doi:10.1016/S1525-0016(03)00169-2

The Effect of Age on Hepatic Gene Transfer with Self-Inactivating Lentiviral Vectors *in Vivo*

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It is known that cellular proliferation, by either compensatory regeneration or direct hyperplasia, can augment lentiviral vector transduction into hepatocytes *in vivo*. For this reason, the present study was designed to determine if adolescent mice (3½ weeks of age), which still have relatively proliferating livers, would have differential transduction compared to older (7 weeks of age) mice. Self-inactivating lentiviral vectors containing the human α_1-antitrypsin (hAAT) promoter driving the expression of either the bacterial lacZ gene or the hAAT cDNA were generated for these studies. We found that adolescent mice given lentiviral vectors expressing lacZ (50 μg p24/mouse) via intravenous administration had a significantly higher level of hepatocyte transduction as measured by X-gal staining of liver sections compared to the 7-week-old mice. In addition, serum hAAT levels were nearly 40-fold higher in 3½-week-old mice administered lentiviral vectors expressing hAAT (50 μg p24/mouse) compared to the 7-week-old mice. Moreover, the incorporation of a matrix attachment region from immunoglobulin κ significantly increased transduction of hepatocytes *in vivo*. Although there was a small reduction in the circulating levels of hAAT, likely due to an immune response against the transgene product, gene expression was sustained for the duration of the study (30 weeks in total). In conclusion, the present study strongly demonstrates that lentiviral vector transduction efficiency and transgene expression were significantly enhanced in adolescent compared to older mice.

Key Words: proliferation, immune system, matrix attachment region, lacZ, human α_1-antitrypsin

INTRODUCTION

Lentiviral vectors (LVs) have promising potential for gene therapy applications to correct inherited genetic diseases and pathological disorders in the liver [1]. One of its main advantages has been its ability to integrate into the host chromosome in the absence of cell division *in vitro* [2]. This is an important property that can be potentially exploited when applying these LVs for *in vivo* clinical applications, particularly due to the fact that nearly all of the cells in the adult mammalian system are differentiated and not actively dividing. For this reason, intensive research has been recently performed to determine the utility of this lentiviral vector system in a variety of differentiated cells *in vivo,* such as the neurons in the brain [3,4], myocytes in the skeletal muscle [5], retinal cells in the eye [6], and hepatocytes in the liver [7–12]. Although many investigators have demonstrated transduction of lentiviral vectors into differentiated tissues *in vivo,* the cell cycle dependence of the cells during LV infection has remained a controversial issue. At present, the only *in vivo* organ that has been studied to address this important issue has been the liver. Our initial studies [9] demonstrated that second-generation LVs had a high dependency for cell cycle progression during the time of lentiviral transduction. The importance of cell cycle progression for efficient LV transduction into early progenitor hematopoietic stem cells has also been suggested [13]. More recent studies by several investigators demonstrated that the incorporation of *cis*-acting DNA elements, such as a DNA flap sequence known as the central polypurine tract sequence (cppt)[1] found in the pol gene of HIV, could significantly increase viral titer and vector

[1] Abbreviations used: cppt, central polypurine tract sequence; VSV-G, vesicular stomatitis virus G protein; hAAT, human α_1-antitrypsin; BrdU, 5′-bromo-2′-deoxyuridine; Igκ, immunoglobulin κ; MAR, matrix attachment region; MLV, Moloney murine leukemia virus.

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1525-0016/03 $30.00

doi:10.1016/S1525-0016(03)00169-2

integration *in vitro* [8,14,15]. We also [8] demonstrated that the requirement for hepatocytes to proceed through the cell cycle in order for lentiviral vectors to integrate into the host genome could be reduced by 50% following the insertion of the cppt into the transfer vector *in vivo*.

Lentiviral-mediated hepatic gene transfer was enhanced regardless of whether proliferation was induced by compensatory regeneration [8–11] or direct hyperplasia [7] prior to the intravenous administration of lentiviral vector particles *in vivo*. Because previous studies have strongly implicated an important role in hepatocellular proliferation prior to LV administration, we hypothesized that young animals, which are in the prematuration phase of their development, would more efficiently transduce LVs compared to their adult animal counterparts.

In addition, host immune responses directed against the transgene products expressed from LVs can result in the loss of expression of circulating levels of protein [10,11]. Studies using first-generation adenoviral vectors have shown that liver-specific promoters, instead of ubiquitous cellular promoters, can alleviate the activation of the host immune system, suggesting that the host response is related to promiscuous transduction of robust antigen-presenting cells [16]. To attempt to avoid gene expression from nonparenchymal cells, for all of the studies described, we used vectors that contained a liver-specific promoter. The present study, therefore, was designed to determine whether enhanced hepatocellular transduction and transgene expression could be achieved in adolescent versus older mice following the intravenous administration of VSV-G-pseudotyped lentiviral vectors containing a liver-specific promoter.

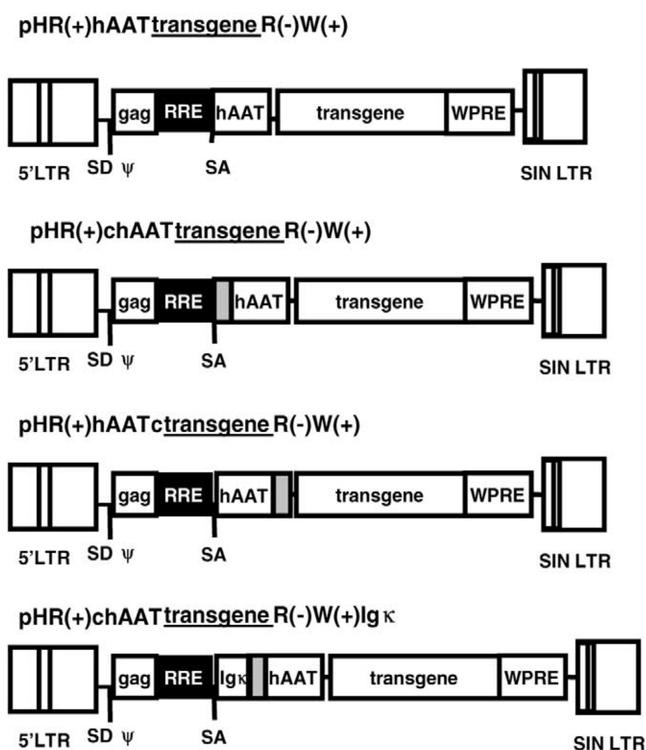

FIG. 1. Schematic of the integrating transfer plasmids for the lentiviral vectors. The transfer plasmids had a 3′ LTR deletion to create a self-inactivating vector. Each of the lentiviral vector constructs contained the Rev-responsive element (RRE) and the woodchuck postregulatory element (WPRE). The central polypurine tract sequence from the HIV *pol* gene (shown as a gray box) was placed either 5′ of the human α_1-antitrypsin promoter (denoted hAAT) or in between the hAAT promoter and the transgene of interest (denoted transgene), which was either sollacZ or hAAT cDNA. The immunoglobulin κ matrix attachment region (denoted Igκ) was placed into the vector 5′ of the cppt.

RESULTS

Effect of Intravenous Infusion of LVs on Mouse Body Weight

We weighed the C57Bl/6 mice prior to tail vein administration of LVs expressing hAAT. We treated all mice with the same vector lot. At the age of 3½ weeks, the weight of the mice averaged 11.0 ± 0.4 (n = 6) and 11.3 ± 0.5 g (n = 6) prior to the administration of LVs expressing hAAT with or without an Igκ MAR, respectively. The mouse weights were not significantly different in the PBS-infused control (11.8 ± 0.1 g; n = 3) groups compared to the experimental vector-infused groups. The average weight in each of the mouse groups increased over the next 3½ weeks to 19.4 ± 0.3 (hAAT vector alone), 19.1 ± 0.5 (hAAT vector with Igκ MAR), and 19.4 ± 0.6 g (PBS-infused control). The weights of the 3½-week-old vector-infused mice at week 7 were not statistically different from those of their age-matched littermate naïve mice. The mouse weights for the groups were as follows: 20.3 ± 0.3 (hAAT vector alone), 20.4 ± 0.6 (hAAT vector with Igκ MAR), and 19.4 ± 0.8 g (PBS-infused control). The similarity in body weight between the different mouse groups and their naïve controls demonstrated that lentiviral administration had no apparent adverse effects on total body growth characteristics of the mice.

From a separate group of mice, we isolated livers at 3½ and 7 weeks of age and determined their wet weights. The mouse livers at 3½ weeks of age weighed about 0.52 ± 0.13 g (n = 4; whole body weight 12.3 ± 0.13 g), while mouse livers at 7 weeks of age weighed 1.09 ± 0.05 g (n = 4; whole body weight 19.9 ± 0.32 g). From these mouse groups, it appears that wet liver weights can be correlated to whole body weight of the individual mice.

Transgene Expression *in Vivo*

We injected LVs expressing human α_1-antitrypsin cDNA (Fig. 1) into C57Bl/6 mice at different ages. It is important to note that all of the mice were from the same litter and that the same LV batch was injected into the mice at

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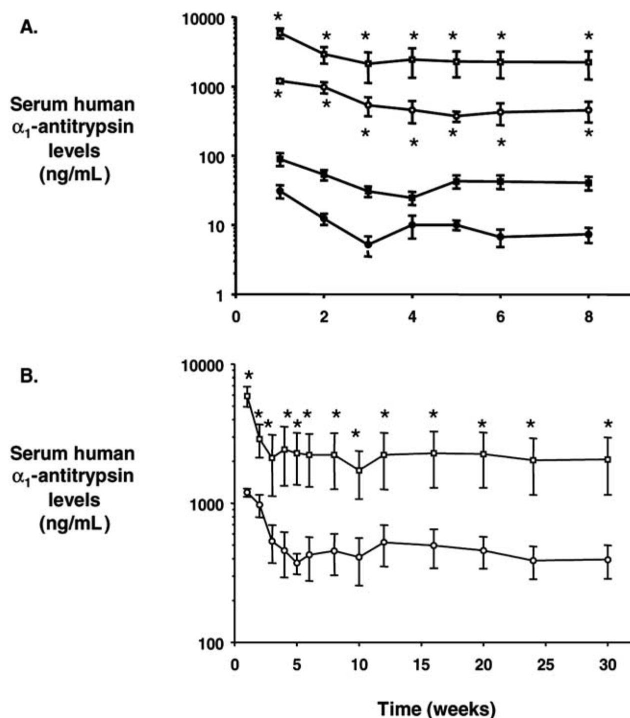

FIG. 2. Serum levels of hAAT expression as measured by ELISA from mice intravenously infused with lentiviral vectors with the hAAT promoter expressing the hAAT cDNA (50 μg p24/mouse). (A) Comparison of serum hAAT levels in C57Bl/6 mice administered lentiviral vectors with (□, ■) and without the Igκ MAR (○, ●). The age of the mice at the time of lentiviral vector administration was either 3½ weeks (○, □) or 7 weeks (●, ■). *$P < 0.05$ between 3½- versus 7-week-old mice. (B) Demonstration of long-term detection of serum hAAT levels in mice intravenously administered lentiviral vectors at 3½ weeks of age. A temporal extension of the lentiviral vector administered to mice shown in A is presented. *$P < 0.05$ between groups with and without the Igκ MAR. n = 6 mice/experimental group.

either 3½ or 7 weeks of age to minimize experimental variability.

C57Bl/6 mice (n = 6) at 3½ and 7 weeks of age were administered LVs expressing human α_1-antitrypsin driven by the hAAT promoter at a dose of 50 μg p24 (Fig. 2). Serum levels of hAAT were obtained over an initial 8-week period to compare the differences in gene expression in animals treated at the two different age groups. Fig. 2 shows the differences in serum hAAT levels in the different mouse groups. We observed peak levels of serum hAAT at week 1 in all of the mouse groups. At week 1, there was an ~40-fold higher level of serum hAAT in mice administered the vector at 3½ versus 7 weeks of age (1195 ± 83 versus 31 ± 7 ng/ml) (n = 6 mice/group). The serum hAAT levels reached a steady state at about week 3 post-vector infusion. Insertion of a matrix attachment region from immunoglobulin κ, a *cis*-acting DNA element, into the LV increased serum hAAT levels by ~5-fold in both age groups compared to their age-matched groups (n = 6 mice/group) that received the non-MAR-containing vector. No serum hAAT was detected in vehicle-infused, age-matched mice (n = 3). Fig. 2B shows the persistence of serum hAAT levels up to 30 weeks (length of study) in the mice administered LVs at 3½ weeks of age. This study confirms not only our present and previous studies, but also other studies that establish the ability of LVs to produce long-term transgene expression.

Transduction Efficiency by Modified Lentiviral Vectors into a Human Hepatoma Cell Line *in Vitro*

Lentiviral transfer plasmids were cloned with the human α_1-antitrypsin promoter including the apolipoprotein E enhancer and the hepatocyte control locus [17] driving the expression of the bacterial marker gene lacZ (see Fig. 1). This promoter is currently being used in a clinical trial for hemophilia B using adeno-associated virus (M. A. Kay, personal communication). We detected no lacZ-positive cells using HeLa cells, an epithelial cell line derived from a human uterus, while we found robust gene expression in hepatoma cell lines, suggesting that the promoter did function in a cell-type-specific manner. As shown in Fig. 3, we found that the incorporation of *cis*-acting elements, specifically the cppt from the HIV *pol* gene, increased the LV transduction efficiency by about twofold in huH7 cells, a human hepatoma cell line, from 545 ± 114 to 1026 ± 190 TU/ng p24 (n = 3 viral different preparations). The centralization of the cppt slightly augmented the transduction efficiency to 1195 ± 60 TU/ng p24 (n = 3), but this was not statistically significant. Moreover, the incorporation of another *cis*-acting DNA element, the Igκ matrix attachment region, further significantly increased the LV transduction efficiency to 3417 ± 287 TU/ng p24 (n = 3; $P < 0.05$), compared to the other three LV groups.

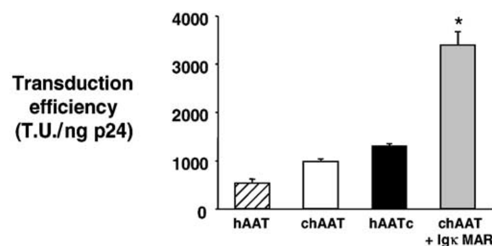

FIG. 3. Transduction efficiency of lentiviral vectors into human hepatoma cell line *in vitro*. Different lentiviral vectors were generated by transient transfection in 293T cells and titered on huH7 cells by X-gal staining and p24 ELISA measurements. All vectors contained the hAAT promoter driving the expression of the lacZ gene (hAAT; hatched bar) with the central polypurine tract sequence 5′ of the hAAT promoter (chAAT; open bar) or in between the promoter and lacZ gene (hAATc; black bar). The insertion of the Igκ MAR (chAAT + Igκ MAR; gray bar) was 5′ of the cppt. *$P < 0.05$ from the Igκ MAR group versus the other three lentiviral vector groups. n = 3 independent lentiviral preparations/group.

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doi:10.1016/S1525-0016(03)00169-2

Transduction Efficiency by Lentiviral Vectors into Hepatocytes *in Vivo*

We administered the LacZ LV to C57Bl/6 littermates at age 3½ or 7 weeks at a dose of 50 or 100 μg p24 (an index of viral titer—see Materials and Methods), sacrificed the mice 8 days following LV administration, and isolated the organs, including the liver, spleen, duodenum, brain, kidney, skeletal muscle, lungs, and heart, for X-gal staining. X-gal-stained liver sections from mice administered 50 μg p24 LV at 3½ weeks of age showed 0.9 ± 0.2% of hepatocytes were β-gal positive ($n = 4$ mice; Fig. 4A). Incorporation of the cppt into the integrating vector between the hAAT promoter and the lacZ gene (Fig. 4A) increased ($P < 0.05$) the hepatic transduction by about twofold (2.0 ± 0.2% of the hepatocytes were X-gal positive; $n = 4$ mice). Insertion of a *cis*-acting DNA element, a fragment of the Igκ MAR, further augmented hepatocyte transduction to 3.2 ± 0.3% ($P < 0.05$; $n = 3$ mice). Mice administered LVs at 7 weeks of age exhibited hepatocyte transduction efficiencies of 0.1–0.35%, which was nearly 10 times lower than that obtained in the 3½-week-old treated mice ($P < 0.001$). Although there was a tendency for the number of X-gal-positive hepatocytes to increase with the inclusion of either the cppt (0.27 ± 0.08%; $n = 4$) or the Igκ MAR (0.35 ± 0.12%; $n = 4$) in the mice administered LV at 7 weeks of age, no statistical significance was noted between the two groups largely due to the small number of X-gal-positive cells. However, there was a significant difference in lacZ-positive cells ($P < 0.05$) in both groups relative to the mice receiving the LV without the *cis*-acting DNA elements (0.1 ± 0.04%; $n = 4$).

In a separate group of mice at 3½ weeks of age, we administered lentiviral vectors expressing the lacZ gene that also included the cppt and Igκ MAR into the portal vein at two different doses (30 and 60 μg p24). Eight days following lentiviral vector administration, we sacrificed the mice ($n = 3$ mice/dose group), sectioned the livers, and scored them for X-gal-positive hepatocytes (Fig. 4B). We found that 0.9 ± 0.2% of the hepatocytes were X-gal positive in mice given 30 μg p24, which significantly increased by about threefold to 2.8 ± 0.2% ($P < 0.05$) in mice given 60 μg p24. This group of mice demonstrates that factors other than the dose of the lentiviral vectors played an important role in enhancing transduction efficiency in the 3½-week-old mice relative to the 7-week-old mice.

We performed further experiments to determine whether the position of the central polypurine tract sequence could influence the transduction efficiency. C57Bl/6 mice (3½ weeks of age) were infused with 100 μg p24 of LVs in which the cppt was either 5′ of the hAAT promoter or between the promoter and the transgene (see Fig. 4C). We found that the centralization of the cppt significantly increased ($P < 0.05$) hepatocyte transduction to 13.2 ± 1.9% ($n = 4$ mice) compared to the livers treated with a LV with the cppt 5′ of the hAAT promoter (7.9 ± 1.6%; $n = 4$ mice). None of the mice administered vehicle solution (PBS) at 3½ ($n = 4$ mice) or 7 weeks ($n = 4$ mice) of age stained for X-gal in the liver sections.

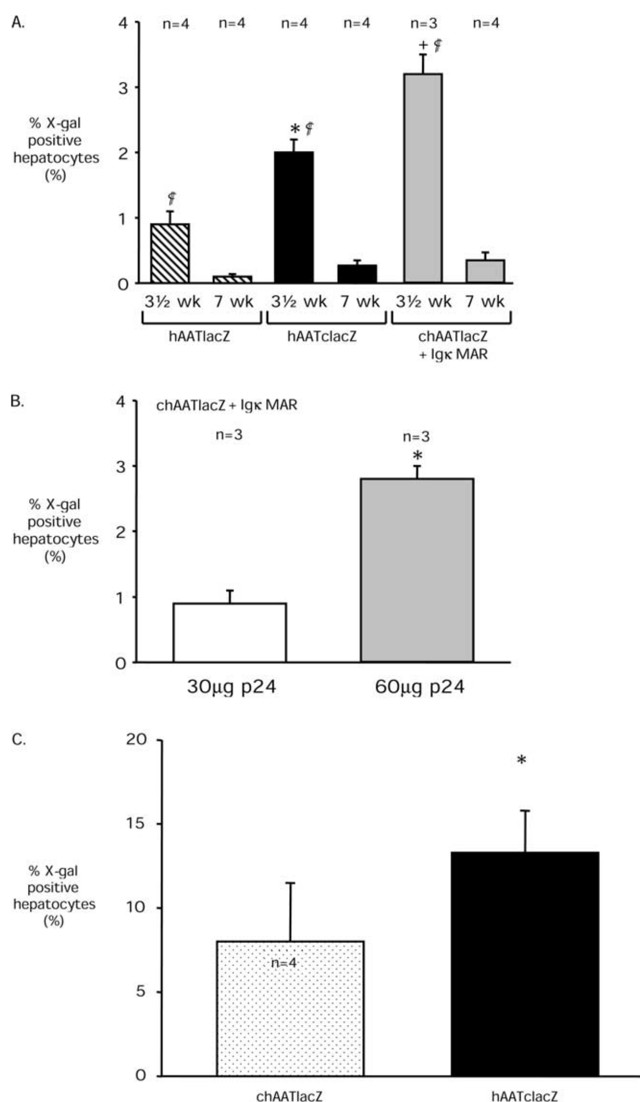

FIG. 4. Transduction efficiency of lentiviral vectors into mouse hepatocytes *in vivo*. (A) A graphic demonstration of the number of X-gal-positive hepatocytes from mice intravenously infused at 3½ or 7 weeks of age with self-inactivating lentiviral vectors with and without *cis*-acting DNA elements. Lentivectors contained the hAAT promoter driving the expression of the lacZ gene (hatched bars) with the central polypurine tract sequence in between the promoter and the lacZ gene (black bars) or with the insertion of the Igκ MAR (gray bars). The dose of the lentivectors administered to the mice in A was 50 μg p24. (B) The effect of dose on hepatocyte transduction in 3½-week-old mouse livers using lentivectors containing the cppt and Igκ MAR. (C) The position effect of the cppt on hepatocyte transduction in 3½-week-old mice administered lentivectors at a dose of 100 μg p24. *$P < 0.05$ between lentiviral vector-infused groups with and without the cppt; +$P < 0.05$ between lentiviral vector-infused groups with and without the Igκ MAR. ¶$P < 0.001$ between 3 ½-week-old and 7-week-old mice. n, number of mice/group.

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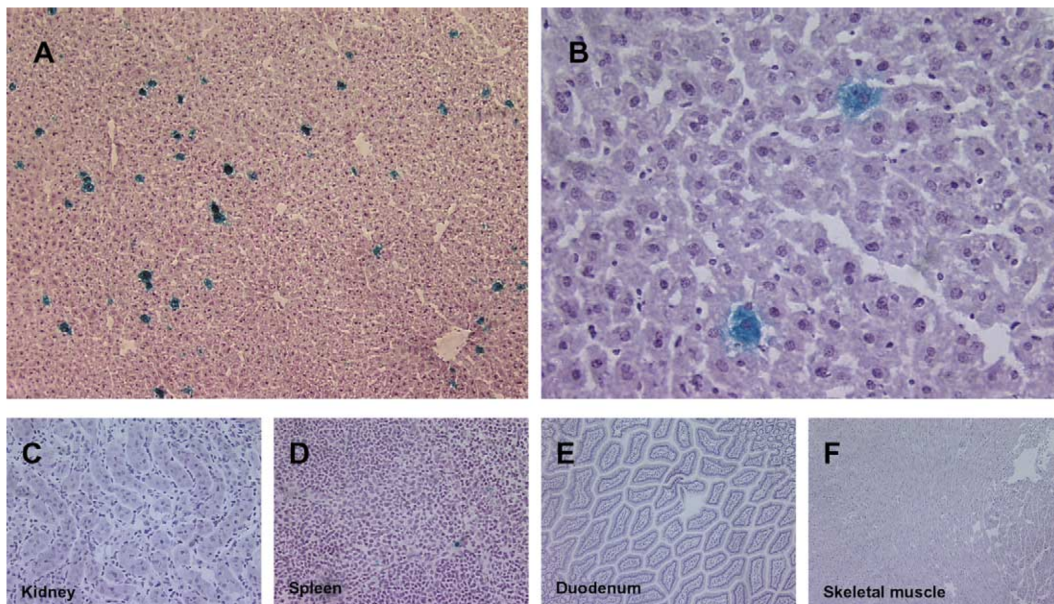

FIG. 5. Micrographs showing tissue sections from C57Bl/6 mice injected intravenously via the tail vein with lentiviral vectors expressing β-galactosidase at 3½ weeks of age. (A and B) Micrographs of liver sections from a C57Bl/6 mouse infused with LV at a dose of 50 μg p24 (A; original magnification 100×) and (B; original magnification 400×). (C–F) Micrographs of extrahepatic organs, including the kidney (C), spleen (D), duodenum (E), and skeletal muscle (F). The dose of the LV administered to the mice in C–F was 50 μg p24.

Specificity of the hAAT Promoter for Liver-Targeted Gene Expression

We isolated mouse organs including the skeletal muscle, lungs, heart, kidney, spleen, duodenum, and brain after intravenously administering lacZ-expressing LVs. Although the hAAT promoter was thought to be a liver-specific promoter, examination of X-gal-stained tissue sections showed slight β-gal activity in the spleen (Fig. 5D), but not in any of the other extrahepatic tissues isolated (Figs. 5C, 5E, and 5F). Qualitatively, there were fewer X-gal-stained cells in the spleen using the hAAT promoter in the present study compared to spleens treated with a constitutively expressed promoter in earlier studies in our lab [7,9]. Our results were consistent with those of Follenzi *et al.* [12], who found "rare GFP cells" in the spleen using a vector driving GFP expression from the hepatocyte-specific albumin promoter [12].

In addition, we isolated the genomic DNA from mice transduced at the age of 3½ weeks with the hAAT-expressing LV (50 μg p24/mouse) from the liver, spleen, kidney, and duodenum. This was to determine the presence of the LV DNA copy number by quantitative PCR analyses. We detected hAAT vector genomes in the liver and spleen, but not in the kidney or duodenum. The LV DNA copies were fivefold higher in the liver than in the spleen (n = 3 mice/organ analyzed). Note that there was no hAAT PCR product amplified in the no-template control reaction. The combination of LV DNA in the liver and spleen and no X-gal staining in the other tissues examined would be consistent with the absence of LV in those tissues.

Hepatocyte Proliferation during Growth Phase of the Mouse

We inserted osmotic minipumps subcutaneously in 3½- and 7-week-old littermates to administer BrdU, a marker for DNA synthesis, continuously for a period of 7 days. We fixed the livers in formalin and embedded them in paraffin prior to immunostaining for BrdU. As shown in Fig. 6, 3½-week-old mice contained 26 ± 1.3% BrdU-positive hepatocytes (n = 6 mice; 1500 total hepatocytes counted/mouse liver). In contrast, 7-week-old mice (n = 8 mice) from the same litter as the 3½-week-old mice had only 6.7 ± 0.7% of their hepatocytes labeled with BrdU. These results are consistent with the quiescence of hepatocytes in older versus younger mice.

Immune Response against the hAAT Transgene Following LV Administration

The results from the present study demonstrate a drop in the serum hAAT in all of the immune-competent C57Bl/6 mice regardless of their age at the time of LV administration. As shown in Table 1, we analyzed transgene expression 6 weeks following the intravenous infusion of LV, at which point the serum levels of hAAT had reached a steady state. The anti-hAAT antibody titer determined by the ELISA was higher in LV- compared to vehicle-infused

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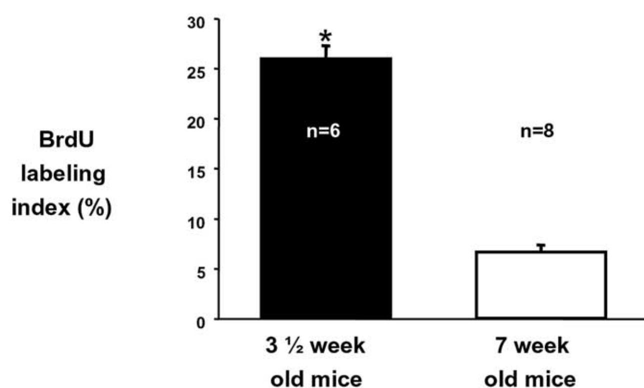

FIG. 6. Rate of liver proliferation during development *in vivo*. 5′-Bromo-2′-deoxyuridine (BrdU) incorporation into mouse hepatocytes at 3½ and 7 weeks of age and presented as percentage of BrdU-positive hepatocytes from a total of 1500 hepatocytes. Osmotic minipumps were subcutaneously implanted for 7 days and delivered 1 mg/day BrdU. Livers were removed and the 1500 hepatocytes/mouse liver (3–5 liver lobes per mouse) were counted for BrdU incorporation. $*P < 0.05$.

mice (mice 3013–3015). Interestingly, the mice that had the biggest drop in serum hAAT from their original week 1 levels concomitantly had the highest anti-hAAT antibodies. We performed a subsequent experiment in which we infused another LV batch into immune-deficient SCID mice (5 weeks of age) with a dose of 50 μg p24 (data not shown). We noted no reduction of serum hAAT, substantiating the likelihood that the initial decrease observed in C57Bl/6 mice was immune response mediated, as demonstrated by our results in Table 1.

DISCUSSION

Methods to promote hepatocellular proliferation, such as compensatory regeneration [8–11] and direct hyperplasia [7], have resulted in significant enhancements in LV transduction into hepatocytes *in vivo*. The present study utilized the natural proliferative ability of the animal, i.e., the adolescent growth phase, to demonstrate the highly efficient capability of LVs to transduce hepatocytes in young (3½ weeks of age) versus more mature (7-week-old) mice. Moreover, we found that younger mice had significantly higher (~40-fold) circulating levels of human α_1-antitrypsin compared with older mice treated in the same way. We also noted that the C57Bl/6 mice that received intravenous LV infusion did not appear to experience any adverse effects on their growth and development. From the results in our present study, we believe that the increased efficiency of LV transduction and transgene expression into the hepatocytes in adolescent mice *in vivo* was likely attributed to the higher number of hepatocytes that traversed through the S phase of the cell cycle as measured by the BrdU immunostaining (Fig. 6). However, there are other factors that may have contributed to the enhanced effects, such as vascular remodeling that may affect LV access to the hepatocytes (or other liver cells), differential expression of transcription factors and DNA repair enzymes, and/or the slightly higher dose of the LV administered to the adolescent mice compared to the adult mice. In terms of the latter point, we performed experiments showing that increased LV doses could lead to enhanced hepatocyte transduction in a relatively linear fashion. Moreover, from our previous work by Ohashi *et al*. [7], mice treated with a specific hepatocyte proliferator, TCPOBOP, prior to LV administration at a dose equal to that given to naïve control mice resulted in significantly higher levels of LV transduction in mouse hepatocytes *in vivo*. These two experiments strongly suggest that the difference in m.o.i. is not the main contributing factor to enhancing the efficiency of LV transduction into the hepatocytes.

Investigators have previously attempted to optimize retroviral tissue transduction in mice during different stages of early development *in vivo*. It has been well characterized that fetal development results in rapid liver growth, whereby successive numbers of cell divisions occur, resulting in an increase in the liver mass [18]. VandenDriessche *et al*. [19], using VSV-G-pseudotyped murine Moloney-based retroviral vectors, demonstrated therapeutic expression of human factor VIII in mouse plasma following intravenous infusion of 2- or 3-day-old mice. More recently, VandenDriessche *et al*. [20] demonstrated qualitatively that newborn mice had a significantly higher level of lentiviral vector transduction in the liver cells, including the hepatocytes and endothelial cells, compared to adult mice. However, no quantitative analyses of the differences in the liver cell transduction were made in this study. Another study showed significant enhancement of LV transduction during the gestational period of the rhesus monkey, when cellular proliferation was highly active [21]. These investigators found

TABLE 1: Anti-hAAT antibody in mouse serum

Mouse	Serum hAAT levels (ng/ml) Week 1	Week 6	Minimum serum dilution at which anti-hAAT IgG is not detected
3001	5157	2367	1:200
3002	4410	3675	1:200
3003	2363	366	1:3200
3004	9464	5968	1:200
3005	5948	460	1:3200
3006	7034	565	1:1600
3013	0	0	1:50
3014	0	0	1:50
3015	0	0	1:50

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doi:10.1016/S1525-0016(03)00169-2

that fetal gene transfer into rhesus monkeys using LVs resulted in a higher level of transduction in the epithelium of the lung at 55 days of gestation versus 70 days of gestation. *In vitro* studies have shown that human fetal hepatocytes may be more susceptible to LV transduction compared to mouse adult primary hepatocytes [22–24]. The results from the present study as well as others clearly demonstrate that the LV system would prove to be a valuable vector for *in utero* applications and disorders in which cellular proliferation was maximally occurring.

Another method to enhance LV transduction is the incorporation of *cis*-acting DNA elements into the integrating vector. Previous studies by several investigators demonstrated that the incorporation of the central polypurine tract sequence [5,8,12,14,15] or matrix attachment regions [8] improved the transduction efficiency and transgene expression in lentiviral vectors *in vitro* and *in vivo*. Consistent with our previous *in vitro* work [8], the insertion of the Igκ MAR into the integrating vector significantly increased the number of X-gal-positive hepatocytes, while enhancing the serum levels of hAAT *in vivo*. MARs are AT-rich sequences of DNA that are thought to regulate chromatin structure and may have a function to open specific transcriptional domains potentially to augment gene expression. It is also possible that the MARs help to bind to the genomic DNA and act as an anchor for the proviral vector to integrate preferentially into the host chromosome. Further studies are necessary to fully elucidate the role of MARs and how they function to enhance lentiviral transduction and transgene expression.

Previous work in our laboratory demonstrated low levels of LV transduction into liver cells (0.1–2%), specifically the hepatocytes, as measured by X-gal staining of liver sections from 7- or 8-week-old C57Bl/6 *scid* mice [7–9]. Other studies using 8-week or older mice found that intravenous infusion of third-generation LVs containing *cis*-acting DNA elements, such as the cppt, showed higher transduction levels in liver cells [11,12,25]. These studies suggested that LV differences, i.e., the insertion of the cppt, resulted in higher transduction of liver cells, but studies in our lab using LVs with the cppt did not achieve comparable transduction levels *in vivo*. A more plausible explanation may be the detection method of the integrated lentiviral transgene used by the different research groups. The present study along with previous work by our group used the bacterial lacZ gene, whereas the other investigators used the green fluorescent protein (GFP) as the reporter to quantify the transduction efficiency in the liver. Previous *in vitro* work by Dull *et al.* [26] demonstrated that the transduction efficiency of GFP-expressing LVs was approximately sixfold higher than that of lacZ-expressing LVs. Moreover, Tsui *et al.* [11] demonstrated variable transduction efficiencies using different methods of lentiviral transgene detection. They demonstrated low levels of hepatocyte transduction (~1%) by human factor IX immunohistochemistry in liver sections, which was in agreement with our previous estimation of transduction efficiency using X-gal staining to detect β-gal expression. On the other hand, their transduction efficiency into liver cells (hepatocytes + nonparenchymal cells) using GFP-expressing LV was significantly higher (~10% of the liver cells). Similarly, we have recently found that GFP can be three- to fourfold more sensitive as a marker in hepatocytes compared to lacZ (data not shown), but the autofluorescence in the liver makes quantification difficult when using this particular marker. Thus, it may be possible to overestimate the true transduction efficiencies in studies using GFP [11]. Regardless of the method used for the detection of LV transduction, it is clear that the present study demonstrated that there was a significant increase in LV-mediated transduction when LVs were intravenously administered at 3½ weeks of age versus 7 weeks of age.

One problem that arose during the study was the gradual loss of hAAT in the mouse serum. The hAAT levels peaked at week 1 and reached a lower steady-state level by week 3, but the serum levels of hAAT maintained a steady-state level for at least 30 weeks, the length of the study. The self-inactivating LVs used in the present study were cloned with a liver-specific promoter to minimize the extrahepatic expression of foreign transgenes, particularly in the immune cells of the spleen. The loss of serum hAAT levels is unlikely due to the transcriptional shut-off of the promoter in cells found within the liver, since earlier gene transfer studies by Okayama *et al.* [27] using Moloney murine leukemia virus-based vector in partially hepatectomized C57Bl/6 mice showed stable, long-term expression of serum hAAT using a similar hAAT promoter. Moreover, mouse studies using naked DNA [17,28] or “gutless” adenoviral vectors [29] incorporating the hAAT promoter have demonstrated extremely long durations of transgene expression following uptake by the liver cells *in vivo*.

The reduction in serum hAAT was likely due to the development of anti-hAAT antibodies since previous studies using viral vectors demonstrated antibody production against hAAT in C57Bl/6 mice [29–31]. Interestingly, C57Bl/6 mice were seemingly ideal animals to express hAAT because in previous studies using this mouse strain, with various vectors including naked DNA [17,28], first-generation adenoviral vectors [16,30,31], MLV-based vectors [27], and AAV [32] did not produce a significant immune response against the circulating hAAT protein. However, a recent study by Schiedner *et al.* [29] found significant anti-hAAT antibody production in C57Bl/6 mice following transduction with a high-capacity gutless adenoviral vector expressing the hAAT gene driven by a constitutive viral promoter. In addition, an earlier study by Schwalter *et al.* [30] found that different mouse strains behaved differently in their ability to mount neutralizing and nonneutralizing antibodies against hAAT.

Antibodies against other transgene products have been

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reported, but few studies have noted the immune responses against transgene products expressed from lentiviral vectors administered intravascularly [10,12]. We initially demonstrated anti-human factor VIII antibody production in C57Bl/6 mice following the administration of VSV-G pseudotyped LV [10]. More recently, Tsui *et al.* found high levels of anti-human factor IX antibodies following intravenous administration of LV. This was an interesting finding since previous studies using a variety of viral and non-viral vectors system in C57Bl/6 mice have not resulted in a loss of circulating hFIX due to the production of antibodies against this particular coagulation factor. The reason for the production of antibodies against the transgene product in the present and previous studies is not fully understood. One explanation may be robust transgene antigen presentation from nonparenchymal cells in the liver. The liver has a cellular composition of 65% hepatocytes and 35% nonparenchymal cells. Previous investigators demonstrated significant LV transduction in nonparenchymal cells, including endothelial, Kupffer, and Ito cells [11,12,25]. Studies in our lab have found that >85% of the LV-transduced cells in the liver were hepatocytes [7]. It was believed that the use of the hAAT promoter in the present study would circumvent the transgene expression in nonparenchymal cells, which were believed to play a role in the immune response. Another possible reason for the immune response against the circulating hAAT protein may be related to the expression of the foreign transgene in the antigen-presenting cells found in the spleen. Although the present study used LVs containing a hepatocyte-specific promoter to circumvent the expression in extrahepatic tissues, we found a small number of X-gal-positive cells in the spleen. The number of X-gal-positive cells in the spleen was much lower than that found in animals transduced with constitutive promoters like PGK or EF1α ([7]; unpublished observations). Coincidentally, Follenzi *et al.* [12] used the albumin promoter, another liver-specific promoter, which resulted in "rare" cellular expression of GFP in the spleen. The vectors used in the present study were similar to those used by Follenzi *et al.* [12], in which the 3′ LTR was deleted such that LTR promoter activity following LV integration would be eliminated as demonstrated by Zufferey *et al.* [33] *in vitro*. Possibly, the *in vivo* integration of a few LVs into a random site or the minimal LTR may still have slight promoter activity in splenic cells resulting in the spurious expression of the transgene and activation of the immune system against the transgene product. More intensive studies are required to further develop liver-specific promoter activity and also elucidate the immunological mechanisms involved in producing the loss of transgene expression in immune-competent animals.

In all, the present study demonstrated dramatic enhancement in transduction efficiency and transgene expression in an age-dependent manner using LVs. The results from the present study clearly demonstrated that adolescent mice can be transduced with lentiviral vectors much more efficiently than older, mature mice. It is likely that hepatocellular proliferation was one important factor resulting in the enhanced transduction, but it is possible that other factors such as viral uptake due to differentially expressed host proteins or liver remodeling during development could have also contributed to the increased effectiveness of LV transduction. Further studies will be necessary to elucidate the role(s) that either plays in the growth phase of live animals.

Materials and Methods

Plasmid constructs for vector production. The packaging construct, pCMVΔR8.74, has four of the viral accessory genes deleted, specifically *vif, nef, vpr,* and *vpu* [26]. pMD.G is the envelope plasmid and encodes the vesicular stomatitis virus G protein. The transfer plasmid encoding the hAAT cDNA driven by the human α_1-antitrypsin promoter with the apolipoprotein E enhancer and the hepatocyte control locus were used and published previously by our lab [8]. All constructs included the central polypurine tract sequence and had a 400-bp deletion in the 3′ LTR creating a self-inactivating vector [8]. The pHR(+)hAATsollacZR(−)W(+) and pHR(+)chAATsollacZR(−)W(+) plasmids were generated when the hAAT cDNA (*Eco*RI/*Eco*RI fragment) was replaced with the lacZ gene (*Eco*RI/*Eco*RI fragment) from pHR2EF1αcytolacZ. The immunoglobulin κ MAR (*Xho*I/*Xho*I fragment) was cloned into the novel *Xho*I site of pHR(+)chAATsollacZR(−)W(+).

Lentiviral vector production and titering assays. The lentiviral vectors were produced as previously described [8,10]. To determine the titer of the HIV vector stocks, serial dilution of concentrated virus was used to infect 5×10^5 HeLa or huH7 cells in a six-well plate in the presence of Polybrene (8 μg/ml). Blue cells were counted following X-gal staining to determine the lentiviral vector titer. HIV p24 Gag antigen concentration was determined by ELISA (Alliance; Dupont–NEN).

Animals. Male C57Bl/6 mice (2½ weeks of age) were purchased from Taconic Farms (Germantown, NY). All animals received humane care according to the institutional guidelines set forth by the Stanford University and Louisiana State University Animal Care Committees and the National Institutes of Health. The mice used in this study were placed in cages within a temperature-controlled room with a 12-h light–dark cycle and *ad libitum* access to food and water. The mice were intravenously infused through the tail vein (0.5 ml) with a variety of lentiviral vectors at a dose of 50 μg p24, which is an index of titer. The mice used for this study were littermates, and some of the mice were injected at the age of 3½ weeks, while the rest of the mice were allowed to age for an additional 3½ weeks before tail vein injection.

Human α_1-antitrypsin ELISA. Mouse serum was obtained intermittently via retro-orbital bleeding over a period of 30 weeks (length of study). The levels of hAAT were measured by ELISA as previously described [34].

Antibody detection against human α_1-antitrypsin. Ninety-six-well NUNC plates were coated with human calibrator serum 4 (120 ng/well; INCStar, Stillwater, MN) for 2 h at room temperature. The plates were then blocked with 4% nonfat dried milk in TBS-T for 60 min prior to the addition of serially diluted mouse serum (1:50–1:12,800) overnight at 4°C. The serum tested for this assay was from mice bled at week 6 infused with the hAAT-expressing LV containing the Igκ MAR. Each serum sample was performed in duplicate. Plates were washed, and secondary antibody (goat anti-mouse IgG; 1:1000) conjugated to horseradish peroxidase was incubated with the samples for 2 h at room temperature. The *O*-phenylenediamine diethyl (Sigma, St. Louis, MO) substrate reaction was activated by the addition of H_2O_2, and the absorbance measurements at 490 nm were determined with a plate reader. There were no detectable anti-hAAT antibody measurements at a dilution of 1:50 in the vehicle-infused and

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doi:10.1016/S1525-0016(03)00169-2

C57Bl/6 *scid* mice. Therefore, the dilution at which the lentiviral vector-infused mouse serum resulted in absorbance measurements similar to those of the control mice was determined.

X-gal staining. Mouse organs, including the liver, spleen, duodenum, kidney, lung, heart, brain, and skeletal muscle, were harvested after the 8th day following the intravenous administration of lentiviral vectors expressing lacZ. The organs were frozen in Tissue Tek OCT compound (Miles, Naperville, IL) on dry ice. Seven-micrometer sections were made and the sections were fixed in glutaraldehyde prior to staining in X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside; Fisher Scientific, Pittsburgh, PA) solution overnight. Tissue sections were subsequently counterstained in hematoxylin, and X-gal-positive nuclei were determined by counting 1000 hepatocytes in 20–25 randomly selected liver fields. The morphological structure of liver cells was determined on the basis of size (normally 30–40 μm), shape of the nucleus (large and round), and general morphology (cells forming liver cell plates).

5′-Bromo-2′-deoxyuridine immunohistochemistry. Cell cycle proliferation in 3½- versus 7-week-old mice was examined by subcutaneously administering BrdU (Sigma) at a dose of 1 mg/day through the use of an osmotic minipump (Model 2001; Alzet, Palo Alto, CA) for a period of 7 days. Mouse livers were then removed from the different aged mice 7 days after the implantation of the osmotic minipumps and fixed in formalin prior to embedding into paraffin as previously described [35]. Liver sections (5 μm) were processed for the detection of BrdU by incubating the tissues with a mouse anti-BrdU monoclonal antibody (Becton–Dickinson, San Jose, CA) at a dilution of 1:200. Sections were counterstained with hematoxylin. Hepatocytes were scored positive for BrdU when clear brown staining of the nucleus could be identified. The brown staining was indicative of DAB precipitation (i.e., antibody binding to BrdU). In each mouse, the BrdU labeling indices of hepatocytes were determined separately by counting a total of 1500 hepatocytes in 20–30 randomly selected liver fields (three different liver lobes) and expressed as a percentage of all positive nuclei. The duodenum was used as a positive control tissue for this experiment.

TaqMan quantitative PCR. Mice expressing hAAT were sacrificed 30 weeks following the injection of lentiviral vectors, and genomic DNA was extracted from the liver, spleen, duodenum, and kidney (n = 3/organ) using Qiagen genomic isolation tips. One microgram of genomic DNA was PCR amplified using the ABI Prism 7700 sequence detector. Primers were purchased from Integrated DNA Technologies (Coralville, IA) as follows: sense primer, 5′-TCAGCCTTGGTCCCCAGGGA-3′, and antisense, 5′-CCCAGAAGACAGATACATCC-3′. PCR was performed using the SYBR green solution from Perkin–Elmer containing AmpliTaq Gold. Standards and experimental samples were run in triplicate (two separate runs were performed for the assay). The PCR conditions were 50°C for 2 min and then 94°C for 10 min (initial melt) and then 40 cycles as follows: 94°C for 15 s and 60°C for 1 min. Negative control sample was PCR amplification of PCR reagents with no template.

Statistical analysis. The significance of differences between groups at the same dose of lentivirus was tested by a one-way ANOVA with the use of StatView 5.0 software (SAS Institute, Inc., Cary, NC). If a probability value of $P < 0.05$ was obtained, the Tukey test was used for comparison for each individual group with the appropriate control.

ACKNOWLEDGMENTS

Frank Park was a recipient of the Judith Graham Pool Postdoctoral Fellowship from the National Hemophilia Foundation. Kazuo Ohashi was a recipient of the Japan Society for the Promotion of Science Fellowship. This work was supported by NIH RO1-HL-64274 (M.A.K.), the Louisiana Gene Therapy Research Consortium (F.P.), and the Career Development Award from the National Hemophilia Foundation (F.P.).

RECEIVED FOR PUBLICATION APRIL 22; ACCEPTED MAY 2, 2003.

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