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# Role of Hepatocyte Direct Hyperplasia in Lentivirus-Mediated Liver Transduction *In Vivo*

KAZUO OHASHI,[1] FRANK PARK,[1,2] and MARK A. KAY[1]


## ABSTRACT

**Lentiviral vectors have been used for gene transfer into the liver, but the ability of these vectors to efficiently transduce quiescent hepatocytes remains controversial. Regardless, lentivirus-mediated gene transfer is greatly enhanced when delivered during hepatocellular cycling. For this reason, the present study was designed to determine the role of hepatocyte proliferation in the enhancement of lentiviral transduction by using three different modes of liver regeneration: (1) compensatory regeneration stimulated by two-thirds partial hepatectomy, (2) direct hyperplasia after intragastric administration of the primary mitogen 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP), and (3) a combination of modes 1 and 2. Vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped lentiviral vector expressing β-galactosidase was administered to mice via the peripheral circulation after a regeneration stimulus. Gene transfer as measured by 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) staining showed 30-fold higher levels of liver transduction in groups 1 and 2 as compared with the non-liver-manipulated control group ($p < 0.005$). The combination of TCPOBOP and partial hepatectomy (group 3) resulted in an ~80-fold increase in transduction efficiency compared with the control animals. The enhanced transduction was consistent with higher levels of hepatocellular proliferation observed in animals that received both treatments compared with either single treatment alone. Importantly, the hepatocytes were the predominant cell type transduced, although transgene expression was observed in a low number of nonparenchymal cells regardless of which liver stimulus was received. Biodistribution studies confirmed that most of the gene transfer was limited to the liver and spleen. Taken together, this study suggests that disease-induced cellular proliferation in the liver will enhance the utility of this vector in treating diseases such as viral hepatitis, liver cirrhosis, and cancer.**


## OVERVIEW SUMMARY

**The present study was designed to understand whether different processes that mediate liver proliferation could enhance VSV-G-pseudotyped lentiviral vector transduction into mouse hepatocytes *in vivo*. Three different modes of liver regeneration (compensatory regeneration, direct hyperplasia, and a combination of the first two modes) were compared, and it was found that lentiviral transduction was significantly enhanced irrespective of the regeneration mode used. Transduction by the lentiviral vectors was found predominantly in hepatocytes rather than nonparenchymal cells in each mouse group. The results from this study demonstrate that liver regeneration can substantially enhance lentiviral transduction into mouse hepatocytes *in vivo*, and for this reason strongly suggest that liver disorders that involve hepatocellular proliferation, such as viral hepatitis, cirrhosis, and/or cancer, may be attractive targets for this viral vector system.**

## INTRODUCTION

LIVER-DIRECTED GENE THERAPY has great potential to reverse genetic causes of many hereditary liver diseases (Ferry *et al.*, 1991; Snyder *et al.*, 1999) and to prevent the progression of acquired liver diseases (Ueki *et al.*, 1999; Rudolph *et al.*, 2000). Of the viral vectors available for gene delivery today, retroviral vectors, such as the prototypical murine Moloney leukemia virus (Mo-MuLV)-based retroviral vectors, have been

[1]Departments of Pediatrics and Genetics, Stanford University, Stanford, CA 94305.
[2]Present address: Department of Medicine and Pharmacology, Louisiana State University Health Sciences Center, New Orleans, LA 70112.

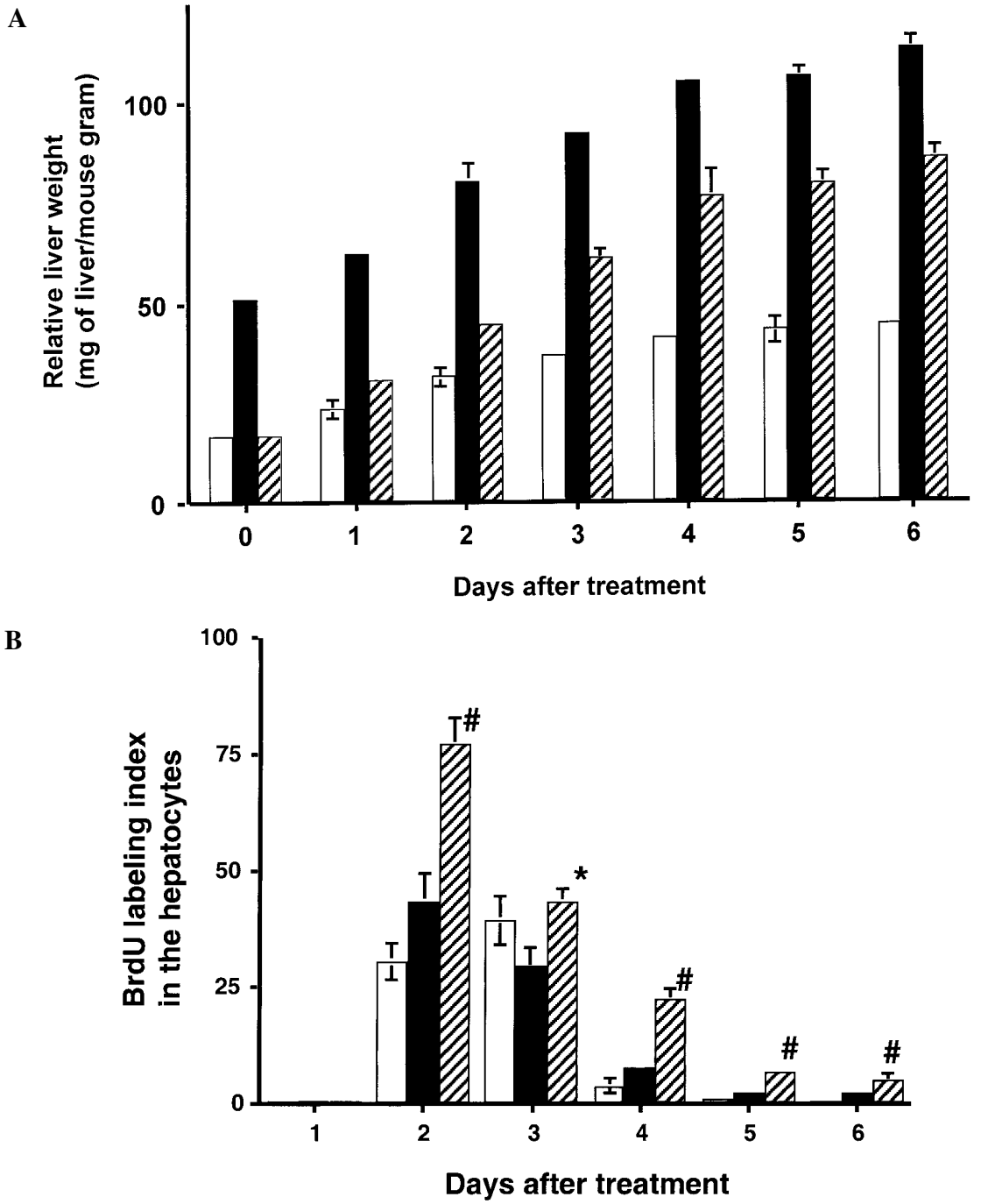


**FIG. 1.** Time course of liver regeneration. (**A**) Changes in relative liver weight; (**B** and **C**) BrdU incorporation in (**B**) hepatocytes and (**C**) nonparenchymal cells. C57BL/6 mice received a two-thirds partial hepatectomy (PH group; open columns), intragastric TCPOBOP (TCPOBOP group; solid columns), or combined TCPOBOP/PH (TCPOBOP/PH group; hatched columns) and were then killed at different time points ($n = 3$/time point). On day 0, livers were excised immediately after the TCPOBOP injection (TCPOBOP group) or after performing PH (PH and TCPOBOP/PH groups). Livers were excised to calculate the relative liver weight (milligrams of liver per gram of body weight; mean ± standard deviation) and BrdU labeling index. $^{\#}p < 0.005$ in TCPOBOP/PH group versus PH and TCPOBOP groups; $^{*}p < 0.005$ in TCPOBOP/PH versus TCPOBOP group.

widely used largely because of their safety and ability to integrate into the host cell genome, which allows for long-term, sustained gene expression (Kay *et al.*, 2001). However, Mo-MuLV vector delivery for liver-directed gene therapy is limited because nuclear membrane dissolution is an essential requirement before integration of retroviral vectors (Miller *et al.*, 1990). For this reason, numerous surgical and chemical strategies have been attempted to augment Mo-MuLV transduction

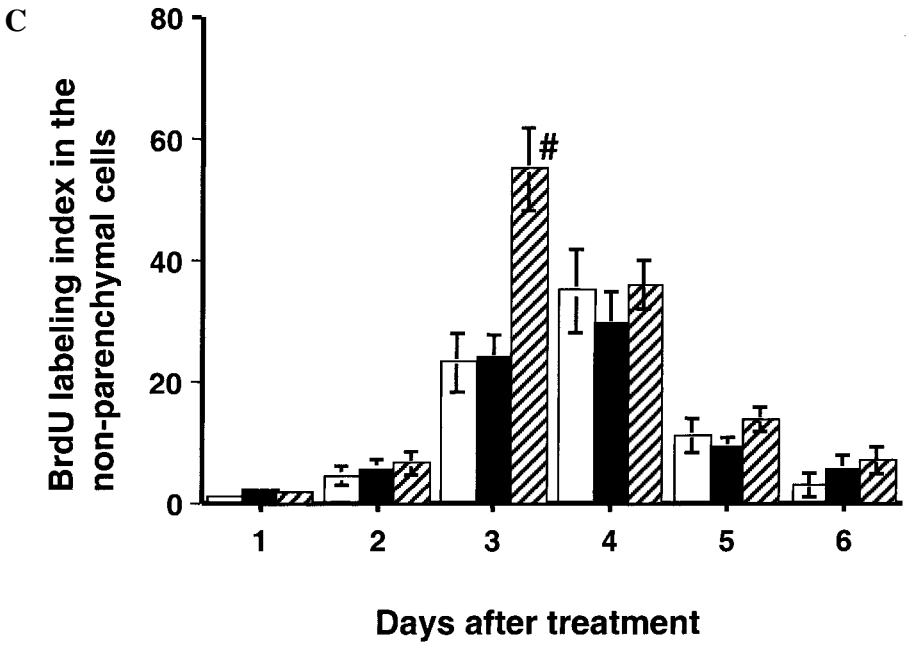


**FIG. 1.** *Continued.*

in the liver (Kay *et al.*, 1993; Bosch *et al.*, 1996; Bowling *et al.*, 1996; Ferry and Heard, 1998; Kosai *et al.*, 1998; Patijn *et al.*, 1998; Gao *et al.*, 2000).

Unlike Mo-MuLV, lentiviruses represent a class of retroviruses with the potential for infecting both cycling as well as noncycling cells (Emerman, 2000), and have been shown to stably transduce a wide variety of quiescent cells *in vitro* as well as *in vivo* (Naldini *et al.*, 1996a,b; Kafri *et al.*, 1997; Miyoshi *et al.*, 1999). Moreover, these lentiviral vectors transduced hepatocytes *in vivo* more efficiently than Mo-MuLV-based vectors (Kafri *et al.*, 1997; Park *et al.*, 2000a), leading to therapeutic production of coagulation factors VIII and IX (Park *et al.*, 2000b; Follenzi *et al.*, 2000; Park and Kay, 2001). However, it is possible that lentiviral vectors require some level of cell cycling *in vivo* to enhance transduction in cells such as hepatocytes. Our previous studies demonstrated that infusion of lentiviral vectors through the portal vein resulted in predominant transduction in cycling hepatocytes (83–91% of the transduced hepatocytes were labeled with cell cycle marker) whether or not liver regeneration was induced (Park *et al.*, 2000a). Hepatocytes were the major transduced cell type, with far fewer nonparenchymal cells transduced (Park *et al.*, 2000a). In addition, hepatocellular regeneration induced by partial hepatectomy significantly increased lentiviral integration and transgene expression *in vivo* (Park *et al.*, 2000a,b). However, the importance of cell cycle progression for efficient lentiviral transduction into cells of the liver remains a controversial issue. A study by Pfeifer *et al.* (2001) reported efficient transduction by lentiviral vectors into livers from naive mice, although the majority of the transduced cells (>78%) were nonhepatocytes. Although the reasons for the discrepancy are not clearly understood, there were differences in the vector and promoters used to drive the expression cassette that could have influenced which cell types expressed the transgene product (Park and Kay, 2001).

Regardless of the importance of cell cycle progression, our previous studies clearly demonstrate the importance of liver regeneration in promoting efficient lentiviral vector transduction to the liver. It is known that there are at least two distinct mechanisms for liver regeneration, specifically compensatory regeneration and direct hyperplasia (Columbano and Shinozuka, 1996). For this reason, the present study was designed to determine which regeneration mode (compensatory regeneration, direct hyperplasia, or a combination of the two proliferative modes) would promote optimal lentiviral vector transduction in mouse hepatocytes *in vivo*.

## MATERIALS AND METHODS

### *Plasmids*

The packaging construct pCMVΔR8.74, and the envelope plasmid pMD.G, are as previously described (Naldini *et al.*, 1996a; Dull *et al.*, 1998). pCMVΔR8.74 is a packaging construct from which four of the accessory genes involved in human immunodeficiency virus (HIV) replication have been deleted: *vif, vpr, vpu,* and *nef*; however, it carries active *rev* and *tat* genes. The transfer plasmid pHR2PGKNLSLacZ encodes nucleus-localized LacZ driven by the mouse phosphoglycerate kinase 1 (*Pgk-1*) promoter as previously described (Park *et al.*, 2000a). This transfer plasmid does not contain the central polypurine tract sequence of HIV.

### *Viral production and titering*

Concentrated lentiviral vectors were produced as previously described (Park *et al.*, 2000a). Viral stocks were titered by serial dilution in the presence of Polybrene (8 $\mu$g/ml) in six-well plates, using $5 \times 10^5$ HeLa cells. The HIV $p24^{gag}$ antigen con-

centration was determined by enzyme-linked immunosorbent assay (ELISA) (Alliance; Du Pont-NEN, Boston, MA). The titer of the vector stocks used in this experiment was $2 \times 10^9$ transducing units (TU)/ml and was $7 \times 10^6$ TU/$\mu$g of $p24^{gag}$ antigen. The absence of replication-competent lentivirus in the vector batch was confirmed by monitoring p24 antigen expression in the culture medium of transduced SupT1 lymphocytes for 3 weeks (detection limit, 3 pg/ml).

### *Animals*

All animal studies were performed in accordance with the institutional guidelines set forth by the Stanford University Animal Care Committee and National Institutes of Health guidelines. In all studies performed, female C57BL/6 (8 weeks old; Taconic Farms, Germantown, NY) were used. Mice were placed in cages within a temperature-controlled room with a 12-hr light–dark cycle and *ad libitum* access to food and water.

### *Methods of hepatocellular proliferation*

Three different methods were used to induce hepatocyte proliferation: (1) PH group—compensatory regeneration was induced by performing a two-thirds partial hepatectomy by surgically removing the median and lateral lobes (Columbano *et al.*, 1997; Park *et al.*, 2000a) under general anesthesia (methoxyflurane [Metafane]; Mallinkrodt, IL); (2) TCPOBOP group—direct hyperplasia was induced by a single intragastric injection of the primary mitogen 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP; kindly provided by B. Diwan, National Cancer Institute, Bethesda, MD) at 3 mg/kg body weight; and (3) TCPOBOP/PH group—combined mode of hepatocyte proliferation (compensatory regeneration and direct hyperplasia) was induced by a single injection of TCPOBOP (3 mg/kg) followed by PH performed 6 hr after TCPOBOP injection.

The cell cycle status of the mouse hepatocytes in the three groups was assessed by administering 5-bromo-2-deoxyuridine (BrdU) (Sigma; St Louis, MO) by an osmotic minipump (model 2001; Alzet, Palo Alto, CA) placed in the subcutaneous space 24 hr before sacrifice. BrdU was administered at a dose of 1 mg/day, and three mice were killed daily in each of the mouse treatment groups during the 6-day study. Mouse livers, kidneys, and spleens were immediately weighed and fixed in a 10% formalin solution for immunohistochemical analyses. Relative liver, kidney, and spleen weights in this study were expressed as tissue weight-to-body weight ratio (milligrams per gram mouse weight).

### *Vector administration*

Lentiviral vectors were injected through the tail vein in 0.4 ml of phosphate-buffered saline (PBS), using a 27-gauge syringe needle. Six separate injections were performed (equal number of lentiviral transducing units in each injection) 12 hr apart, commencing 24 hr after the administration of TCPOBOP (TCPOBOP and TCPOBOP/PH group) or after two-thirds partial hepatectomy (PH group) (see Fig. 3A). Tissues were analyzed for the lentiviral transduction 84 hr after the final vector injection.

### *Histological analyses*

Cell proliferation status was assessed by producing formalin-fixed, paraffin-embedded liver sections as previously described (Ohashi *et al.*, 1996a). Briefly, 5-$\mu$m sections were deparaffinized and incubated with mouse anti-BrdU monoclonal antibody (Becton Dickinson, San Jose, CA) at a dilution of 1:200 for 2 hr at room temperature followed by administration of avidin–biotin–peroxidase complex (Hsu *et al.*, 1981). Sections were lightly counterstained with hematoxylin. Cells were considered positive for BrdU when clear brown staining of the nucleus could be identified. In each mouse, the BrdU labeling indexes (LI) of hepatocytes and nonparenchymal cells were determined separately by counting a total of 2000 hepatocytes in 20 randomly selected liver fields and expressed as a percentage of all positive nuclei. The duodenum was used as a positive control tissue for this experiment.

To analyze the biodistribution of lentiviral vector transduction, liver, spleen, brain, heart, lung, kidney, duodenum, and skeletal muscle samples were isolated for $\beta$-galactosidase ($\beta$-Gal) expression, using snap-frozen tissue in Tissue Tek O.C.T. compound (Miles, Naperville, IL). Ten-micrometer sections were fixed with 0.5% glutaraldehyde and stained overnight for $\beta$-Gal, using 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactoside (X-Gal; Fisher Scientific, Pittsburgh, PA). Sections were lightly counterstained with hematoxylin and X-Gal-positive nuclei were determined by counting at least 2000 hepatocytes in 20 randomly selected liver fields. The morphological structure of liver cells was determined as described by MacSween *et al.* (1994), in which hepatocytes were histologically differentiated from nonparenchymal cells on the basis of size (normally 30–40 $\mu$m), shape of the nucleus (round), and general morphology (cells forming liver cell plates).

### *Alanine aminotransferase assay*

As a marker for hepatic injury, serum alanine aminotransferase (ALT) levels were determined by using a colorimetric diagnostic kit (Sigma), as described previously (Patijn *et al.*, 1998; Park *et al.*, 2000a,b).

### *Statistical analysis*

With the use of StatView 5.0 software (SAS Institute, Cary, NC), one-way analysis of variance (ANOVA) was used for statistical analysis of significance. If a probability value of $p < 0.05$ was obtained, the Tukey test was then used for comparison for each group with the appropriate control.

## RESULTS

### *Hepatocellular proliferation during regeneration*

The changes in liver mass relative to body weight, otherwise known as the relative liver weight (RLW), and BrdU labeling index (LI) measured as a function of time after the initiation of liver regeneration are shown in Fig. 1. Initial RLWs measured immediately after the initiation of liver regeneration were $50.3 \pm 1.5$, $16.7 \pm 1.0$, and $16.8 \pm 1.1$ mg/g body weight in the TCPOBOP, PH, and TCPOBOP/PH groups, respectively. In the partially hepatectomized compensatory regeneration

group (PH group), the RLW rapidly reached ~90% (44.6 ± 1.7 mg/g body weight) of the initial RLW by day 6 (Fig. 1A). This represented a 167 ± 9.7% increase in liver weight compared with the time of the partial hepatectomy (16.7 to 44.6 mg/g body weight). In comparison, the TCPOBOP-induced direct hyperplasia group (TCPOBOP group) had a 127 ± 5.0% increase in RLW between days 0 and 6, reaching a final weight of 114 ± 2.8 mg ($p < 0.001$ vs. initial RLW). In the mouse group in which we induced both modes of liver regeneration (TCPOBOP/PH group), the RLW reached 86.1 ± 2.7 mg/g body weight by day 6, or an increase of 413 ± 16.2% (16.8 to 86.1 mg/g body weight; Fig. 1A). Relative spleen and kidney weights were not different among the groups (data not shown). The gross appearance of the livers in the TCPOBOP and TCPOBOP/PH groups was markedly enlarged compared with the nontreated and PH groups, respectively (Fig. 2).

To determine the time course of hepatocyte and nonparenchymal (notably Kupffer, endothelial, and stellate) cell proliferation, the number of cell nuclei that entered the S phase of the cell cycle was determined by BrdU labeling (Fig. 1B and C). With respect to hepatocyte proliferation, the BrdU labeling index (LI) during the first 24-hr period after the initiation of the liver regeneration in all mouse groups was low (0.12 to 0.29%). In the PH group, the BrdU LI peaked at 39.4% between 48 and 72 hr and then decreased over time. In comparison, the TCPOBOP group had its LI peak between 24 and 48 hr, reaching 43.3%, which was slightly higher than the peak value observed in the PH group. The peak LI in the TCPOBOP/PH group was significantly higher than that of the other two groups, reaching 77.3% by 24 to 48 hr after treatment ($p < 0.005$). The temporal rise in cellular proliferation of nonparenchymal cells in each group lagged ~24 hr compared with hepatocyte proliferation, because the nonparenchymal cells were found to be actively proliferating between 48 and 96 hr.

### *Lentiviral transduction of mouse liver after hepatocellular proliferation*

Single high doses of lentiviral vectors administered via the portal vein resulted in transient hepatocellular injury lasting 1 day after vector infusion (Park *et al.*, 2000a,b; Park and Kay, 2001). To circumvent this problem, we attempted to deliver a therapeutic dose of lentivirus by dividing the dose over multiple injections via the tail vein. Six injections were performed 12 hr apart at a dose of $1 \times 10^7$ TU/injection (total of $6 \times 10^7$ TU/mouse) or $3.3 \times 10^7$ TU/injection (total of $2 \times 10^8$ TU/mouse) (Fig. 3A). In both groups of animals, no hepatic injury as determined by the serum ALT was observed (Table 1). However, at the doses administered, transduction into the hepatocytes was observed in 0.1 ± 0.03 and 0.23 ± 0.03% of the hepatocytes in the lower and higher dose groups, respectively (Fig. 3B and Fig. 4A and B).

Because of the low level of lentiviral transduction observed in the hepatocytes *in vivo*, we promoted liver regeneration by three different modes of treatment (compensatory regeneration, direct hyperplasia, and a combination of the first two modes) before the administration of the vectors in an attempt to increase transduction efficiencies *in vivo*. Previous studies in our laboratory have used compensatory regeneration to increase lentivirus-mediated gene transfer (Park *et al.*, 2000a,b). Because

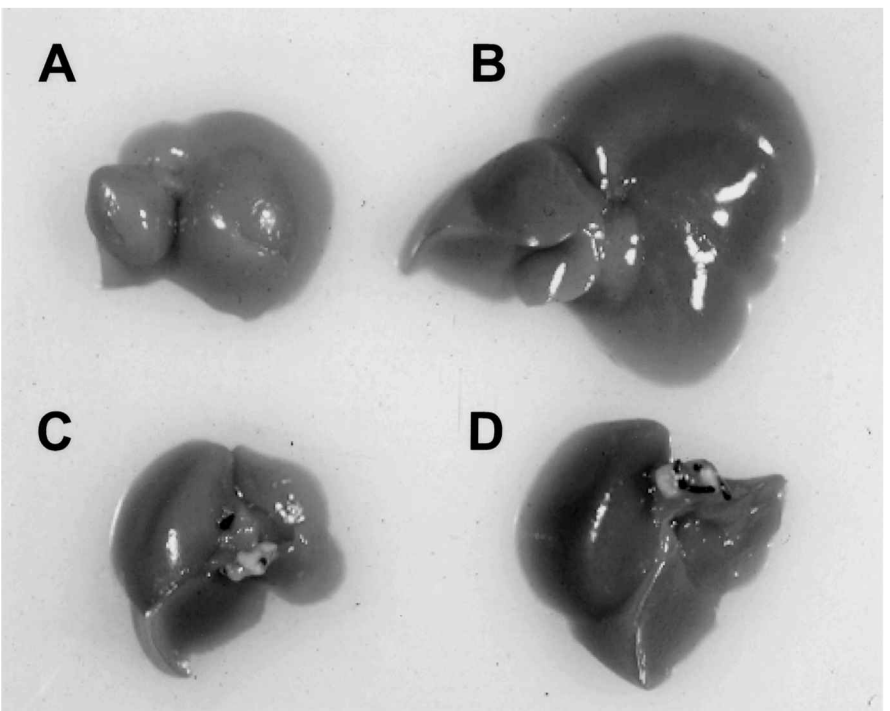


**FIG. 2.** Representative gross appearance of the mouse livers excised on day 6 after the induction of different modes of liver regeneration. (**A**) Control mouse (liver unmanipulated); (**B**) TCPOBOP group; (**C**) PH group; (**D**) combined (TCPOBOP/PH) group. Note enlarged liver mass in TCPOBOP and TCPOBOP/PH groups as compared with their counterparts to their left.

our initial experiment demonstrated that proliferation activities of hepatocytes and nonparenchymal cells were more active between 24 and 96 hr after the initiation of liver regeneration (Fig. 1), six injections (12 hr apart) of lentiviral vector were administered into the tail vein, commencing 24 hr after initiation of liver regeneration (Fig. 3A). Infusion of $6 \times 10^7$ TU resulted in $\beta$-galactosidase expression in $3.0 \pm 0.4$ and $2.9 \pm 0.3\%$ of the hepatocytes in the PH and TCPOBOP groups, respectively (Fig. 4A). In the combined TCPOBOP/PH group, lentiviral transduction was significantly higher, with $7.6 \pm 0.9\%$ of the hepatocytes being X-Gal positive ($p < 0.005$ vs. liver-unmanipulated, PH, and TCPOBOP groups).

Increasing the dose of the lentivirus to $2 \times 10^8$ TU increased the transduction efficiency in mouse hepatocytes in each of the liver regeneration groups tested compared with the lower dose groups (Fig. 4B). In the groups for which only one proliferative stimulus was performed (PH or TCPOBOP administration), the transduction efficiencies were $7.1 \pm 1.0$ and $6.5 \pm 0.9\%$, respectively. In the combined TCPOBOP/PH group, the number of X-Gal-positive hepatocytes reached $17.3 \pm 2.8\%$ (Fig. 4B). On the other hand, the transduction efficiencies of nonparenchymal cells in each of the proliferative modes were significantly lower as compared with hepatocytes, resulting in $0.07 \pm 0.05$, $1.0 \pm 0.4$, $1.2 \pm 0.3$, and $2.0 \pm 1.0\%$ in naive (control) liver, PH, TCPOBOP, and combined TCPOBOP/PH groups, respectively (Fig. 4C). Because the liver is normally composed of 65% hepatocytes and 35% nonparenchymal cells, these results showed that transduction occurred predominantly (>85%) in hepatocytes in all the groups, including the naive (control) liver group. To confirm that liver regeneration was not affected by vector administration, the livers were excised and weighed. We found that the increase in RLW in each vector treatment group was similar to the control, non-vector-treated livers (data not shown). Moreover, the excised livers showed relatively normal histology with no observable liver injury.

### *Biodistribution of lentiviral transduction after intravenous administration*

Intravenous infusion of lentiviral vectors expressing $\beta$-Gal resulted in transduction into few splenic cells, but the cell type(s) were not identified (Fig. 3F). There was no detectable X-Gal staining in the other extrahepatic tissues examined, including the brain, lung, heart, kidney, duodenum, and skeletal muscle in any of the experimental groups (Fig. 3G–I).

## DISCUSSION

The present study demonstrated that lentiviral transduction was significantly enhanced (~30-fold) when liver regeneration was activated by either compensatory regeneration (i.e., two-thirds partial hepatectomy) or direct hyperplasia (i.e., single intragastric injection of TCPOBOP) compared with a naive (control) liver group. Interestingly, the combination of compensatory regeneration with direct hyperplasia further augmented (~80-fold) lentivirus-mediated transduction in mouse hepatocytes *in vivo*. Moreover, transduction occurred predominantly in hepatocytes, and less frequently in nonparenchymal cells.

### *Different modes of liver regeneration: effect on lentiviral transduction* in vivo

Liver regeneration is known to be induced through two distinct pathways: (1) compensatory regeneration and (2) direct hyperplasia (Columbano and Shinozuka, 1996; Michalopoulos and DeFrances, 1997; Ledda-Columbano *et al.*, 1998). Studies have elucidated that each mode of liver regeneration has a different molecular mechanism(s) involved to promote its function. For compensatory liver regeneration, induced by partial hepatectomy or carbon tetrachloride administration, enhanced expression of immediate-early genes, such as c-*fos*, c-*jun*, *LRF-1*, and c-*myc* in conjunction with the activation of other transcription factors such as AP-1 or NF-$\kappa$B has been observed (Thompson *et al.*, 1986; Cressman *et al.*, 1994; Michalopoulos and DeFrances, 1997). Studies have shown that cytokines, such as tumor necrosis factor $\alpha$ and interleukin 6 (IL-6), which are responsible for NF-$\kappa$B and STAT3 (signal transducer and activator of transcription 3) activation, respectively, were critical factors in mediating compensatory regeneration (Cressman *et al.*, 1996; Yamada *et al.*, 1997; Taub *et al.*, 1999). In contrast, direct hyperplasia produces liver regeneration through a unique mechanism, which is distinct from compensatory liver regeneration, because none of the factors mentioned above have been observed to accumulate in this model of regeneration (Columbano *et al.*, 1997; Ledda-Columbano *et al.*, 1998; Patijn *et al.*, 1998). The detailed mechanisms involved in TCPOBOP-induced direct hyperplasia of the liver has not been fully elucidated, but a study has shown that TCPOBOP is a direct agonist of the nuclear receptor, constitutive androstane receptor (CAR), whereby the function of CAR has been implicated as a mediator of TCPOBOP-induced liver regeneration (Tzameli *et al.*, 2000; Wei *et al.*, 2000). Herein, we have shown that hepatocellular regeneration can be significantly enhanced by combining these two modes of liver regeneration. In fact, the two modes of liver regeneration were additive because the liver mass and BrdU labeling were higher compared with either liver regeneration method performed alone.

The similarity in lentivirus-mediated gene transfer despite different mechanisms of hepatocellular regeneration is an important finding because it demonstrates that the vector may be useful for the treatment of liver diseases in which different pathological conditions activate hepatic regenerative processes. For example, chronic hepatitis or liver cirrhosis resulting from hepatitis virus infection would be one of the targeted liver diseases for which lentiviral vectors may be applied. In hepatitis viral infection, cells undergo compensatory regeneration because of the high incidence of virus-mediated cell death (Ohashi *et al.*, 1996b). It has been demonstrated that, on average, hepatocytes from individuals infected with hepatitis virus undergo 40 times more cell divisions compared with those from noninfected individuals (Ohashi *et al.*, 1996b). Hepatocytes with preneoplastic (i.e., hyperplastic lesions in the liver) and/or neoplastic phenotypic alterations, which occur during the course of chronic infection with hepatitis virus, would more likely be in cell cycle, making them good targets for antiviral or tumor-based gene therapy. In clinical trials, once the proliferative status of the liver is known through biopsy specimens, it may be possible to calculate the approximate amount of lentivirus that

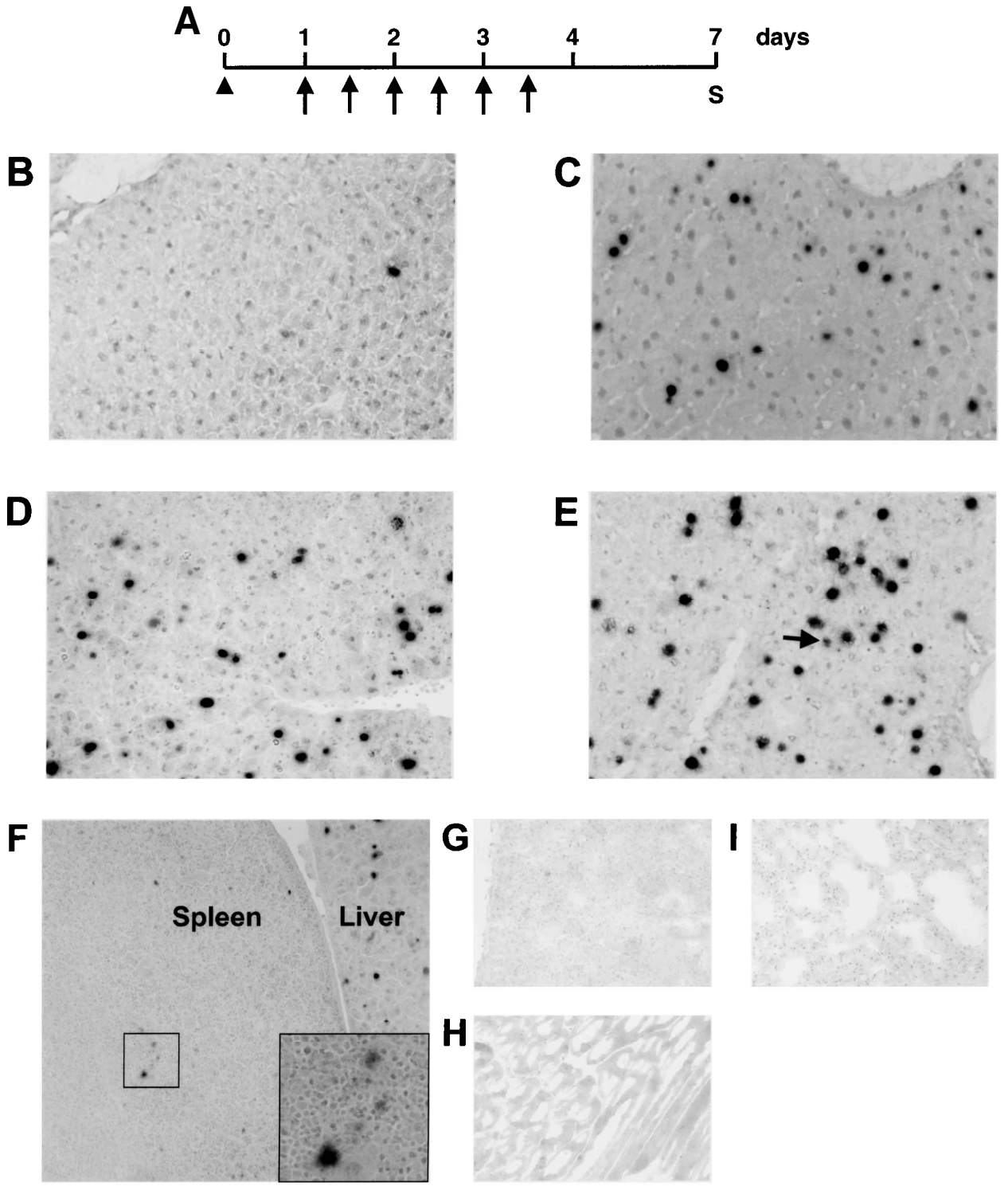


**FIG. 3.** Lentiviral transduction in the mouse liver and other organs after hepatocellular proliferation. (**A**) Schematic of experimental protocol. Arrowhead, initiation of liver regeneration (PH, TCPOBOP, TCPOBOP followed by PH at 6 hr or nontreated); arrows, tail vein injection of lentiviral vector; S, sacrifice. (**B–I**) Representative photomicrographs from X-Gal-stained liver sections (**B–E**) and other organs (**F–I**) in animals receiving $3.3 \times 10^7$ TU in each injection (a total of $2 \times 10^8$ TU) of lentivirus. Sections were lightly counterstained with hematoxylin. (**B**) Naive liver (control) group; (**C**) PH group; (**D**) TCPOBOP group; (**E**) combined (TCPOBOP/PH) group. Note predominant transduction in the hepatocytes and infrequent transduction in the nonparenchymal cells (arrow). (**F**) Spleen; (**G**) kidney; (**H**) skeletal muscle; and (**I**) lung in the TCPOBOP/PH group. Original magnification: (**F**) ×100 (inset, ×400); (**B–E** and **G–I**) ×200.

Table 1. Alanine Aminotransferase Activity in Mice Receiving Multiple Infusions of Lentiviral Vectors[a]

| *Vector dose infused (TU)* | *No. of mice* | *Alanine aminotransferase activity (IU)* | |
|---|---|---|---|
| | | *Day 1* | *Day 3* |
| $6 \times 10^7$ | 3 | 60.7 ± 5.8 | 68.7 ± 7.4 |
| $2 \times 10^8$ | 3 | 68.0 ± 6.2 | 62.3 ± 6.5 |
| Control (PBS) | 3 | 68.3 ± 7.4 | 67.3 ± 7.1 |

[a]Three different groups of mice ($n = 3$ for each group) received six separate infusions, 12 hr apart, at two different doses of lentiviral vector expressing $\beta$-galactosidase (equal amount of vector was injected during each infusion) through the tail vein. Serum analysis was performed on days 1 and 3 after the completion of vector infusion. Values represent means ± SD.

would be needed for efficient transduction without producing significant amounts of liver injury.

Because administration of TCPOBOP consistently induced liver regeneration in each mouse studied, we believe that TCPOBOP can be a practical chemical for use in gene transfer studies related to liver regeneration in mice. However, because the activity of TCPOBOP is species specific (i.e., active in mice but not in rats), and because TCPOBOP is categorized as a carcinogen (Diwan *et al.*, 1992), the practical application(s) of this chemical agent would not be for clinical use, but more for unraveling basic biological questions.

### *Cellular transduction in the liver*

Our results demonstrated that after intravenous infusion of lentivirus, the hepatocyte was the predominant cell type (>85% of the cells being transduced) transduced in the liver. This finding was consistent regardless of whether vectors were infused into quiescent or regenerating cells (Fig. 4B and C). This finding is also consistent with our previous report, and other reports, in which lentiviruses were infused into the portal circulation (Park *et al.*, 2000a) or into the liver by intraparenchymal needle injection (Kafri *et al.*, 1997). Because nonparenchymal cells, including Kupffer cells, are known to play important roles in immunological networks, the predominant transduction of hepatocytes and infrequent transduction into nonparenchymal cells offer advantages for achieving gene therapy-based applications in the liver. However, one study by Pfeifer *et al.* (2001) reported that predominant transduction occurred in nonparenchymal cells, with infrequent transduction into hepatocytes (>78% of the cells being transduced were nonparenchymal cells). No biological stimulus was implemented in their study to activate liver proliferation. At present, the reason for the discrepancy between these results and ours is unclear, but it may be related to the difference in lentiviral constructs, such as the incorporation of *cis*-acting DNA elements like the central polypurine tract (cPPT; Park and Kay, 2001) or their use of the cytomegalovirus (CMV) promoter, which is relatively inactive in hepatocytes *in vivo* (Kay *et al.*, 1992a,b, 1993; Snyder *et al.*, 1997). Further studies will be needed to address these issues.

### *Biodistribution of the vector*

An optimal gene therapy vector should be tissue specific regardless of the route of administration. In the present study, we determined the biodistribution profiles of lentiviral vectors after intravenous administration. We found predominant transduction within the liver and, to a lesser extent, the spleen, which was consistent with our previous study (Park *et al.*, 2000a). Even though we used the ubiquitous *Pgk-1* promoter (McBurney *et al.*, 1994), no lentivirus-mediated transgene expression was observed in any of the other organs examined for all of the experimental groups. No episomal forms of lentivirus were detected in the brain by Southern blot analysis (Park *et al.*, 2000a). In addition, we have found that direct injection into the brain, skeletal muscle, and kidney resulted in the expression of $\beta$-galactosidase with the *Pgk-1* promoter (our unpublished observations). From this information, we speculate that the lentiviral particles delivered through the vascular system may not effectively penetrate the endothelial cell layer, resulting in its inability to transduce other organs.

Regardless, the transduction of splenic cells by the lentiviral vectors may be problematic because this could result in antigen presentation and produce immune responses against the transgene product. The potential immunological problems could be avoided by using hepatocyte-specific promoters. In the case of hepatic delivery of adenovirus, a vector known to transduce splenic and nonparenchymal liver cells in addition to hepatocytes, an immune response against a secreted transgene protein was avoided with the use of a hepatocyte-specific promoter (Pastore *et al.*, 1999). Our present study utilized a ubiquitous promoter (*Pgk-1*), which is relatively weak compared with other ubiquitous promoters, such as EF1$\alpha$ (Park *et al.*, 2000b). For these reasons, development of liver-specific promoters with various *cis*-acting regulatory sequences, such as human $\alpha_1$-antitrypsin promoter with the apolipoprotein E enhancer and hepatocyte control region (Miao *et al.*, 2000), into lentiviral constructs may allow for higher levels of gene expression *in vivo*.

### *Liver toxicity profile*

Safety profiles are of concern in the application of newly developed viral vectors. In our earlier observations, a single intraportal injection of lentiviral vectors induced dose-dependent liver injuries (Park *et al.*, 2000a,b; Park and Kay, 2001). In the initial study, $2 \times 10^8$ TU of the lentiviral vector infused as a bolus via the portal vein resulted in serum ALT levels of about 200 IU on day 1, but lower doses did not produce any significant changes compared with control saline-infused mice. On

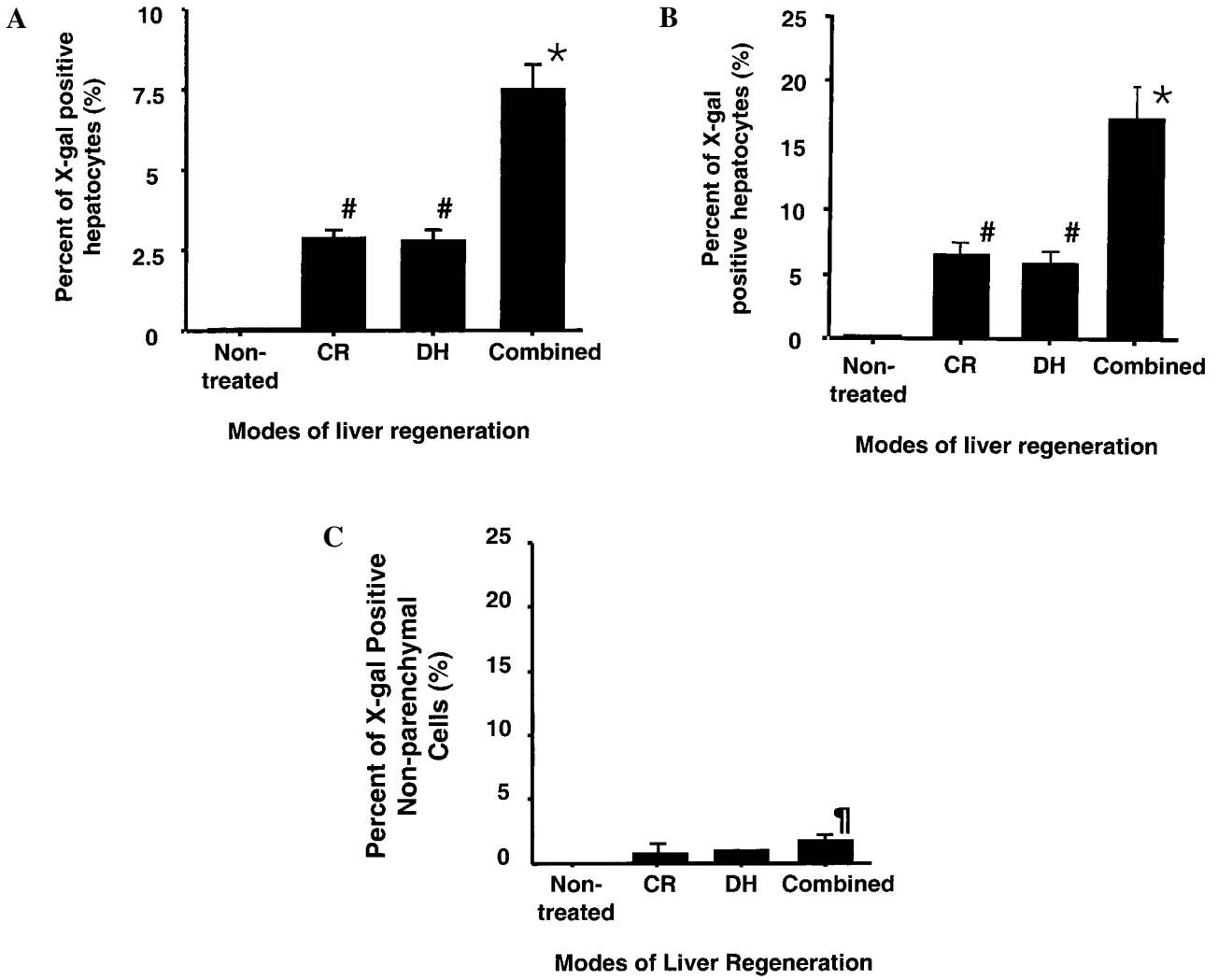


**FIG. 4.** Lentivirus-mediated transduction efficiency in mouse liver after hepatocellular proliferation. Transduction efficiencies were determined by scoring X-Gal-positive cells as a percentage of the total number of cells counted. (**A** and **B**) Transduction efficiency in hepatocytes from mice treated with a total of (**A**) $6 \times 10^7$ TU or (**B**) $2 \times 10^8$ TU of vector. (**C**) Gene transduction efficiency in nonparenchymal cells from mice treated with a total of $2 \times 10^8$ TU of vector. Values are shown as means ± standard deviation, $n = 3$ or 4 mice per group. $^*p < 0.005$ in combined (TCPOBOP/PH) group versus naive, nontreated liver group (control), compensatory regeneration (CR, equivalent to PH) group, and direct hyperplasia (DH, equivalent to TCPOBOP) group. $^{\#}p < 0.001$ in PH and TCPOBOP groups versus naive liver (control) group. $^{\P}p < 0.001$ in combined (TCPOBOP/PH) group versus naive, nontreated liver (control) group.

the other hand, the present study demonstrates that multiple, lower dose infusions into the general circulation minimized liver injury even at a total dose of $2 \times 10^8$ TU, while maintaining its ability to efficiently transfer genes. It is likely that the split doses of vector infusion administered through the general circulation diluted the vector or contaminants, which led to the minimal vector-related injury observed in the present study. Moreover, there may be vector preparation heterogeneity in terms of contaminants, which could lead to various levels of hepatic injury leading to different transduction efficiencies by the lentiviral vectors.

From this study, we conclude that liver regeneration significantly enhanced lentiviral transduction of mouse livers *in vivo*. Because the present study demonstrated the dependence of lentiviral transduction on hepatocyte cell cycling, future clinical applications with these vectors may be especially useful in liver disease states in which active proliferation plays a prominent role, such as viral hepatitis, liver cirrhosis, and cancer.

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Address reprint requests to:
*Mark A. Kay, M.D., Ph.D.*
*Departments of Pediatrics and Genetics, Stanford University*
*300 Pasteur Dr., Room G305*
*Stanford, CA 94305*

*E-mail:* markay@leland.stanford.edu

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