specific proteins, such as dynein (23). Studies in our laboratory using fluorescein-labeled anti-α tubulin antibodies and confocal microscopy indicate that hepatocyte microtubules are rapidly depolymerized after 66% hepatectomy. This may result in the failure of the endosomes to be translocated to the lysosomes. Modification of the surface proteins of the endosomal vesicles may prevent subsequent attachment to microtubules following their reformation. Since the transfected DNA harbored within the endosomal vesicles is protected from DNase (Fig. 4), the transgene may persist for the life of the hepatocyte, which has been estimated to be around 90 days in rats (24).

There was a general correlation between the amount of the transgene persisting in hepatocytes and its expression. Although the present studies show that virtually all internalized plasmid persisting in the liver after the first 24 h exists within cytoplasmic vesicles, it is likely that transgene expression requires translocation to nuclei. Transgenes located in nuclei, either as episomes or integrated with chromosomal DNA, may be present at copy numbers too low to be detected by Southern analysis. This notion is consistent with the observation that the level of expression of the transgene is very low compared to the copy number of the transgene persisting in the liver (3).

Implications for Gene Therapy—Carrier-mediated targeting of genes to specific cells in vivo provides an attractive method for gene therapy for the treatment of inherited diseases. We have achieved temporary amelioration of hyperlipidemia in low density lipoprotein receptor-deficient Watanabe heritable hyperlipidemic rabbits (and for familial hypercholesterolemia in man) by receptor-mediated targetted delivery of normal low density lipoprotein receptor cDNA to the liver (4). Short term persistence and expression of the transgene is a major limitation to application of this technique in gene therapy. Prolonged persistence and expression of the human serum albumin cDNA has been achieved in analbuminemic rats (Nagase strain) by performing 66% hepatectomy after gene targeting (3). Based on the data presented here on the mechanism of persistence of endocytosed DNA following partial hepatectomy, it is likely that pharmacological agents can be used to achieve transgene persistence, thus making partial hepatectomy unnecessary.

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