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In Vivo Hepatocyte Transduction with Retrovirus during In-flow Occlusion

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Gene therapy research would be facilitated by a technically simple procedure for transducing hepatocytes *in vivo*. Previously reported methods have employed partial hepatectomy followed 24 hr later by asanguineous perfusion of the regenerating liver with retrovirus. We have developed a simpler method of *in vivo* transduction in which we deliver an intraportal bolus of retrovirus to the regenerating rodent liver during a brief period of hepatic in-flow occlusion. On Day 0, adult male Sprague–Dawley rats ($N = 19$) underwent 70% hepatectomy to induce hepatocyte replication. On Day 1, retroviral supernatant was harvested from an amphotropic retroviral packaging cell line that packaged an LNL6-derived vector containing the cytomegalovirus promoter driving expression of the *Escherichia coli* β-galactosidase (βgal) gene. Twenty-four hours after partial hepatectomy, experimental rats ($N = 17$) received 6×10^5 colony-forming units of retrovirus by intraportal injection during a 3-min occlusion of the hepatic artery and portal vein. Control rats ($N = 2$) received intraportal medium (without retrovirus), also during in-flow occlusion. The procedure required 20–25 min, and the survival rate was 84%. Cryostat sections were prepared from liver biopsies obtained on Post-transduction Days 8 and 15 and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside to detect βgal expression. Light microscopic examination of Day 8 sections from surviving experimental rats ($N = 14$) revealed 0.10–1.00% blue (i.e., transduced) hepatocytes per low power field, while sections from control rats ($N = 2$) exhibited no blue cells. Day 15 sections from experimental rats revealed a somewhat lower frequency of hepatocytes expressing βgal. We conclude that retroviral transduction during in-flow occlusion is a rapid, reliable, low-mortality method of attaining stable genetic modification of regenerating rodent hepatocytes. © 1993 Academic Press, Inc.

INTRODUCTION

Research on the gene therapy of inherited metabolic and hematologic disease would be facilitated by a reliable, technically simple method for the *in vivo* retroviral transduction of rodent hepatocytes. To date, animal studies on the genetic modification of hepatocytes have relied on protocols involving: (1) partial hepatectomy with isolation of recipient hepatocytes, followed by *in vitro* retroviral transduction and reimplantation of those hepatocytes into the same animal [1, 2] or (2) partial hepatectomy followed 24 hr later by asanguineous liver perfusion with retrovirus [3–5]. The laboratories of J. Wilson and J. R. Chowdhury have used the first of these methods to ameliorate hypercholesterolemia in the Watanabe rabbit model of low-density lipoprotein receptor (LDLR) deficiency [2]. However, this *ex vivo* approach is cumbersome, time-consuming, and suffers from the uncertainties (e.g., infection, hepatocyte damage) inherent to the isolation, culture, and *in vitro* transduction of hepatocytes. Ferry *et al.* have used a retroviral vector, containing the *Escherichia coli* β-galactosidase (βgal) gene, and the asanguineous perfusion method to attain hepatocyte transduction frequencies of 1–5% [4]. However, their procedure is technically difficult and exposes the recipient animal to the stresses of retroperitoneal dissection, 20 min of hepatic ischemia (in-flow and out-flow occlusion), and prolonged cannulation of the portal vein and inferior vena cava.

We reasoned that a simpler surgical technique would allow more rapid testing of multiple retroviral vectors. Consequently, we have devised a quick, low-mortality method of *in vivo* hepatocyte transduction in which we deliver an intraportal bolus of infection-competent, but replication-deficient, retroviral construct to the regenerating rodent liver during a brief (3-min) period of hepatic in-flow occlusion.

MATERIALS AND METHODS

Animals

Age-matched adult male Sprague–Dawley rats (outbred, 200–275 g) were purchased from Sasco, Inc. (Omaha, NE) and maintained on Rodent Laboratory Chow 5001 (Ralston Purina, St. Louis, MO) and tap water *ad libitum*. A 12-hr light/dark cycle was employed, and the rats were allowed 5 days to acclimatize to their quarters before use. All experiments were performed in adherence with protocols approved by the Washington University Animal Studies Committee.

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*Construction of IRES–*dhfr Expression Cassette*

An expression cassette comprising the encephalomyocarditis virus internal ribosome entry site (IRES) [6] and a methotrexate-resistant form of murine dihydrofolate reductase (*dhfr) [7] was constructed as follows. The plasmid pFR400, which contains the *dhfr cDNA, was digested with *Nco*I, and the resulting linear *dhfr plasmid was blunt-ended with T4 polymerase. The blunt-ended *dhfr plasmid was further digested with *Hin*dIII and gel purified on sea plaque agarose (FMC Bioproducts, USA). The purified *Hin*dIII/blunt-end *dhfr fragment was cloned into pBluescript(BS)-II-KS (Stratagene, La Jolla, CA), which had been previously digested with *Hinc*II/*Hin*dIII. A plasmid containing the *dhfr cDNA in the pBS-II-KS vector was isolated and called pBS-II–*dhfr. This plasmid was digested to completion with *Xba*I and *Hin*dIII and gel purified as above. Next, the *Xba*I/*Hin*dIII partial fragment of pLZ1C1, which contained the IRES [6], was cloned into the *Xba*I/*Hin*dIII-digested pBS-II–*dhfr plasmid. The resulting plasmid was subsequently digested with *Not*I and blunt-ended with T4 polymerase. Finally, *Bgl*II linkers (New England Biolabs, No. 1066) were inserted to produce the pBS-II–IRES–*dhfr plasmid. This was digested with *Bgl*II/*Xho*I to yield a pBS-II–IRES–*dhfr *Bgl*II/*Xho*I fragment suitable for insertion into our retrovirus (see below).

Retrovirus

Retroviral constructs were derived from the LNL6 Moloney murine leukemia virus vector provided by Dr. Dusty Miller (Fred Hutchinson Cancer Research Center, Seattle, WA) [8]. An initial construct (RS8391) was prepared from LNL6 as follows. The *Eco*RI site downstream from the extended packaging sequence (Ψ^+) was mutated to a *Bgl*II site by linker insertion. The neomycin phosphotransferase (*neo*) gene and the majority of the polylinker were removed as a *Bgl*II/*Sal*I fragment and replaced with the pBS-II–IRES–*dhfr *Bgl*II/*Xho*I fragment, thus regenerating the *Bgl*II and *Xho*I sites. This fragment contained the IRES from the 5′-untranslated region of the encephalomyocarditis virus [6] upstream from the gene for a methotrexate resistant form of murine *dhfr [7] (see above). The IRES allows the downstream *dhfr gene to initiate translation in a cap-independent fashion.

A second construct (RS10991, Fig. 1) was made by inserting a cassette containing the human cytomegalovirus (CMV) immediate-early enhancer/promoter [9] driving expression of the *E. coli* βgal gene [6] into the unique *Bgl*II site of RS8391. The orientation of the CMV enhancer/promoter–βgal cassette was confirmed by restriction digestion.

Amphotropic Retroviral Packaging Cell Line

The amphotropic GP + *env*AM12 retroviral packaging fibroblast line [10] was grown to 50% confluency in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% heat-inactivated calf serum (HICS, Hyclone Laboratories, Logan, UT), penicillin (PCN, 10^5 U/liter), and streptomycin (SCM, 10^5 μg/liter). The packaging cells were transfected with 10 μg of the RS10991 construct by the calcium phosphate method [11]. After transfection, the medium was changed every 2 to 3 days.

Methotrexate-resistant clones packaging the RS10991 construct were picked after 14 days of *in vitro* selection in medium containing 250 n*M* methotrexate (Sigma Chemical Co., St. Louis, MO). Each methotrexate-resistant clone was expanded and then titered for retroviral activity.

The retroviral titer for each RS10991 packaging clone was determined by using retroviral supernatant to transduce murine NIH 3T3 fibroblasts which had been plated in DMEM with 10% HICS/PCN/SCM at a density of 1×10^6 cells per 60-mm dish (50% confluency). On Day 1, the cells were repleted with 5 ml fresh medium that contained serial dilutions of retroviral supernatant (from a single packaging clone) and 8 μg/ml hexadimethrine bromide (Polybrene, Sigma), a polycation which enhances interactions between retroviral particles and target cell membranes. *In vitro* transduction of the NIH 3T3 cells with the RS10991 construct was allowed to proceed for 24 hr prior to medium change. On Day 3, the transduced NIH 3T3 cells were split 1:10 and plated into 100-mm dishes containing either medium or medium plus 250 n*M* methotrexate. After 48 hr, the cells plated in medium alone were stained overnight, at 37°C, with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Stratagene) [12]. By low-power light microscopy, the number of blue (i.e., transduced) cells was counted and multiplied by the dilution of packaging medium added to that plate to yield the retroviral titer in blue cell-forming units (BCFUs)/ml packaging medium. The cells plated in medium and methotrexate were maintained in culture for 2 weeks, with medium/methotrexate changes every 2 to 3 days. On Day 14, the number of surviving (i.e., methotrexate-resistant) colonies was counted and multiplied by the dilution of packaging medium added to that plate to give the retroviral titer in colony forming units (CFUs)/ml packaging medium.

Finally, the high-titer clone packaging the RS10991 construct was grown to confluency on 100-mm Primaria plastic plates (Becton–Dickenson Labware, Lincoln Park, NJ) and proven free of replication-competent helper virus activity by amplification and assay for marker rescue on fresh NIH 3T3 cells [10, 13].

Retroviral Supernatant Harvest

On Day 0, confluent plates of the high-titer GP + *env*AM12 clone packaging the RS10991 construct were repleted with fresh medium (lacking methotrexate) and allowed to produce infection-competent/replication-deficient retroviral particles for 18 hr. Early on Day 1, the

retroviral supernatant was harvested and filtered through 0.45-μm cellulose acetate paper (Schleicher & Schuell, Keene, NH), spiked with Polybrene (8 μg/ml, final concentration), and stored on ice until use.

In Vivo Hepatocyte Transduction Technique

On Day 0, 19 age-matched adult male rats underwent standard 70% hepatectomy [14] to induce hepatocyte DNA replication [15, 16]. Under ether anesthesia, the left lateral and median liver lobes were removed. Postoperatively, 5 ml lactated Ringer's solution was injected intraperitoneally as fluid replacement. Twenty-four hours after partial hepatectomy, 17 experimental rats underwent *in vivo* hepatocyte transduction with the RS10991 retroviral construct during in-flow occlusion. Two control rats received medium alone, also under in-flow occlusion. The details of the operative procedure were as follows (see also Fig. 2):

1. Repeat midline laparotomy was performed under ether anesthesia.
2. The bowels were reflected to the left and wrapped in normal saline soaked gauze.
3. The hepatic artery (HA) was dissected free.
4. The proximal portal vein (PV) was dissected from its bifurcation at the porta hepatis to its most proximal tributary (the gastroduodenal vein).
5. The HA and PV were clamped in succession with atraumatic microaneurysm clips.
6. Twenty seconds were allowed for blood to drain from the liver.
7. Using a $\frac{1}{2}$-in. 30-gauge needle, the PV was cannulated proximal to the clip, and injected with 3 ml RS10991 retroviral supernatant that had been incubated on ice with Polybrene (8 μg/ml) for 2 to 4 hr. Approximately 6×10^5 CFUs were delivered over 60 sec.
8. After 3 min the in-flow occlusion clamps were removed in the same order they had been applied.
9. Hemostasis was attained using point pressure and topical thrombin (Parke–Davis, Morris Plains, NJ) at the PV cannulation site.
10. The abdominal wound was closed in two layers with running 4.0 nylon suture.
11. Each animal recovered from anesthesia on a heating pad and was fully awake and ambulatory before being returned to its quarters.

On Day 8 (14 surviving experimental rats and 2 control rats) and Day 15 (14 surviving experimental rats only), each rodent underwent open wedge biopsy of the caudal portion of the regenerated right lateral liver lobe. Hemostasis was attained with electrocautery. Biopsy specimens were immediately frozen in liquid nitrogen and stored at −70°C.

Liver Sections and Histochemical Staining

Liver biopsy specimens were imbedded in O.C.T. compound (Miles, Inc., Elkhart, IN) and cut into cryostat sections 8 μm thick. Each section was mounted on a glass slide, fixed for 10 min in 1.25% glutaraldehyde (Sigma) on ice, and stained overnight at 37°C in X-gal (0.5 mg/ml) [12]. The next day, sections (10 per animal) were evaluated by light microscopy, the number of blue hepatocytes per low-power field was counted, and the arithmetic mean was calculated.

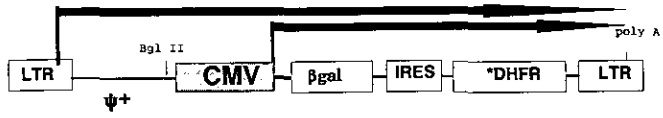

FIG. 1. RS10991 retroviral construct (derived from the LNL6 Moloney murine leukemia virus vector). LTR, retroviral long terminal repeat sequence; Ψ +, extended encapsidation (packaging) signal for retroviral genomic RNA; CMV, human cytomegalovirus immediate-early enhancer/promoter; IRES, encephalomyocarditis virus internal ribosome entry site; βgal, *Escherichia coli* β-galactosidase gene; *dhfr, gene-encoding methotrexate-resistant form of murine dihydrofolate reductase.

RESULTS

Production of High-Titer Retroviral Vector

Using the GP + *env*AM12 line to package the RS10991 retroviral construct (Fig. 1), we attained high levels of *in vitro* expression of the βgal and *dhfr genes. Light microscopic analysis of NIH 3T3 fibroblasts that had been transduced with the RS10991 construct and stained with X-gal 48 hours later (to detect βgal expression), revealed a titer of 2×10^5 BCFUs/ml retroviral supernatant. X-gal staining of nontransduced NIH 3T3 fibroblast monolayers resulted in no blue cells. Similarly, transduction of NIH 3T3 fibroblasts with the RS10991 construct, followed by methotrexate selection for 14 days (to detect *dhfr expression), resulted in a titer of 2×10^5 CFUs/ml retroviral supernatant.

In Vivo Hepatocyte Transduction during In-flow Occlusion

We also used the RS10991 retroviral construct to transduce regenerating rat hepatocytes *in vivo* by the intraportal bolus injection of retroviral supernatant during hepatic in-flow occlusion. Results were assessed by X-gal staining of liver biopsies obtained on Post-transduction Days 8 and 15.

Light microscopic examination of X-gal-stained liver biopsies obtained on Day 8 from the 14 surviving experimental rats revealed 3 to 40 (0.10–1.00%) blue (i.e., transduced) hepatocytes per low-power field (Table 1 and Fig. 3A). Day 8 X-gal-stained liver sections from two control rats exhibited no blue cells (Fig. 3B). Liver biopsies taken from experimental rats on Day 15 revealed a preserved, although somewhat lower, frequency of hepatocytes expressing βgal (Table 1). Under high

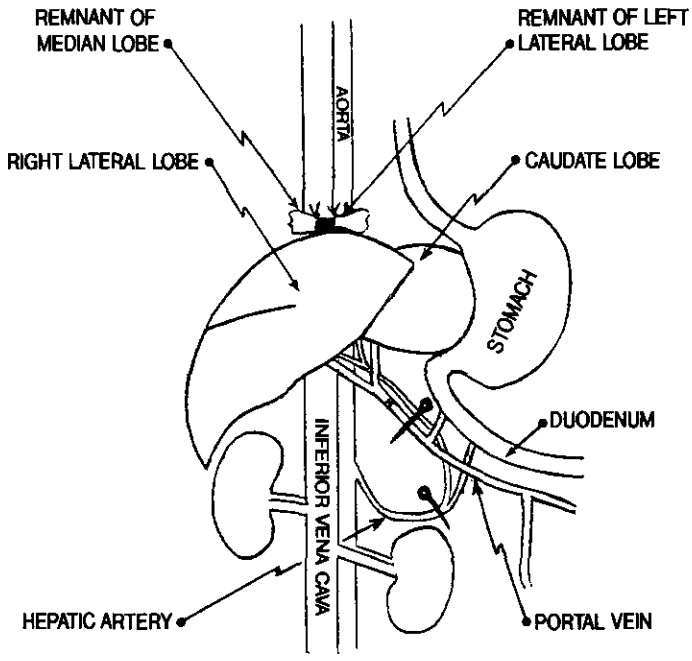

FIG. 2. Schematic of hepatic in-flow occlusion/portal vein injection method of *in vivo* hepatocyte transduction with retrovirus. Clamps are present on the hepatic artery and the proximal portal vein. An "×" marks the point of portal vein cannulation.

power, blue hepatocytes were noted to occur in small clusters on Post-transduction Day 15 sections (Fig. 3C).

Our method of 70% hepatectomy followed 24 hr later by hepatic in-flow occlusion and PV injection of retroviral supernatant (or medium alone) required only 20–25 min (for the in-flow occlusion procedure) and resulted in 84% survival (16 of 19 rats). In subsequent experiments, this technique has been employed in a total of 85 rats with an overall long-term survival of 84%.

DISCUSSION

Gene therapy for inherited disease remains a tantalizing possibility. In-born errors of metabolism such as phenylketonuria, α_1-antitrypsin deficiency, and familial hypercholesterolemia are obvious candidates for liver-directed gene therapy. Some hematologic disorders, such as Factor IX deficiency and Protein C deficiency, may also be treatable by genetic alteration of hepatocytes. However, the clinical application of liver gene therapy will require a reliable method of generating stably transduced hepatocytes in genetically deficient subjects [17]. To date, animal studies in this area have met with two problems: (1) developing optimum hepatocyte transduction vectors which result in the stable integration and long-term expression of foreign genes and (2) devising a rapid, reliable, low-morbidity surgical procedure for accomplishing this liver-directed gene transfer.

Several hepatocyte transduction techniques have been explored, including retroviral vectors [18–20], adenoviral vectors [21], and plasmid DNA complexed with polylysine-conjugated asialoglycoproteins [22, 23] or liposomes [24, 25]. However, not all of these methods consistently result in the long-term expression of transduced genes. For example, the adenoviral [21] and liposome-mediated [24, 25] techniques do not result in the stable integration of foreign genetic material into hepatocyte chromosomal DNA and yield only transient gene expression [17]. Similarly, asialoglycoprotein receptor-mediated transduction does not result in sustained plasmid gene expression [22] unless performed in conjunction with partial hepatectomy [23]. Moreover, the level of sustained expression in these partially hepatectomized animals is quite low [23]. Only retroviral vectors consistently result in the stable integration of transferred genes into hepatocyte chromosomal DNA [20, 26, 27]. Depending on the retroviral construct's promoter elements, this can lead to long-term expression of transduced genes [17].

Infection-competent/replication-deficient retroviral constructs are most promising as vectors for human gene therapy [17]. The advantages of retroviral vectors include their high efficiency of gene transfer into replicating cells, their known mechanism for the integration of foreign genetic material into target cell DNA, and their inability to replicate and produce infectious prog-

TABLE 1

***In Vivo* Hepatocyte Transduction Frequency**

Animal	Mean number of blue hepatocytes per low-power field	
	Day 8	Day 15
Experimental rats		
1	20	8
2	5	2
3	20	15
4	3	<1
5	9	ND[a]
6	13	ND
7	15	ND
8	30	5
9	4	1
10	13	ND
11	15	10
12	40	37
13	15	10
14	5	2
Control rats		
A	0	ND
B	0	ND

[a] ND, not done.

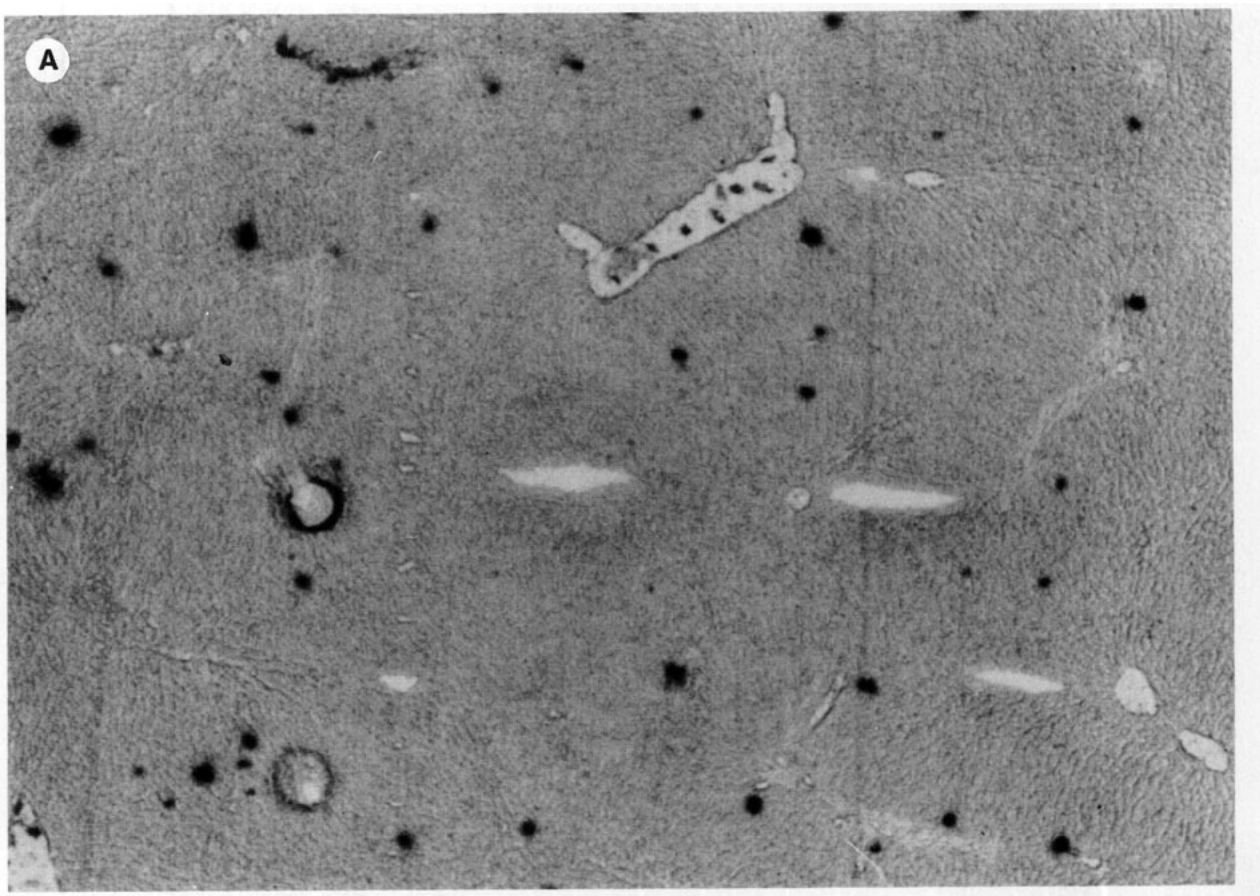

FIG. 3. (A) Representative X-gal-stained liver section taken from a rat that had undergone *in vivo* transduction with the RS10991 retroviral construct 8 days before. Under low power, approximately 1% of hepatocytes are blue. (B) Representative X-gal-stained liver section taken from a rat that had received medium alone (no retrovirus) during hepatic in-flow occlusion 8 days before. No blue cells are present. (C) Representative X-gal-stained liver section taken from a rat that had undergone *in vivo* transduction with the RS10991 retroviral construct 15 days before. Under high power, several small clusters of blue (i.e., transduced) hepatocytes are visible. (N.B. Blue cells appear black in these half-tone black and white photomicrographs.)

eny once integration has occurred [17, 27, 28]. Lingering concerns about retroviral vectors include their very infrequent activation of cellular oncogenes and the remote chance that the packaging clone will generate a replication-competent retrovirus by recombination [10, 13, 17]. The latter difficulty has been avoided by the development of retroviral packaging lines (e.g., GP + *env*AM12) which have defective encapsidation (Ψ) and replication signals, and which incorporate the retroviral packaging genes (*gag, pol, env*) on separate plasmids [10, 27, 29]. The activation of cellular oncogenes, on the other hand, remains a possibility but has not been demonstrated to date with replication-deficient retroviral vectors [17].

In conjunction with these genetic engineering methods, at least two general approaches to liver gene therapy have been developed: (1) an *ex vivo* approach, which involves *in vitro* transduction of isolated hepatocytes followed by reimplantation, and (2) an *in vivo* approach, which allows direct delivery of foreign genes to replicating hepatocytes. In detail, the *ex vivo* approach entails partial hepatectomy in a recipient animal, collagenase digestion of the specimen to yield a hepatocyte suspension, primary culture and *in vitro* transduction of the hepatocytes with the gene of interest, followed by reimplantation into the recipient animal in a manner that will ensure hepatocyte survival and maintenance of liver-specific function [1, 2, 30]. Wilson and co-workers have used this method and a retroviral construct containing the chicken β-actin promoter and a normal rabbit LDLR gene to successfully treat hypercholesterolemia in Watanabe rabbits [2]. However, this technique is cumbersome, time-consuming, and suffers from the uncertainties (e.g., infection, hepatocyte damage) inherent to the isolation and *in vitro* processing of liver cells. Moreover, due to rheologic considerations, reimplantation of more than 2% of the hepatocyte mass is not possible.

In contrast, *in vivo* methods allow direct delivery of

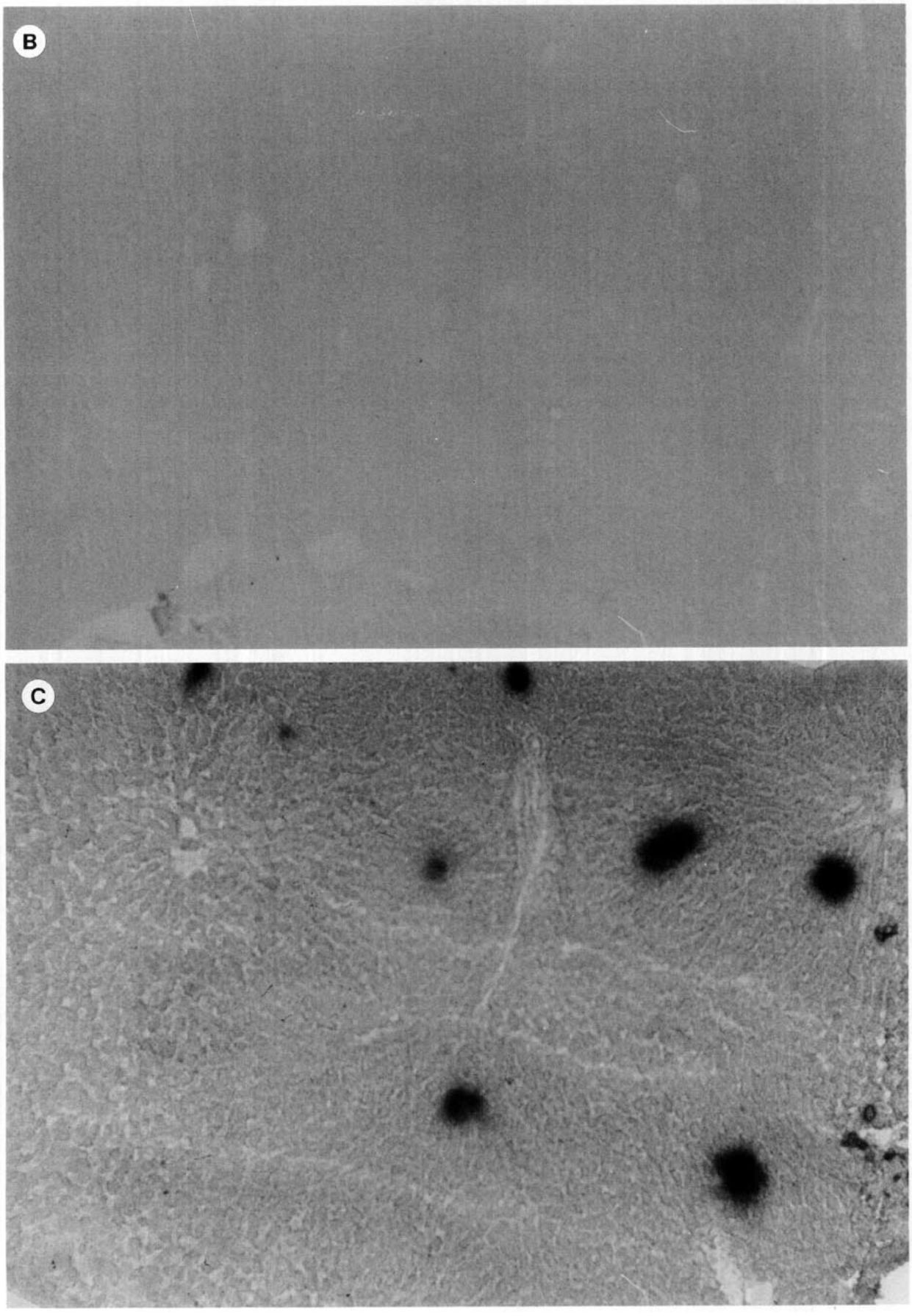

FIG. 3—*Continued*

foreign genes to the liver. Using retroviral vectors, this has been accomplished by intravenous injection [3], parenchymal injection [31], and asanguineous perfusion [4, 5] of the regenerating adult rodent liver. Injection of retroviral vectors into the unclamped PV results in low transduction efficiencies [3], possibly due to complement inactivation of retroviral particles and inadequate contact between recipient hepatocytes and circulating retrovirus. Direct injection of retrovirus into the liver parenchyma suffers from uncertainties regarding the effects of local injury on the uptake of vector from the interstitial space and the limited distribution of the foreign gene in the liver. In the setting of carbon tetrachloride liver injury, Kaleko *et al.* used this technique to deliver a retroviral vector carrying the *neo* gene. They demonstrated "low levels" of *in vivo* transduction by polymerase chain reaction analysis of liver biopsy specimens obtained 10 weeks to 6 months after operation [31]. In contrast, asanguineous perfusion involves isolating the regenerating liver from the rest of the circulation for a extended period while a retroviral vector is infused into the PV [4, 5]. The two asanguineous perfusion methods reported to date have relied on 70% hepatectomy, done 24 hr before retrovirus infusion, to induce hepatic regeneration [14–16] and thereby allow stable integration of foreign genetic material into hepatocyte chromosomal DNA [26]. Using this method and a retroviral vector carrying the βgal gene, Ferry *et al.* attained 1–5% hepatocyte transduction efficiencies, as determined by X-gal staining of liver sections obtained 21 days after transduction [4]. The advantages of this method include direct delivery of the gene of interest to the target cell and the elimination of all hepatocyte harvest and *in vitro* processing steps. However, this mode of gene delivery also requires extensive retroperitoneal dissection (to position the necessary out-flow clamps) and prolonged cannulation of the inferior vena cava and PV. Moreover, it entails significant hepatic ischemia (20 min) in an animal that has already undergone extensive liver resection 24 hr previously.

Herein, we report a novel technique of *in vivo* hepatocyte transduction in rodents. Like the asanguineous liver perfusion method, our method requires partial hepatectomy to induce hepatic regeneration. However, our in-flow occlusion operation is less physiologically demanding than complete vascular exclusion of the liver. We occlude hepatic in-flow for only a brief period (3 min) and deliver retrovirus directly into the proximal PV. Since there is no blood flowing into the liver, the hepatocytes are essentially bathed in retroviral supernatant. Theoretically, in-flow occlusion also minimizes "flow-through" and the inactivation of retroviral particles by complement proteins encountered within the hepatic sinusoids. Using our method and the RS10991 construct shown in Fig. 1, we have attained hepatocyte transduction frequencies of 0.10–1.00% and an 84% rodent survival rate. This technique will allow rapid testing of multiple retroviral constructs for purposes of assessing relative *in vivo* promoter strengths. This should facilitate the development of optimized retroviral vectors for hepatic gene therapy of inherited metabolic and hematologic disease.

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