Unrestricted Hepatocyte Transduction with Adeno-Associated Virus Serotype 8 Vectors in Mice

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Recombinant adeno-associated virus (rAAV) vectors can mediate long-term stable transduction in various target tissues. However, with rAAV serotype 2 (rAAV2) vectors, liver transduction is confined to only a small portion of hepatocytes even after administration of extremely high vector doses. In order to investigate whether rAAV vectors of other serotypes exhibit similar restricted liver transduction, we performed a dose-response study by injecting mice with β-galactosidase-expressing rAAV1 and rAAV8 vectors via the portal vein. The rAAV1 vector showed a blunted dose-response similar to that of rAAV2 at high doses, while the rAAV8 vector dose-response remained unchanged at any dose and ultimately could transduce all the hepatocytes at a dose of 7.2 × 1015 vector genomes/mouse without toxicity. This indicates that all hepatocytes have the ability to process incoming single-stranded vector genomes into duplex DNA. A single tail vein injection of the rAAV8 vector was as efficient as portal vein injection at any dose. In addition, intravascular administration of the rAAV8 vector at a high dose transduced all the skeletal muscles throughout the body, including the diaphragm, the entire cardiac muscle, and substantial numbers of cells in the pancreas, smooth muscles, and brain. Thus, rAAV8 is a robust vector for gene transfer to the liver and provides a promising research tool for delivering genes to various target organs. In addition, the rAAV8 vector may offer a potential therapeutic agent for various diseases affecting nonhepatic tissues, but great caution is required for vector spillover and tight control of tissue-specific gene expression.

Liver-directed gene transfer with viral and nonviral vectors has been explored for the treatment of a variety of inherited and acquired diseases, including hemophilia (51), various metabolic diseases such as mucopolysaccharidosis (42), hyperlipidemia (24), tyrosinemia (41), and diabetes mellitus (25), and chronic viral hepatitis (29). Among the vectors used to deliver genes to hepatocytes in vivo, recombinant adeno-associated virus (rAAV) vectors are one of the most promising vehicles because they are based on nonpathogenic viruses, transduce both dividing and nondividing cells, and achieve long-term stable transgene expression with minimal toxicity and cellular immune response in animals.

AAV is a small, nonpathogenic, replication-defective parvovirus with a single-stranded DNA genome. Among the various serotypes of AAV, rAAV vectors based on AAV serotype 2 (rAAV2) have been most extensively investigated as gene delivery vectors in vivo, demonstrating efficacy and safety. Based on successful results in a series of preclinical studies for rAAV2-mediated gene therapy, several clinical trials were initiated for the treatment of inherited diseases, including hemophilia B (22).

Despite such recent advances, rAAV2-mediated hepatic gene transfer has still been suboptimal and transduction efficiency in the liver remains unsatisfactory, particularly in cases that require higher transduction efficiency. One major drawback in this system is that only ~10% of hepatocytes are stably transducible with rAAV2 vectors (5, 39, 59). In other words, it is not possible to increase transduction efficiency in mice (either number of transduced hepatocytes or expression of transgene products) in proportion to given doses when vector doses of 3.0 × 1011 vector genomes (vg) or more are administered per mouse. Liver transduction becomes saturated at higher doses, with transduction efficiencies of around 10% of total hepatocytes (39). The mechanism of this restricted liver transduction has not been elucidated but is not related to impaired uptake of vector particles by hepatocytes because rAAV2 vector genomes are found in a majority of hepatocytes within 24 h after vector infusion (32). We have reasoned from our observations that, in the liver, there are two distinct hepatocyte subpopulations with different metabolic states. That is, only a small subset of hepatocytes have all the machinery required for establishing stable rAAV2 transduction, while the other subset is devoid of some machinery for the process or has some inhibitory machinery that prevents the process (32). Recently, Thomas et al. proposed a model in which the rate of capsid uncoating determines the transduction efficiency with rAAV vectors in the liver (54).

In the past 5 years, there have been several major breakthroughs in rAAV vector technologies that include productions of rAAV vectors derived from alternative serotypes (6, 7, 15, 17, 46, 47, 60) and the development of self-complementary (or double-stranded) rAAV vectors (13, 30, 31, 57). Over a hundred different AAV sequences have been isolated thus far from human and nonhuman primates (14). Their recombinants have been investigated extensively for tissue tropism and transduction efficiency, enabling a dramatic increase in transduction efficiency in various target tissues.
efficiency (4, 14, 15) and a change of tissue or cell type tropism or vector distribution patterns in a given tissue (7, 53, 56). Now, finding the optimal AAV serotypes for efficient and tissue-specific transduction has become imperative for successful gene therapy. The other breakthrough, packaging of double-stranded vector genomes into virions, i.e., self-complementary rAAV2, has greatly enhanced transduction efficiency, although the vectors can only hold half of the genome.

We and others have established a method by which rAAV2 vector genomes can be cross-packaged into heterologous capsid proteins derived from alternative serotypes, making chimeric virions, so-called pseudo-serotyped rAAV vectors (17, 46). This allowed us to conduct a thorough side-by-side study to compare liver transduction efficiency among different pseudo-serotyped rAAV vectors, types 1 to 6, in mice. Although the rAAV1, -2, and -6 vectors achieved similar expression levels and none of the other serotypes resulted in a dramatic increase in stable liver transduction efficiency (17), the rAAV1, -2, and -6 vectors each exhibited distinct dose-response profiles (17). We have established in two independent studies that rAAV2-mediated stable liver transduction is proportional to given vector doses ranging from 2 to 4 x 10^9 to 3 x 10^11 vg/mouse (17, 39). Interestingly, and unlike the rAAV2 vector, rAAV1 vector administration into the liver resulted in a disproportionately greater increase in stable liver transduction, as vector doses increased within the same dose range (17), although this dose-response profile for rAAV1 was blunted at doses higher than 4 x 10^13 vg/mouse. Since we quantified the transgene product and not the number of transduced hepatocytes in the previous study, it is not known if rAAV1-mediated liver transduction is also confined to a small population of hepatocytes.

In the present study, we investigated whether restricted liver transduction is also the case for pseudo-serotyped rAAV1 and rAAV8 vectors. The rAAV1 vector was selected because it exhibited a distinct dose-response profile, while the rAAV8 vector was chosen because it has been shown to transduce mouse hepatocytes better than rAAV2 (15, 49, 54). As a result, in contrast to rAAV2 vectors, we find that all the hepatocytes are permissive to stable rAAV8 transduction and ~100% hepatocyte transduction is achievable by portal vein injection at a dose of 7.2 x 10^12 vg/mouse. In addition, and unlike the situation with rAAV2 vectors, we find that such high transduction efficiency is achievable by a single tail vein injection of rAAV8 vectors. Finally, we elucidate that the rAAV8 vector can transduce skeletal muscle throughout the body, the entire cardiac muscle, and substantial numbers of pancreatic cells, smooth muscle cells, and brain cells after intravenous injection. These observations not only help us understand the mechanisms of rAAV vector transduction but also emphasize both the utility and promiscuity of rAAV8 vectors.

**MATERIALS AND METHODS**

**Construction of rAAV vectors.** The construction and production of the rAAV vectors AAV2-EF1α-nsLacZ, AAV2-hF.IX16, and AAV2-CMV-lacZ were described elsewhere (23, 37, 39, 40), although we did not clearly denote the serotype in the vector names in our previous publications. Briefly, AAV2-EF1α-nsLacZ is a bacterial β-galactosidase-expressing rAAV2 vector harboring the human elongation factor 1α (EF1α) enhancer-promoter, the Escherichia coli lacZ gene with a nuclear localization signal (nls), and the simian virus 40 poly(A) signal. AAV2-hF.IX16 is a human coagulation factor IX (hF.IX)-expressing rAAV2 vector comprising a liver-specific promoter (the apolipoprotein E hepatic locus control region-human α1-antitrypsin gene promoter) (32), hF.IX minigene (containing a 1.4-kb DNA fragment of the first intron from the hF.IX gene), and the bovine growth hormone poly(A) signal. AAV2-CMV-lacZ is a β-galactosidase-expressing rAAV2 vector harboring the human cytomegalovirus enhancer-promoter with an intron from the human growth hormone gene, the cytolic lacZ gene, and the simian virus 40 poly(A) signal.

For AAV1-EF1α-nsLacZ, AAV8-EF1α-nsLacZ, and AAV8-CMV-lacZ, rAAV2 vector genomes were cross-packaged into capsids derived from AAV serotype 1 or 8 with the corresponding AAV helper plasmids (15, 17) (the AAV8 helper plasmid was kindly provided by James M. Wilson). All the vectors were produced by the triple transfection method, purified by two cycles of cesium chloride gradient centrifugation, and concentrated as outlined elsewhere (3, 17). The final viral preparations were kept in phosphate-buffered saline (PBS) containing 5% sorbitol. The physical particle titers were determined by a quantitative dot blot assay.

**Animal procedure.** Six- to 8-week-old male C57BL/6 and C57BL/6 Rag-1 mice were purchased from Jackson Laboratory (Bar Harbor, Maine). The portal vein and tail vein injections of rAAV vector preparations were performed as previously described (34, 35). Controls were naive uninfected mice or mice injected with the excipient (PBS–5% sorbitol) only. Blood samples were periodically collected from the retroorbital plexus. All the animal experiments were performed according to the guidelines for animal care at Stanford University.

**Protein analysis.** We extracted total liver proteins and determined expression levels of β-galactosidase in rAAV vector-transduced mouse livers with a β-galactosidase enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals, Indianapolis, Ind.) as previously described (37). We normalized β-galactosidase levels with the total protein concentration determined by the Lowry assay using a DC protein assay kit (Bio-Rad, Hercules, Calif.), and described the values as picograms of β-galactosidase per milligram of protein. We measured human coagulation factor IX levels in mouse plasma by an enzyme-linked immunosorbent assay specific for human coagulation factor IX. We measured levels of serum alanine aminotransferase (ALT) with the ALT reagent set (Teco Diagnostics, Anaheim, Calif.).

**Histological analysis.** Pieces of mouse liver lobes were embedded in Tissue Tek Optimal Cutting Temperature compound (Sakura Finetek USA, Inc., Torrance, Calif.) and frozen on dry ice. In some instances, various mouse tissues other than the liver, i.e., brain, lung, heart, spleen, kidney, intestine, testis, pancreas, and skeletal muscle (quadriiceps, tibialis anterior, or tongue), were also collected and processed in the same way. For histochemical detection of β-galactosidase expression, 10-μm sections were cut, fixed with 1.25% glutaraldehyde, stained with 5-bromo-4-chloro-3-indolylphosphate (X-Gal) as described (21), and counterstained with light hematoxylin or nuclear fast red. To determine transduction efficiency in the liver, at least 2,000 nuclei per section were examined for β-galactosidase expression from each animal.

In order to determine cell types in the brain transduced with the rAAV8 vector, we performed immunohistochemical analysis of brain sections. The blocks were cut into coronal sections 12-μm thick with a cryostat. The sections were air dried, mounted on gelatin-coated slides, and dried at room temperature before staining.

The sections were fixed in 4% paraformaldehyde for 15 min and washed three times in PBS for 5 min. The sections were blocked with PBS containing 5% goat serum at room temperature for 30 min. The sections were incubated with primary antibodies against β-galactosidase (1:200; rabbit immunoglobulin G fraction; Invitrogen, Carlsbad, Calif.), with a mixture of mouse monoclonal anti-NeuN antibody (1:200; Chemicon International Inc.), or mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:200; Chemicon International Inc.) in PBS containing 5% goat serum at room temperature for 1 h. NeuN and GFAP served as markers for neurons and astrocytes, respectively. The sections were washed three times in PBS for 5 min. Then they were incubated with Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G (2 μg/ml; Invitrogen) and Alexa Fluor 594-conjugated goat anti-mouse immunoglobulin G (2 μg/ml; Invitrogen) in PBS at room temperature for 30 min. The sections were washed three times with PBS for 5 min. Finally, the sections were mounted in Vectashield (Vector Laboratories, Burlingame, Calif.). Immunoreactivity was assessed and viewed under a confocal laser-scanning microscope (TCS NT; Leica).

For detection of pancreatic islet cells, we performed X-Gal and insulin double staining as previously described (35). For detection of Kupffer cells in the liver, we performed X-Gal and F4/80 double staining. Briefly, frozen sections in Optimal Cutting Temperature compound were cut 10-μm thick, air dried for 10 min, and stored at –20°C. Samples were thawed for 1.5 h and then fixed in chilled acetone at –20°C for 20 min. Samples were dried for 6 min and rehydrated in PBS for 10 min. Slides were stained for X-Gal overnight and washed three times in PBS for 5 min. Samples were incubated in 0.5% hydrogen peroxide.
TABLE 1. Hepatocyte transduction with AAV1-, AAV2-, or AAV8-EF1α-nlslacZ 6 weeks postinjection

<table>
<thead>
<tr>
<th>Vector</th>
<th>Dose (vg/mouse)</th>
<th>No. of mice</th>
<th>Mean transduction efficiency (%) ± SD</th>
<th>Mean β-galactosidase expression (pg/mg of protein) ± SD</th>
<th>Mean vector copy no. per transduced cell (ds-vg/dge) ± SD</th>
<th>Mean net vector copy no. per transduced cell (ds-vg/dge) ± SD</th>
<th>Mean β-galactosidase expression rate (pg/mg of total protein) ± SD</th>
<th>Mean vector genome activity (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV1</td>
<td>5.0 × 10^10</td>
<td>4</td>
<td>4.2 ± 1.0</td>
<td>426 ± 110</td>
<td>1.1 ± 0.2</td>
<td>27.0 ± 3.1</td>
<td>100.5 ± 9.5</td>
<td>376.7 ± 67.4</td>
</tr>
<tr>
<td></td>
<td>3.0 × 10^11</td>
<td>6</td>
<td>7.7 ± 0.7</td>
<td>1215 ± 263</td>
<td>9.3 ± 0.9</td>
<td>1216.1 ± 142</td>
<td>160.1 ± 40.5</td>
<td>133.3 ± 38.4</td>
</tr>
<tr>
<td></td>
<td>1.8 × 10^12</td>
<td>4</td>
<td>13.2 ± 2.5</td>
<td>2078 ± 389</td>
<td>54.9 ± 18.1</td>
<td>4135.3 ± 83.1</td>
<td>159.7 ± 27.8</td>
<td>40.1 ± 12.1</td>
</tr>
<tr>
<td></td>
<td>7.2 × 10^12</td>
<td>3</td>
<td>23.5 ± 1.0</td>
<td>2057 ± 399</td>
<td>92.3 ± 12.2</td>
<td>4121.2 ± 69.6</td>
<td>88.9 ± 8.0</td>
<td>21.5 ± 1.6</td>
</tr>
<tr>
<td>AAV2</td>
<td>3.0 × 10^11</td>
<td>6</td>
<td>3.9 ± 0.5</td>
<td>888 ± 222</td>
<td>5.1 ± 2.0</td>
<td>132.2 ± 47.5</td>
<td>235.8 ± 72.4</td>
<td>197.0 ± 92.5</td>
</tr>
<tr>
<td>AAV8</td>
<td>5.0 × 10^10</td>
<td>4</td>
<td>8.1 ± 1.8</td>
<td>1669 ± 201</td>
<td>7.2 ± 1.5</td>
<td>90.6 ± 5.3</td>
<td>211.7 ± 31.5</td>
<td>223.5 ± 23.5</td>
</tr>
<tr>
<td></td>
<td>3.0 × 10^11</td>
<td>6</td>
<td>14.9 ± 3.4</td>
<td>2963 ± 517</td>
<td>58.2 ± 10.9</td>
<td>413.1 ± 134.8</td>
<td>208.2 ± 63.5</td>
<td>53.0 ± 14.6</td>
</tr>
<tr>
<td></td>
<td>1.8 × 10^12</td>
<td>3</td>
<td>65.8 ± 9.0</td>
<td>9052 ± 1541</td>
<td>207.5 ± 56.0</td>
<td>318.4 ± 87.5</td>
<td>140.8 ± 40.2</td>
<td>44.8 ± 7.4</td>
</tr>
<tr>
<td></td>
<td>7.2 × 10^12</td>
<td>4</td>
<td>97.4 ± 0.3</td>
<td>21686 ± 3051</td>
<td>620.6 ± 42.8</td>
<td>637.2 ± 42.6</td>
<td>222.7 ± 31.4</td>
<td>35.3 ± 7.2</td>
</tr>
<tr>
<td>None (excipient)</td>
<td>3</td>
<td>0.0</td>
<td>&lt;4</td>
<td>0.0</td>
<td>4.0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

a X-Gal-positive nuclei/total hepatocyte nuclei counted (at least 2,000 hepatocyte nuclei were counted).
b β-Galactosidase antigen levels in liver extracts were normalized to the amount of total protein in samples.
c Net vector copy number is defined as the number of double-stranded vector genomes per transduced hepatocyte; i.e., (vector copy number per cell [vg/dge]/transduction efficiency [%]) × 100.
d β-Galactosidase expression rate is defined as picograms of β-galactosidase protein per milligram of cellular protein produced from transduced hepatocytes corresponding to 1% of total hepatocytes; i.e., β-galactosidase expression divided by transduction efficiency. The values reflect β-galactosidase production per transduced hepatocyte.
NA, not applicable.

RESULTS

All the hepatocytes were permissive to stable rAAV8 vector transduction. We previously demonstrated that only a small portion of hepatocytes are permissive to stable rAAV2 vector transduction, which restricts a linear vector dose-response at doses higher than 3.0 × 10^11 vg/mouse (39). The maximum number of stably transducible hepatocytes may vary depending on the experimental settings, but it normally plateaus at ~10% of total hepatocytes. In order to investigate the correlation between vector dose and transduction efficiency with other pseudo-serotyped rAAV vectors, we injected mice with AAV1-EF1α-nlslacZ or AAV8-EF1α-nlslacZ via the portal vein at four different doses ranging from 5.0 × 10^10 to 7.2 × 10^12 vg/mouse, i.e., 5.0 × 10^10, 3.0 × 10^11, 1.8 × 10^12 and 7.2 × 10^12 vg/mouse (n = 3 to 6 per group). As a reference, we also injected six mice with 3.0 × 10^11 vg of AAV2-EF1α-nlslacZ. Control mice were injected with the excipient (PBS–5% sorbitol) only (n = 3). Six weeks after vector injection, we harvested the liver samples and determined the transduction efficiency by counting β-galactosidase-expressing hepatocytes, measuring β-galactosidase antigen levels by enzyme-linked immunoassay, and measuring the double-stranded vector genome copy numbers in the liver by Southern blot. The results are summarized in Table 1 and Fig. 1, and representative microscopic pictures are shown in Fig. 2.

As demonstrated, all the hepatocytes were permissive to stable transduction with the rAAV8 vector, reaching ~100% hepatocyte transduction at 7.2 × 10^12 vg/mouse. β-Galactosidase-positive cells with a small nucleus were occasionally found in the rAAV8-transduced liver (Fig. 2B). X-Gal/Kupffer cell double staining revealed no transduction in Kupffer cells (Fig. 2D). The origin of β-galactosidase-positive small nuclei could not be determined conclusively. These might be portions of hepatocyte nuclei or represent rAAV8-transduced nonparenchymal cells besides Kupffer cells. The number of hepatocytes transduced with the rAAV1 vector was 24% at a dose of 7.2 × 10^11 vg/mouse. Since injection of a vector dose higher than 10^13...
providing adenovirus helper functions (11, 12), genotoxic treat-
nomes into transgene-expressible double-stranded genomes
transduction. Nonetheless, the results suggest that all the hepa-
tomato the maximum number of hepatocytes permissive to stable rAAV1
vg/mouse was not feasible, it was not possible to determine the
maximum number of hepatocytes permissive to stable rAAV1
transduction. Nonetheless, the results suggest that all the hepa-
tocytes can process incoming single-stranded rAAV vector ge-
nomes into transgene-expressible double-stranded genomes
without extrinsic assistance to augment transduction, such as
providing adenovirus helper functions (11, 12), genotoxic treat-
ment (1, 2), or forced biochemical modification of single-
stranded DNA binding proteins (43-45, 61).

Dose-response profile with the rAAV8 vector did not change
until all the hepatocytes were transduced. In rAAV2-mediated
liver transduction, a proportional dose-response profile does not change at doses of less than $3.0 \times 10^{11}$ vg/mouse, but the vector dose-response reaches saturation at higher doses (39). Likewise, our previous and present studies showed that the dose-response profile in rAAV1-mediated liver transduction changes between a low dose range (showing a disproportio-
ately greater dose-response) and a high dose range (showing a
blunted dose-response) (17) (Fig. 1B).

In the rAAV8-mediated liver transduction, log/log plots of the
vector doses and the number of transduced hepatocytes
(Fig. 1A) or the level of transgene expression (Fig. 1B) exhib-
ted linearity throughout the given vector doses, with regres-
sion coefficients ($r$) of 0.945 (Fig. 1A) and 0.995 (Fig. 1B). The
log $y$/log $x$ slopes of the dose-response curves were 0.450 (Fig.
1A) and 0.585 (Fig. 1B). The log $y$/log $x$ slope for a propor-
tional dose-response should be $\approx 1.0$. Therefore, neither the
dose-response determined by measuring the number of trans-
duced hepatocytes nor that determined by measuring the
transgene protein product was directly proportional to various
vector doses. The similar values of both slopes (both are
around 0.5) imply that the total amount of transgene product and the number of transduced hepatocytes correlate with each other. Although the dose-response with rAAV8 was not pro-
portional, both the number of transduced hepatocytes and
transgene expression levels predictably increased with a con-
stant factor of $\exp(-0.5)$. In other words, when the AAV8-
EF1α-nlslacZ vector is injected into mice at doses of $5.0 \times 10^{10}$
vg/mouse or higher, an $x$-fold increase in the vector dose re-
results in $x^{1.05}$-fold increase in the number of transduced hepa-
tocytes and transgene expression until all the hepatocytes are
transduced.

Total number of stably transduced double-stranded vector
genes in the liver increased in proportion to given vector
doses irrespective of serotype. Next we determined double-
stranded vector genome copy numbers per diploid genomic
Equivalent (ds-vg/dge) in the livers by Southern blot analysis.
The results are summarized in Table 1 and Fig. 1C. In Fig. 1C,
all vectors showed similar log $y$/log $x$ slopes close to 1.0 (i.e.,
0.775, 1.005, and 1.119 for rAAV8, rAAV1 and rAAV2, re-
spectively) with a regression coefficient ($r$) of 0.999 in each case
until the slopes of the dose-response curves started decreasing.
This demonstrates that the total number of stably transduced double-stranded vector genomes in the liver increased propor-
tionally irrespective of serotype until the saturation dose was
reached. Considering that each of the three serotypes display
distinct dose-response profiles, it is conceivable that the quality
or state but not the absolute quantity of the double-stranded
vector genomes in hepatocytes determines the dose-response
profiles.

Decreased specific activity of vector genomes in the liver was
concordant with the emergence of vector genome concatemers.
It is possible to deduce the specific activity of the double-
stranded vector genomes by dividing $\beta$-galactosidase expres-
sion levels by vector genome copy numbers (Table 1). In rAAV1 or rAAV8-mediated liver transduction, vector genome
specific activity decreased as the vector dose increased. This

![Figure 1](http://jvi.asm.org/)

**FIG. 1.** Vector dose-response profiles in AAV1-, AAV2-, and
AAV8-EF1α-nlslacZ-transduced mouse livers. The percentage of
transduced hepatocytes in the livers (A), total $\beta$-galactosidase antigen
levels (B), and number of double-stranded vector genomes per diploid
genomic equivalent (ds-vg/dge) (C) are shown as a function of injected
vector doses. Solid markers represent the values obtained from the
present study. The dose-response profiles in AAV2-EF1α-nlslacZ-me-
diated liver transduction were obtained from our previous study (39)
for comparison and are depicted with open circles. Values are means
± standard deviation.

![Figure 2](http://jvi.asm.org/)

**FIG. 2.** Liver transduction with $7.2 \times 10^{12}$ vg of AAV8-EF1α-
nslacZ delivered via the portal vein. The liver was harvested 6 weeks
postinjection and stained with X-Gal and light hematoxylin. A repre-
sentative result is shown (A and B). Virtually all hepatocytes were
transduced with rAAV8 throughout the liver. The liver was stained
heterogeneously with X-Gal, with central vein areas being less intense.
(C) X-Gal-stained hepatocytes around a central vein area. Although
genome expression near central veins was not as strong as in portal areas,
most of the hepatocytes express the transgene. (D) X-Gal and F4/80
double staining. None of the Kupffer cells (brown) were transduced.
Small nuclei positive for $\beta$-galactosidase are indicated with arrows in
panels B and D. These might be portions of hepatocyte nuclei or
represent rAAV8-transduced nonparenchymal cells besides Kupffer
cells. Scale bars, 100 $\mu$m.
FIG. 3. Southern blot analysis of rAAV vector genomes in liver transduced with AAV1- or AAV8-EF1α-nls lacZ at various doses. The left and right panels show the results obtained with AAV1-EF1α-nls lacZ- and AAV8-EF1α-nls lacZ-injected mice. Total genomic DNA was extracted from the livers harvested 6 weeks postinjection and separated on 0.8% agarose gels following BamHI or KpnI digestion. BamHI cleaves the vector genome only once at nucleotide position 1362, while KpnI does not cut the 4,828-base genome. The vector genomes were detected with a 2.1-kb lacZ probe (nucleotide positions 1518 to 3639). Each lane represents an individual mouse. Injected vector doses (vg per mouse) are indicated above each lane. For the results obtained from the mice injected with 5.0 × 10^{10} vg/mouse, strips from overexposed blots are also shown to demonstrate the presence or absence of concatemers. They are indicated with thicker lines above the lanes. Open and solid arrows indicate head-to-tail and tail-to-tail molecules, respectively. Open and solid arrowheads indicate supercoiled double-stranded circular monomer vector genomes and concatemers, respectively. Head-to-tail molecules include both circular monomer genomes and concatemers, while tail-to-tail molecules represent concatemers exclusively. Therefore, the intensity of tail-to-tail molecules well correlates with the abundance of concatemers.

was also the case for rAAV2 vectors (39). The mechanism of this decreased genome specific activity is not yet clear but is likely related to the formation of less active double-stranded vector molecules, presumably concatemers (39). The present study, combined with the results from our previous study (39), showed that decreased vector genome specific activity is concordant with the emergence of concatemers. At the lowest dose, 5.0 × 10^{10} vg/mouse, the rAAV8 vector genomes formed substantial concatemers, while rAAV1 and rAAV2 formed exclusively circular monomers (Fig. 3) (39). The vector genome specific activities of rAAV1, rAAV2, and rAAV8 at this dose were 377, 489, and 223 (pg/mg of protein)/(ds-vg/dge), respectively, which is consistent with our presumption that concatamer formation decreases vector genome specific activity.

Comparison of tail (peripheral) vein and portal vein injection of rAAV8 vectors. Recently, Sarkar et al. reported that tail vein injection was as efficient as portal vein injection in the context of the canine coagulation factor VIII-expressing rAAV8 vector at a dose of 1.0 × 10^{11} vg/mouse (49). In our previous studies, we found that, with multiple serotypes, rAAV transduction was more efficient when delivered by the portal vein compared to the tail vein (17, 35). To further address this issue, we injected male C57BL/6 mice with 3.0 × 10^{11} vg of the rAAV8-hF.IX16 vector via the portal vein or the tail vein and monitored plasma human coagulation factor IX levels. Figure 4A summarizes the results. Both routes of injection resulted in rapid and robust expression of human coagulation factor IX in mouse plasma with no significant difference in transgene expression (Student’s t test, P = 0.28, with 4-week time point values). Human coagulation factor IX expression peaked 4 weeks postinjection at levels of around 200 μg/ml, one log higher than that obtainable with the corresponding rAAV2 vector, AAV2-hF.IX16 (17, 39). However, transgene expression declined thereafter to levels of 40 to 50 μg/ml. Such a decline in human coagulation factor IX expression has not been observed with AAV2-hF.IX16. The mechanism of the decline is currently unknown.

Next, in order to determine the transduction efficiency of rAAV8 in the liver, we injected male C57BL/6 rag-1 mice with two different doses of AAV8-EF1α-nls lacZ (3.0 × 10^{11} and 7.2 × 10^{12} vg/mouse) via two different routes (tail vein and portal vein, n = 4 each), and determined liver transduction efficiency 6 weeks postinjection through histochemical and molecular analyses. Control mice received excipient only. Serum samples were collected for measurement of ALT levels at days 1, 3, and 10 after injection. As summarized in Table 2, liver transduction efficiency was comparable between the two routes of injection at any vector dose (Student’s t test, P = 0.27 and 0.47 for the doses of 3.0 × 10^{11} and 7.2 × 10^{12} vg/mouse, respectively), achieving ~90% transduction efficiency with 7.2 × 10^{12} vg/
at 7.2 galactosidase-expressing transduced cells were observed out-

ever found to be strongly positive (Fig. 5D). In the pancreas, most of the positive cells were acinar cells and found outside the Langerhans islets. There was no difference in the distribution between tail vein and portal vein injections (data not shown).

In the brain, immunohistochemical analyses demonstrated that both neurons and glial cells were transduced (Fig. 5C). The transduced cells were distributed throughout the brain, including the cerebral cortex, striatum, hippocampus, thalamus, and cerebellum. There were several small foci where transduced cells were clustered (Fig. 5B). These foci include the median eminence and arcuate nucleus of the hypothalamus and basolateral nucleus of the amygdala. Purkinje cells in the cerebellum were regionally well transduced (Fig. 5Bf), as previously reported by others using rAAV2 vectors (13, 20).

In the testis, β-galactosidase-positive cells were occasionally observed but restricted to cells residing in the interstitial space. None of the cells in the seminiferous tubules were positive for β-galactosidase activity.

Southern blot analysis of DNA extracted from these tissues revealed that double-stranded vector genomes were detected in all tissues analyzed at relatively high levels (Fig. 6). The average double-stranded vector copy numbers in each tissue were 19.6 ds-vg/dge in the heart, 19.0 ds-vg/dge in the skeletal muscle, 14.4 ds-vg/dge in the lung, 13.7 ds-vg/dge in the kidney, 7.6 ds-vg/dge in the testis, 5.1 ds-vg/dge in the intestine, 4.2 ds-vg/dge in the spleen, 2.7 ds-vg/dge in the brain, and 2.1 ds-vg/dge in the pancreas (from the highest to the lowest). It should be noted that even in the tissues with few positive cells, such as the spleen, testis, and skeletal muscle, double-stranded rAAV8 vector genomes were detected at levels comparable to those in the tissues with many β-galactosidase-expressing cells, suggesting that the extent of β-galactosidase expression does not necessarily correlate with the level of vector genome dissemination.

Peripheral injection of a rAAV8 vector transduced all the skeletal and heart muscles and a majority of pancreatic cells in the context of the cytomegalovirus promoter. To further address the discrepancy of the histological and Southern blot analyses in the context of the AAV8-EF1α-nlslacZ vector, we repeated the tissue distribution study with the AAV8-CMV-
lacZ vector. We injected C57BL/6 rag-1 male mice via the tail vein with the AAV8-CMV-lacZ vector at a dose of 3.0 × 10^{11} or 7.2 × 10^{12} vg/mouse (n = 2 each). Three weeks after vector injection, we analyzed various tissues by X-Gal staining. At a dose of 3.0 × 10^{11} vg/mouse, hepatocytes were transduced at levels comparable to that with AAV8-EF1α-nlslacZ. However, interestingly, the best-transduced organ was not the liver but the heart. A majority of cardiomyocytes were transduced with AAV8-CMV-lacZ at a dose of 3.0 × 10^{11} vg/mouse (Fig. 5E), although not many β-galactosidase-expressing positive cells were observed in other nonhepatic tissues, including skeletal muscle.

At a dose of 7.2 × 10^{12} vg/mouse, the whole liver was transduced (Fig. 5E). Amazingly, AAV8-CMV-lacZ transduced the heart and skeletal muscles with an extremely high efficiency at this dose. The entire heart muscle was transduced, and virtually all the myofibers in skeletal muscles were trans-
FIG. 5. Representative photomicrographs of sections of various mouse tissues 6 weeks after tail vein injection of AAV8-EF1α-mlslacZ at a dose of $7.2 \times 10^{12}$ vg/mouse (A to D) or 3 weeks after tail vein injection of AAV8-CMV-lacZ at a dose of $3.0 \times 10^{11}$ or $7.2 \times 10^{12}$ vg/mouse (E). The sections were either X-Gal stained (A, B, D, and E) or stained with designated antibodies (C). (A) Tissue distribution of β-galactosidase-positive cells: lu, lung; h, heart; s, spleen; k, kidney; i, intestine; t, testis; p, pancreas; and m, skeletal muscle (quadriceps). The top row represents tissues from a mouse injected with excipient only, while the bottom row shows samples from vector-injected mice. (B) Brain transduction with rAAV8. (a) Cerebral cortex. Positive cells are scattered throughout the region. (b) Hippocampus. Positive cells are observed in both granule and pyramidal cell layers. (c) Striatum. (d) Amygdala. (e) Hypothalamus. β-Galactosidase-positive neurons and glial cells are clustered in the arcuate nucleus and median eminence. Some ependymal cells of the third ventricle are also positive. (f) Cerebellum. Purkinje cells are regionally well transduced. (C) Confocal microscopy to assess colocalization of β-galactosidase and either NeuN (a marker for neurons) or GFAP (a marker for astrocytes) to determine rAAV8-transduced cell types in the cerebral cortex of the brain. Both neurons and glial cells were transduced with rAAV8. Scale bars, 5 μm. (D) Transduction of vascular smooth muscle cells in the walls of a branch of the coronary artery (a) and a branch of the splenic artery (b). (E) Tissue distribution of β-galactosidase-positive cells in mice injected with AAV8-CMV-lacZ via the tail vein. The vector doses (vg/mouse) are indicated above the pictures. The section of the pancreas was also stained with anti-insulin antibody (brown cells). Scale bars (duplicated lines), 250 μm. lv, liver; t.a., tibialis anterior limb muscle. The tissues in panels A, B, D, and E were counterstained with nuclear fast red or light hematoxylin. Scale bars represent 100 μm unless otherwise noted.
efficiency, of a given target tissue. A novel vector can yield widespread transduction, with considerable uptake in nontarget tissues, particularly the heart, skeletal muscles, smooth muscles, pancreas, and brain. However, this opens a new possibility that systemic administration of the rAAV8 vector becomes promiscuous, leading to undesirable transduction in nontarget tissues, including liver, skeletal muscles, smooth muscles, pancreas, and brain. In each set of tissues, the left and right lanes represent samples from mice injected via the tail vein and portal vein, respectively. For densitometric analysis, see the Results section.

DISCUSSION

The present study was conducted to investigate whether rAAV vectors of alternative serotypes can stably transduce all hepatocytes by administration of a high vector dose. To address this issue, we have chosen the rAAV1 and rAAV8 vectors and performed a dose-response study with a nuclear-localizing LacZ-expressing vector. Both vectors have been shown to have liver transduction kinetics distinct from that of rAAV2 vectors (15, 17, 54). Although the rAAV1 vector failed to transduce all the hepatocytes due to a blunted dose-response at high vector doses, the present study clearly demonstrated that the dose-response profile of rAAV8 remained unchanged throughout a wide range of vector doses until all the hepatocytes were stably transduced at 7.2 × 10^{12} vg/mouse. Excluding the self-complementary vectors discussed below, this is the first report that a rAAV vector can yield ~100% hepatocyte transduction with no obvious toxicity after a simple, noninvasive peripheral vein injection. In addition, we demonstrated that intravenous injection of the rAAV8 vector into mice could transduce entire skeletal muscles throughout the body, the entire heart muscle, and substantial numbers of pancreatic cells, smooth muscle cells, and brain cells.

Our knowledge about the mechanisms of rAAV transduction is still very limited. We have been investigating why rAAV2 vectors can stably transduce only a subset of hepatocytes. Impaired vector uptake in a subset of hepatocytes cannot explain this observation because vector genomes were found in most of the hepatocytes a day following vector administration (32). Therefore, blocks at the level of post-vector entry processing should contribute to the restricted liver transduction with rAAV2. Such barriers include endosomal processing and viral coat modification, which involves the ubiquitin/proteasome system (9, 19), cytoplasmic trafficking (48, 50), nuclear entry, uncoating (54), conversion from single-stranded to double-stranded vector genomes (11, 12, 38), processing of double-stranded vector genomes into transcriptionally active molecules via vector genome recombination (8, 36), and stable residence in the nuclear environment, which allows transgene expression. It is not easy to reconcile various observations with some conflicts from different laboratories at this time, but several recent observations or innovations have provided several important clues to this issue.

Recently, self-complementary or double-stranded rAAV vectors have been developed (13, 30, 31, 57). Self-complementary rAAV vectors possess half-sized hairpin-like double-stranded vector genomes. The important feature of this vector is that it skips the requirement for duplex DNA formation from single-stranded vector genomes, which is one of the fundamental limiting steps for rAAV vector transduction (11, 12). It has recently been shown that ~90% of hepatocytes could be stably transduced when the self-complementary rAAV serotype 2 vector carrying a marker gene was injected into mouse livers (57). This suggests that release of vector genomes from viral virions occurs in most of the hepatocytes, and therefore the mechanisms for restricted liver transduction should be related to the inefficiency of duplex DNA formation from single-stranded vector genomes. However, and importantly, this does not necessarily exclude the possibility that factors upstream of duplex DNA formation may contribute to the inefficiency of liver transduction with rAAV2.

At least two models have been proposed to address the inefficient duplex DNA formation in rAAV2-mediated liver transduction. The first model involves cellular machinery that directly regulates duplex vector genome formation. Recently, Zhong et al. demonstrated that, in T-cell protein tyrosine phosphatase transgenic mice and FKBP52-knockout mice, the rAAV2 vector transduced 12 to 16 times more hepatocytes than the wild-type counterparts (61). In these mouse models, phosphorylated forms of FKBP52, known to bind to the AAV inverted terminal repeat and block second-strand synthesis (43), are downregulated or deficient. From their observations, they claimed that impaired duplex DNA formation by second-strand synthesis (11, 12) precludes efficient transduction. The second model involves an upstream factor, i.e., the rate of capsid uncoating indirectly determines the efficiency of duplex DNA formation. In this model proposed by Thomas et al. (54), slower capsid uncoating of rAAV2 than of rAAV8 limits the formation of duplex DNA in hepatocytes through annealing of complementary plus and minus single-stranded rAAV2 genomes (38), resulting in inefficient liver transduction with rAAV2 vectors. At present, however, we do not have a clear answer that explains why stable liver transduction with rAAV2, but not rAAV8, is restricted to a fraction of hepatocytes.

Nonetheless, the present study demonstrated that all hepatocytes are capable of processing single-stranded rAAV2 genomes (delivered with AAV8 capsids) into duplex DNA. This implies that viral capsid proteins, and not cellular factors by
themselves, substantially influence the efficiency of duplex DNA formation in each hepatocyte, at the level of post-vector entry processing of rAAV vectors. In other words, in rAAV2-nonpermissive hepatocytes, which account for ~90% of total hepatocytes and can take up rAAV2 vectors but not express transgene product, duplex rAAV2 vector genome formation is impaired at the level of post-vector entry when vector genomes are delivered with AAV2 capsids but not impaired when they are delivered with AAV8 capsids.

The tissue biodistribution profile after systemic administration of the rAAV8 vector has been reported in a study with a hemophili A mouse model (49). They injected mice with 10^{10} to 10^{11} vg/mouse. At a dose of 3.0 \times 10^{11} vg/mouse, and the heart was the best-transduced tissue among all the tissues analyzed including the liver at 3.0 \times 10^{11} vg/mouse. At a dose of 7.2 \times 10^{12} vg/mouse, 100% of cardiomyocytes were transduced. Although we could not determine the minimum rAAV8 vector dose required for 100% cardiomyocyte transduction, it is presumed to be much less than 7.2 \times 10^{12} vg/mouse, given that 3.0 \times 10^{11} vg/mouse was sufficient to transduce a majority of cardiomyocytes. Skeletal muscles were also well transduced at a high vector dose. At a dose of 7.2 \times 10^{12} vg/mouse, virtually all the myofibers in the entire skeletal muscle system throughout the body were transduced, although they were less susceptible to rAAV8 than cardiac muscle, given that not many myofibers were transduced at a dose of 3.0 \times 10^{11} vg/mouse. Recently, Gregorevic et al. have shown that intravascular administration of rAAV6 vectors resulted in widespread skeletal muscle transduction and entire cardiac muscle transduction, as we have observed with rAAV8 in our present study, and they also have established proof of principle that systemic administration of a rAAV6 vector can be used to treat Duchenne muscular dystrophy (16). It should be noted that in order to increase the permeability of the peripheral microvasculature, their method required simultaneous injection of vascular endothelium growth factor, which was not needed in the context of the rAAV8 vectors.

It is also intriguing that we observed extensive transduction in the pancreas with rAAV8 without any histological evidence suggestive of cell damage or inflammation. In agreement with the previous report on rAAV8 (55), pancreatic acinar cells were the major target, but insulin-producing pancreatic islet cells were also transduced to a certain extent. Our study has demonstrated that systemic administration of rAAV8 vectors could achieve pancreatic transduction at levels equivalent to or even higher than that achievable with adenovirus vectors (55).

It was surprising to us that tail vein or portal vein injection of the rAAV8 vector could transduce broad regions of the brain, since in general it is not possible to transduce this organ by systemic intravenous administration of viral vectors due to the presence of the blood-brain barrier. rAAV vector shedding with negligible levels in the brain has occasionally been reported in tissue distribution preclinical studies (10, 17, 32, 49, 58). However, none of these studies have investigated the origins of the PCR-positive signals. Whether rAAV traversed the blood-brain barrier and transduced neurons and glial cells or remained in the connective tissues including blood vessels has not been addressed. It is intriguing that the median eminence and arcuate nucleus of the hypothalamus and basolateral nucleus of the amygdala were focally transduced with high efficiency. The mechanism(s) underlying focal high transduction is not clear but may be related to a rich blood supply. Interestingly, the hypothalamus is known to have fenestrated capillaries that have numerous small pores increasing vascular permeability.

Dissemination of the viral vectors to the brain is a serious concern in terms of liver gene therapy, but gene delivery to neurons and glial cells by viral vectors holds great promise for gene therapy for central nervous system diseases. Direct intracranial injection of vectors allows efficient transduction of brain tissue, but the transduction is normally limited to the vicinity of the injection site. Many central nervous system diseases broadly affect brain tissue, and therefore global brain transduction by alternative approaches is often preferred. However, the presence of the blood-brain barrier has precluded widespread transduction of the brain. Recently, two strategies, in utero gene transfer (26, 27) and systemic or regional viral administration after mannitol infusion (13, 28), have been shown to successfully overcome this hurdle. The former approach takes advantage of the immaturity of the fetus’s blood-brain barrier with increased permeability, and the latter transiently disrupts the blood-brain barrier by making a hyperosmotic environment in the brain capillaries. The mechanisms by which rAAV8 could efficiently traverse the intact blood-brain barrier have yet to be elucidated, and whether rAAV8 particles were actively escorted by a not-yet-defined system or the high dose of rAAV8 vector infusion itself damaged the blood-brain barrier needs to be addressed.

It should be noted that the viral preparation we used for this study contained 5% sorbitol in PBS. Sorbitol is a carbohydrate with the same molecular weight as mannitol and is used clinically to introduce a hyperosmotic environment. Fu et al. reported that, in order to open the blood-brain barrier and transduce mouse brain tissue with intravenously administered rAAV2, preinfusion of 200 \mu l of 25% mannitol (corresponding to 50 mg of mannitol) was required, and simultaneous infusion of the same amount of 12.5% mannitol (corresponding to 25 mg) had no effect. In our study, we injected 300 \mu l of vector preparations (equivalent to 15 mg of mannitol), and therefore it is unlikely that our excipient contributed to the transient disruption of the blood-brain barrier. Nonetheless, our study clearly demonstrated that intravenous administration of rAAV8 vectors can transduce neurons and glial cells in broad regions of the adult mouse brain without any treatment that disrupts the blood-brain barrier. Although the mechanism is not clear, rAAV8 will offer an alternative approach to global central nervous system gene delivery in combination with currently available strategies.
Except for the liver, direct injection into the target tissue is a standard approach for transduction with rAAV vectors. This approach is desirable because it can minimize the possibility of vector dissemination to remote organs. However, it has often suffered from the confinement of vectors to the injection site, precluding widespread transduction in a target organ. In this regard, rAAV8 may be applied for global transduction in a given nonhepatic target organ. All the tissues analyzed had double-stranded rAAV8 vector genomes at levels of at least 2 ds-vg/dge. It is important to emphasize that vector dissemination was determined by genomic DNA Southern blot analysis and not a PCR-based assay. This method is superior in detecting double-stranded vector genomes formed within cells. It should be noted that transduction efficiency determined by transgene expression and vector genome copy numbers were not correlated. Presumably the promoter activities vary among recombinant adeno- associated virus type 2, 4, and 5 vectors: transduction of variant cell types and regions in the mammalian central nervous system. Proc. Natl. Acad. Sci. USA 97:3428–3432.


