DNA delivered to the liver by asialoglycoprotein receptor-mediated endocytosis is degraded in lysosomes within 48 h. To test the hypothesis that microtubular disruption should promote transgene persistence by interrupting endosomal translocation to lysosomes, plasmids containing bacterial chloramphenicol acetyltransferase (pSV2-CAT) or human bilirubin-UDP-glucuronosyltransferase-deficient homozygous Gunn rats (10) were complexed with asialoglycoprotein-polylysine conjugates, and 1 mg of the complexed DNA was injected intravenously into bilirubin-UDP-glucuronosyltransferase-deficient Gunn rats. 30 min before DNA injection, one group received 0.75 mg of colchicine/kg of body weight intraperitoneally, which was shown by immununofluorescent confocal microscopy to disrupt the microtubular network. Control rats received normal saline. In colchicine-pretreated rats receiving pSV2-CAT, hepatic chloramphenicol acetyltransferase activity persisted for 9–14 weeks, whereas in the saline-pretreated group the activity was detectable for 48 h only. In colchicine-pretreated Gunn rats receiving pSVK3-hBUGT, the DNA persisted in liver for 10 weeks, bilirubin glucurononides were excreted in bile, and serum bilirubin levels declined by 25–35% in 2–4 weeks and remained reduced for 8 weeks. Without colchicine pretreatment, the DNA was detectable in liver for 2 days only, and serum bilirubin levels were not reduced. Thus, microtubular disruption provides a noninvasive method for prolonging the effect of liver-targeted gene therapy.

Galactose-terminated (asialo-) glycoproteins (ASG) are internalized via the asialoglycoprotein receptor (ASGR), which is expressed in large numbers only in hepatocytes. Following endocytosis, the endosomal vesicles containing the ligand are translocated to lysosomes where the ligands are degraded. During this translocation, the ligand is uncoupled from the receptor, which returns to the cell surface and is reutilized (1). Because of the high level of expression of ASGR in hepatocyte surface membranes, ASGR-mediated endocytosis has been utilized for targeted delivery of macromolecules, including DNA, to the liver (2). For this purpose, ASGs are conjugated to polycations, such as polylysine. Under appropriate conditions, these conjugates form a soluble complex with DNA. After intravenous infusion, the ASG-polycation-DNA complex is internalized by hepatocytes via ASGR-mediated endocytosis. As expected, DNA internalized by this pathway is rapidly degraded, presumably in lysosomes. However, a small fraction of the DNA apparently reaches the nucleus and is expressed transiently (3, 4). In an effort to prolong the transgene expression, 66% hepatectomy has been performed after DNA delivery. This procedure results in persistence and expression of the endocytosed DNA for many weeks (5, 6). The great majority of the persisting DNA exists in a pool of cytoplasmic vesicles (7) as undegraded input plasmid (6, 7). The mechanism of persistence of the endocytosed DNA in hepatocellular cytoplasm is not clear. The wave of hepatocellular mitosis that occurs 16–48 h after partial hepatectomy cannot explain this persistence, because a great majority of the endocytosed DNA is rapidly degraded in nonhepatotomized recipients within the first 4 h after DNA delivery (6, 7).

We have observed recently that the microtubular network in a significant proportion of hepatocytes is transiently disrupted after 66% hepatectomy (8). Because intact microtubules are required for translocation of endosomes to lysosomes (9), it is possible that the early disruption of microtubules after partial hepatectomy may play a role in transgene persistence after this procedure. Based on these observations, we hypothesized that transient pharmacological disruption of microtubules may also prolong the persistence and expression of DNA endocytosed into hepatocytes. In this paper, we have used bilirubin-UDP-glucuronosyltransferase-deficient homozygous Gunn rats (10) to evaluate this hypothesis. Two plasmids, one expressing bacterial chloramphenicol acetyl transferase and another expressing human bilirubin-UDP-glucuronosyltransferase-1, were transferred by ASGR-mediated endocytosis in vivo. The effect of microtubular disruption by colchicine administration on the persistence and expression of these gene were determined.

MATERIALS AND METHODS

Animals—Homozygous Gunn rats and congeneic normal Wistar-RHA rats of both genders (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. All animals received humane care in accordance with guidelines of the National Institutes of Health and the Animal Research Committee of the Albert Einstein College of Medicine.

Chemicals—Anti-α-raft brain β-tubulin monoclonal antibody, a fluorescein isothiocyanate-conjugated anti-mouse IgG antibody, and colloidal gold (15 nm) were obtained from Sigma. All other reagents were of the highest purity available.

Gene Therapy Aided by Microtubular Disruption

- Infusion of Colchicine and DNA-Carrier Adducts—In initial experiments we determined the maximum tolerated dose of colchicine in Gunn rats and congenic normal Wistar RHA rats. In both groups, significant mortality was observed with a dose of 2 mg/kg of body weight injected intraperitoneally. Therefore, we limited the maximum dose to 1 mg/kg of body weight for subsequent studies. To determine the minimum dose required for microtubular disruption, colchicine (0.25–1.0 mg/kg of body weight) was dissolved in normal saline and injected intraperitoneally. At this proportion, the electrophoretic mobility of the plasmid on agarose gel electrophoresis was fully inhibited (2). The complex was filtered through a 0.2-μm filter and was stored at 0–4°C for up to 1 week before infusion into rats.

- Determination of the Dose of Colchicine and Time Course of Its Action—In initial experiments we determined the maximum tolerated dose of colchicine in Gunn rats and congenic normal Wistar RHA rats. In both groups, significant mortality was observed with a dose of 2 mg/kg of body weight injected intraperitoneally. Therefore, we limited the maximum dose to 1 mg/kg of body weight for subsequent studies. To determine the minimum dose required for microtubular disruption, colchicine (0.25–1.0 mg/kg of body weight) was dissolved in normal saline and injected intraperitoneally (4 rats in each group). Controls (n = 4) received normal saline intraperitoneally. The rats were anesthetized with ether, and the livers were prepared for immunofluorescence confocal microscopy as described below. To determine the time course of action of colchicine, we injected 0.75 mg/kg of colchicine (which was sufficient for effective disruption of the microtubules) into five groups of rats (two in each group). Controls were left untreated. The livers were prepared for immunofluorescence confocal microscopy as described below. To determine the optimal temporal relationship between administration of colchicine and infusion of the DNA-carrier complex, 100 pmol DNA in 5.0 ml, was infused into tail veins of Gunn rats anesthetized with ether. Because the disruption of microtubules results in the depletion of cell surface ASGR over time, preliminary studies we determined the optimum temporal relationship between administration of colchicine and infusion of the DNA-carrier complex. Injection of colchicine 30 min before DNA administration resulted in a combination of efficient internalization of the DNA by the liver and its persistence in hepatocytes (data not shown). For determination of the effect of colchicine on serum bilirubin, six Gunn rats were injected with the carrier pSVK3-hBUGT, without colchicine pretreatment, and six others were injected with the DNA-carrier complex 30 min after colchicine pretreatment.

- Infusion of Colchicine and DNA-Carrier Adducts—The DNA-carrier complex (100 pmol DNA in 5.0 ml) was infused into tail veins of Gunn rats anesthetized with ether. Because the disruption of microtubules results in the depletion of cell surface ASGR over time, in preliminary studies we determined the optimal temporal relationship between administration of colchicine and infusion of the DNA-carrier complex. Injection of colchicine 30 min before DNA administration resulted in a combination of efficient internalization of the DNA by the liver and its persistence in hepatocytes (data not shown). For determination of the effect of colchicine on serum bilirubin, six Gunn rats were injected with the carrier pSVK3-hBUGT, without colchicine pretreatment, and six others were injected with the DNA-carrier complex 30 min after colchicine pretreatment.

- Fourteen other Gunn rats in each group were used for determining the time course of DNA persistence, human bilirubin-UDP-glucuronosyltransferase-1 expression, bilirubin-UDP-glucuronosyltransferase activity, and bile pigment analysis. For these studies, two rats in each group were killed at various intervals. For determination of hepatic ASGR, four Gunn rats were injected with the carrier pSVK3-hBUGT, without colchicine pretreatment, and six rats were injected with the DNA-carrier complex 30 min after colchicine pretreatment.

- Preparation of the Liver and Conflonal Immunofluorescence Microscopy—Fixation of the liver was carried out in control animals (no colchicine administration) and at various doses or time intervals after colchicine injection as described above. Technical details of tissue preparation, immunofluorescence staining, and confocal microscopy have been described (8). In brief, the rats were anesthetized with ether, and the livers were fixed by in situ perfusion with 4% paraformaldehyde. Processing for a given series were completed within a 24-h period. Because microtubules disassemble at low temperatures, care was taken to fix the livers in situ at 37°C. After fixation, all procedures were carried out at room temperature. Tissue sections (45 μm) were prepared with an Oxford Vibratome (Ted Pella, Testin, CA). The tissue sections were permeabilized by exposure to 0.1% Triton X-100 for 1 min and then reacted with an anti-β-tubulin antibody and subsequently with an anti-mouse IgG-fluorescein isothiocyanate conjugate. Confocal microscopy was carried out with a Bio-Rad MRC 600 scanning confocal microscope outfitted with a krypton/argon laser using a Nikon planapo 60× N.A. 1.40 objective. Coded sections were scanned at 0.5-μm intervals to a maximum depth of 3–6 μm, with the observers not aware of the experimental conditions. When comparing control and experimental sections, observations were made at the same depth. The laser units and filter combinations were kept consistent throughout and were checked for stability at the end of the study. Within 20 and 30 fields of each section were scanned, and an overall assessment of the extensiveness of the fluorescently labeled microtubular network was made. At the end of the examination of series, the code was broken.

RESULTS

- Dose of Colchicine and Time Course of Its Effect—In control sections, a delicate and extensive system of microtubules was seen throughout the hepatocytes and nonparenchymal cells. Because the degradation of the majority of the internalized DNA occurs within the first hours after endocytosis (6, 7), we wanted to determine the dose of colchicine that is adequate for microtubular disruption shortly after its administration. Therefore, the dose-response relationship was determined 2 h after colchicine injection. Disruption of microtubules was minimal at a dose of 0.25 mg/kg of body weight (Fig. 1B). The microtubules were significantly disrupted at higher doses (Fig.
Microtubular network was fully regenerated. Partially regenerated, and 48 h after colchicine administration, the microtubular network had partially regenerated, and maximum microtubular disruption was observed in 2 h. By 24 h, the microtubular network had partially regenerated, and 48 h after colchicine administration, the microtubular network was fully regenerated.

Time Course of Degradation of pSVK3-hBUGT1 Internalized via ASGR-mediated Endocytosis—To correlate our studies of microtubule integrity with the degradation of internalized DNA, we digested the DNA extracted from liver homogenates with Apal and performed Southern blot analysis using an oligonucleotide probe specific for the unique 5’ domain of human bilirubin-UDP-glucuronosyltransferase-1 (Fig. 2). Densitometric analysis of the Southern blots showed that in 20 min, 70–80% of the infused DNA was internalized by the liver 20 min after injection, representing about 30,000–35,000 copies of the plasmid per hepatocyte. This calculation was based on our previous finding that 80% of the DNA internalized by the liver is present in hepatocytes (7). In control rats (without colchicine pretreatment), only about 10% of the initial DNA load remained in the liver 4 h after administration (Fig. 2A); by 24 h, the plasmid DNA was undetectable by Southern blot. In contrast, in rats that were pretreated with 0.75 mg of colchicine/kg of body weight, 30% of the endocytosed plasmid was retained by the liver at 4 h (Fig. 2B). By 24 h after DNA administration, the plasmid concentration had decreased to 8–12% of the level at 20 min. The DNA concentration remained nearly at this level for 8–10 weeks, after which it progressively declined and became undetectable in 14 weeks.

Hepatic CAT Activity—CAT activities in homogenates of livers removed from rats at various intervals after infusion of the plasmid-carrier complex are shown in Fig. 3. CAT activity was undetectable in the liver samples at 20 min or 4 h after DNA administration. At 24 h, CAT activity was detected at approximately 1.0 microunits/mg of protein in the group that received colchicine. In the colchicine-pretreated group, CAT activity persisted at approximately this level for 8 weeks, after which it progressively declined and became undetectable by the end of the study (14 weeks).

Expression of Human B-UDP-glucuronosyltransferase—Expression of the human bilirubin-UDP-glucuronosyltransferase-1 protein was determined by immunoblot using an isoform-specific anti-peptide antibody (Fig. 4). This antibody did not recognize any protein from livers from untreated Gunn rats. In the group of rats that were pretreated with colchicine, the immunoreactive human bilirubin-UDP-glucuronosyltransferase-1 was detectable from 24 h to 10 weeks. In the nonpretreated group, the enzyme protein was detectable only at the 24-h point after DNA administration. Bilirubin-UDP-glucuronosyltransferase Activity—UDP-glucuronosyltransferase activity toward bilirubin was detectable in liver microsomal fractions from both colchicine-pretreated and nonpretreated groups at 24 h after administration of pSVK3-hBUGT1 (Table I). At subsequent time points, the en-

![Figure 1](https://example.com/f1.jpg)

**Fig. 1.** Effect of colchicine on hepatocyte microtubular network. 2 h after the administration of colchicine or normal saline (control), rats were anesthetized and liver were fixed in situ by perfusion with paraformaldehyde as described in the text. Immunofluorescence studies were performed by scanning confocal microscopy using an anti-β-tubulin antibody. Two sets of experiments are shown. Set 1 (upper row): A, normal saline; B, 0.25 mg of colchicine/kg of body weight; C, 1 mg of colchicine/kg of body weight. Set 2 (lower row): D, normal saline; E, 0.75 mg of colchicine/kg of body weight.

![Figure 2](https://example.com/f2.jpg)

**Fig. 2.** Southern blot analysis of DNA internalized by the liver. Liver samples were collected at various time points from Gunn rats that were administered pSVK3-hBUGT1 without (A) or after (B) pretreatment with colchicine. Liver homogenates were treated with proteinase K and sodium dodecyl sulfate, and DNA was extracted as described in the text. The DNA was digested with the restriction enzyme Apal, which has a single recognition site on the plasmid pSVK3-hBUGT1. After electrophoresis on 0.8% agarose gels, Southern analysis was performed using a 32P-labeled probe specific for human bilirubin UDP-glucuronosyltransferase-1. Liver samples were collected at 4 h (lanes 1), 24 h (lanes 2); 2 weeks (lanes 3), 5 weeks (lane 4), 8 weeks (lane 5), 10 weeks (lane 6), and 14 weeks (lane 7). Each data point is from a single rat, representative of three experiments.
zyme activity was detectable in only in the colchicine-pre-
treated group for up to 8 weeks after DNA administration. For
comparison, we determined bilirubin-UDP-glucuronosyltrans-
ferase activity in human liver microsomes. The specific enzyme activity in the hepatic microsomes of colchicine pretreated Gunn rats was approximately 2–4% of that in human liver microsomes (10–20 nmol/mg of protein/min).

Bilirubin Glucuronides Excreted in Bile—To directly deter-
mine bilirubin-UDP-glucuronosyltransferase activity in vivo, we analyzed pigments excreted in the bile. The bile of untreated Gunn rats (not shown) or Gunn rats treated with pSV2CAT (Fig. 5A) did not contain significant amounts of bilirubin glucuronides, the majority of the pigments excreted in bile being unconjugated bilirubin. 24 h after administration of pSVK3-hBUGT1, significant amounts of bilirubin monogluco-
should interfere with the later phases of receptor-mediated endocytosis it may be predicted that microtubular disruption associated with motor proteins (17). Based on the above considerations it may be predicted that microtubular disruption would be considered toxic in humans. However, other microtubule-disrupting agents, such as Vinca alkaloids, may be more clinically appropriate. Alternatively, receptor-mediated delivery of colchicine to the liver may be used to limit its systemic toxicity.

Persistence of Endocytosed DNA Depends on Early Events Following Endocytosis—We have shown previously that normally most of the DNA internalized by hepatocytes via ASGR is degraded during the first 4 h after endocytosis (6). Therefore, any maneuver that results in persistence of a significant fraction of the internalized DNA must be effective during the first few hours after DNA administration. Partial hepatectomy results in persistence of endocytosed genes in cytoplasmic vesicles of hepatocytes and prolongs their expression (7). The time course of degradation of the endocytosed DNA indicates that such persistence cannot be the result of DNA synthesis, karyokinesis, and mitosis of hepatocytes that occur 16–24 h after partial hepatectomy (7). Therefore, the protective effect of 66% hepatectomy on the internalized DNA appears to result from events that occur within the first few hours after partial hepatectomy. In previous studies, we have observed a significant degree of disruption of the microtubular network 2–6 h after 66% hepatectomy. This event is rapid enough to affect translocation of endosomes to lysosomes, thereby inhibiting the degradation of endosomal contents, which may explain the observed transgene persistence (8). To directly test the hypothesis that microtubular disruption should result in persistence of the endocytosed DNA, in this study we used colchicine pretreatment to disrupt hepatocellular microtubules. Our finding that following microtubular disruption the endocytosed DNA persists in the cytoplasm is consistent with the postulate that during the period required for regeneration of the disrupted microtubules, the “dereailed” endosomal vesicles containing the internalized DNA may undergo changes that interfere with their association with microtubules or functional motor proteins.

Optimum Time of Colchicine Injection in Relation to the DNA Delivery—Because microtubule-disrupting agents interfere with the return of ASGR to the plasma membrane, the receptor is redirected from the cell surface to the cytoplasm. Over a period of time, this leads to depletion of cell surface receptors (23), thereby reducing endocytosis. Therefore, initial experiments were conducted to determine the time course of microtubular depolymerization after colchicine administration to identify the correct time of colchicine administration in relation to DNA delivery. These experiments showed that injection of colchicine up to 30 min before ASGR-mediated DNA delivery did not significantly reduce internalization of the plasmid by the liver. Because microtubules were markedly disrupted within 2 h after colchicine administration, this allowed the opportunity to study the effect of microtubule disruption on the persistence of the endocytosed plasmid.

Significance for Liver-directed Gene Therapy—Receptor-mediated liver-targeted delivery of therapeutic genes in vivo is an attractive method for gene therapy for inherited diseases. However, attempts to use this method for treating metabolic liver diseases, such as hyperlipidemia in low density lipoprotein receptor-deficient Watanabe heritable hyperlipidemic rabbits resulted in only short term effects (4). Prolonged persistence of the transgene has heretofore required partial hepatectomy after gene delivery (5), which obviously limits the potential clinical applicability of this method. Dramatic prolongation of transgene expression by pharmacological disruption of microtubules provides a novel noninvasive technique for achieving transgene persistence. The dose of colchicine used in this study would be considered toxic in humans. However, other microtubule-disrupting agents, such as Vinca alkaloids, may be more clinically appropriate. Alternatively, receptor-mediated delivery of colchicine to the liver may be used to limit its systemic toxicity.

**Fig. 6. Serum bilirubin concentrations.** Blood samples were collected at the indicated times, and serum bilirubin was determined as described in the text. Each data point is the mean of six experiments ± S.E. c, DNA without colchicine; o, DNA with colchicine.

Serum Bilirubin Concentrations—In the colchicine-pretreated group, serum bilirubin concentrations declined by 25–35% in 2–4 weeks, remained at the reduced levels for 4–6 weeks, and then gradually returned to pretreatment levels by 14 weeks (Fig. 6). Without colchicine pretreatment, serum bilirubin remained unchanged from the pretreatment levels.

**DISCUSSION**

Translocation of Endocytotic Vesicles to Lysosomes Requires Microtubules—After endocytosis, the ligand-containing vesicles exhibit a saltatory movement at 30 nm/s, which is followed by a rapid vectorial movement at 50 nm/s, until the vesicles fuse with lysosomes (22). In mammalian liver, the initial phase of endocytosis does not require microtubules (23). However, during the vectorial translocation to lysosomes, endosomes remain associated with microtubules (24, 25). After the endosome is acidified, the receptor dissociates from the ligand (26) and the receptor-containing domain segregates from the ligand-containing domain. The receptor-containing membranous domain returns to the cell surface, whereas the ligand-containing vesicle translocates to the lysosome, where its contents are degraded. Movement of the ligand-containing vesicle to the lysosome along microtubules may be powered by the ATP-utilizing motor, cytoplasmic dynein (25). The receptor-containing domain returns to the cell surface also along microtubules, probably powered by the other motor molecules, such as kinesin. Depolymerization of microtubules by colchicine treatment causes redistribution of ASGR from the cell surface to the cytoplasm (23). The directionality of movement of vesicles along microtubules may depend on activation of specific groups of motor proteins by phosphorylation of proteins that are associated with the motor proteins (17). Based on the above considerations it may be predicted that microtubular disruption should interfere with the later phases of receptor-mediated endocytic pathway by inhibiting receptor-ligand segregation (23, 24) and progression of endosomes to lysosomes.

Serum bilirubin concentrations.

| TREATMENT | TREATMENT |
|-----------|-----------|
| DETECTION | DETECTION |
| 20        | 80        |
| 40        | 100       |
| 60        | 120       |
| 80        | 140       |
| 100       | 160       |

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Microtubular Disruption Prolongs the Expression of Human Bilirubin-uridinediphosphoglucuronate-glucuronosyltransferase-1 Gene Transferred into Gunn Rat Livers

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