Dose Response in Rodents and Nonhuman Primates After Hydrodynamic Limb Vein Delivery of Naked Plasmid DNA

Christine I. Wooddell,¹ Julia O. Hegge,¹ Guofeng Zhang,^{2,*} Magdolna G. Sebestyén,¹ Mark Noble,¹ Jacob B. Griffin,¹ Loretta V. Pfannes,^{1,†} Hans Herweijer,¹ James E. Hagstrom,¹ Serge Braun,³ Thierry Huss,⁴ and Jon A. Wolff^{2,*}

Abstract

The efficacy of gene therapy mediated by plasmid DNA (pDNA) depends on the selection of suitable vectors and doses. Using hydrodynamic limb vein (HLV) injection to deliver naked pDNA to skeletal muscles of the limbs, we evaluated key parameters that affect expression in muscle from genes encoded in pDNA. Short-term and long-term promoter comparisons demonstrated that kinetics of expression differed between cytomegalovirus (CMV), muscle creatine kinase, and desmin promoters, but all gave stable expression from 2 to 49 weeks after delivery to mouse muscle. Expression from the CMV promoter was highest. For mice, rats, and rhesus monkeys, the linear range for pDNA dose response could be defined by the mass of pDNA relative to the mass of target muscle. Correlation between pDNA dose and expression was linear between a threshold dose of 75 $\mu\text{g/g}$ and maximal expression at approximately 400 $\mu\text{g/g}$. One HLV injection into rats of a dose of CMV-LacZ yielding maximal expression resulted in an average transfection of 28% of all hind leg muscle and 40% of the gastrocnemius and soleus. Despite an immune reaction to the reporter gene in monkeys, a single injection transfected an average of 10% of all myofibers in the targeted muscle of the arms and legs and an average of 15% of myofibers in the gastrocnemius and soleus.

Introduction

AN ATTRACTIVE APPROACH for the treatment of muscle diseases is nonviral delivery of naked plasmid DNA (pDNA) expressing therapeutic genes (Sebestyén *et al.*, 2007; Braun, 2008; Duan, 2008; Zhang *et al.*, 2010). Plasmid DNA is a stable molecule that is relatively inexpensive to produce in large quantities. Unlike viral vectors, repeat deliveries of pDNA for chronic diseases are possible because pDNA does not induce an immune response toward the vector (Jiao *et al.*, 1992; Le *et al.*, 2000; Zhang *et al.*, 2001; Hagstrom *et al.*, 2004; Romero *et al.*, 2004). The pDNA delivered to skeletal muscles of rodents or primates is retained in muscle fibers and expresses the encoded gene product for many months, if not years (Wolff *et al.*, 1992; Danko *et al.*, 1993, 1997; Zhang *et al.*, 2004, 2010; Sebestyén *et al.*, 2007). Long-term expression of foreign genes can be obtained from naked pDNA without

chromosomal integration if the target cell is postmitotic (e.g., muscle fibers) or slowly mitotic (e.g., hepatocytes) and if there is no immune response to the expressed gene (Wolff *et al.*, 1992; Miao *et al.*, 2000; Herweijer *et al.*, 2001; Zhang *et al.*, 2001; Wooddell *et al.*, 2008).

Initially pDNA was delivered to muscles by intramuscular injection (Wolff *et al.*, 1992; Danko *et al.*, 1993, 1997). Vascular delivery procedures were then developed to deliver pDNA to skeletal muscles of a whole limb by injection into an artery (Budker *et al.*, 1998; Zhang *et al.*, 2001, 2004; Danialou *et al.*, 2005) or a vein (Hagstrom *et al.*, 2004; Kamimura *et al.*, 2010). The hydrodynamic limb vein (HLV) injection procedure is a facile method by which to deliver naked nucleic acids to skeletal muscles throughout the limbs of rodents and nonhuman primates (Hagstrom *et al.*, 2004). A tourniquet is used to delimit the delivery area in the limb. Naked pDNA is rapidly injected into the vein in the antegrade direction

¹Roche Madison, Madison, WI 53711.

²Departments of Pediatrics and Medical Genetics, Waisman Center, University of Wisconsin-Madison, Madison, WI 53705.

³Association Française contre les Myopathies, 91002 Evry, France.

⁴Transgene, 67405 Illkirch Graffenstaden, France.

*Present address: Roche Madison, Madison, WI 53711.

†Present address: Medical College of Wisconsin, Milwaukee, WI 53226.

with a volume of saline that is sufficient to facilitate extravasation of the pDNA from the vasculature and into the muscle tissue. This is a nonsurgical procedure in large animals, as the injection is transcutaneous. In rodents minor surgery is required to visualize the vein during injection. Multiple injections can be performed to increase expression levels or to extend the duration of expression (Hagstrom *et al.*, 2004; Sebestyen *et al.*, 2007; Zhang *et al.*, 2010). The HLV injection procedure is safe in dystrophic muscle, and the pDNA is delivered equally well to muscles of dystrophic *mdx* mice as to those of wild-type mice (Zhang *et al.*, 2010). This procedure has an excellent safety profile (Hagstrom *et al.*, 2004; Toumi *et al.*, 2006; Vigen *et al.*, 2007; Hegge *et al.*, 2010) and is a compelling candidate for the treatment of neuromuscular diseases.

Using an effective dose of pDNA to express a therapeutic gene will yield optimal results from gene therapy treatments, both in efficacy of the treatment and cost of the therapy. We performed these studies to understand how skeletal muscles of rodents and primates respond to the pDNA dose delivered by HLV injection and to address factors that affect gene expression from the pDNA. First, we compared the ubiquitously expressed cytomegalovirus (CMV) promoter with muscle promoters for short-term and long-term expression levels in mouse muscle and for muscle-specific expression. We then evaluated effects of pDNA size, multiple pDNA injections, and dose relative to target muscle mass to optimize pDNA-encoded gene expression in rodents and non-human primates. Using a dose of pDNA optimized in this way, we then determined the percentages of target limb muscles that were transfected in rats and rhesus monkeys.

The purity of pDNA preparations is important for consistency of expression. Most experiments presented here were performed with pDNA that had low levels of bacterial DNA contamination and that caused no muscle damage when tested in mice (Wooddell *et al.*, 2010). Some of the luciferase-expressing pDNA used in monkey studies early on was not evaluated in this regard, which could have led to greater variability in these data.

Materials and Methods

Animals

Outbred ICR mice and inbred C57BL/6 wild-type mice were 8–12 weeks old. Sprague-Dawley rats were 130–157 g in weight. These rodents were obtained from Harlan Laboratories (Indianapolis, IN). Breeding pairs of B6Ros. Cg-Dmd^{mdx-4Cv}/J (*mdx4Cv*) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred at the University of Wisconsin-Madison (Madison, WI). Male mice and female rats were used in experiments. Animals had access *ad libitum* to water and Teklad rodent diet (Harlan Laboratories) or mouse diet 5015 chow (PMI Nutrition International, Brentwood, MO). Nonhuman primate studies were performed in adult rhesus monkeys. Monkeys were 2.5 to 34 years of age and 3.9 to 11.5 kg in weight. Fourteen were males and 2 were females. All procedures were carried out in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals* and were approved by the Mirus Bio (Madison, WI), Roche Madison (Madison, WI), or University of Wisconsin-Madison Animal Care and Use Committee.

Plasmid DNA

CMV-Lux, DCR-Lux, and hCK-Lux include a hybrid 16S/19S simian virus 40 (SV40) intron (Okayama and Berg, 1983), the *Photinus pyralis* (firefly) peroxisomal luciferase (Lux) coding sequence (GenBank accession number M15077), the SV40 late polyadenylation [p(A)] signal, and a kanamycin resistance gene. Promoters include the human cytomegalovirus immediate-early enhancer and promoter (CMV) sequence from position –749 to +7 with respect to the transcription start site (GenBank accession number AY315197), the 9546-bp human desmin promoter and truncated locus control region (DCR) as previously described (Zhang *et al.*, 2004), and the human muscle creatine kinase (hCK) promoter sequence from position –2628 to +49 (GenBank accession number M21487, locus HUMCKMM1). CMV-Luc (5.7 kb) was constructed by inserting the codon-optimized firefly luciferase gene (Promega, Madison, WI) into the pCI mammalian expression vector (Promega). This vector contains the CMV promoter, a β -globin-IgG hybrid intron, the SV40 late poly(A) signal, and the ampicillin resistance gene. CMV-Luc (11.4 kb) and CMV-Luc (17.1 kb) were constructed from CMV-Luc (5.7 kb) by insertion of stuffer sequences from λ phage to increase their size, as previously described (Zhang *et al.*, 2004). The expression cassette in CMV-Luc (pMIR48) is the same as in CMV-Luc, but pMIR48 is kanamycin resistant. CMV-LacZ contains the β -galactosidase (β -Gal) coding sequence in pCI. mMCK-Luc was constructed by replacing the CMV promoter in CMV-Luc (5.7 kb) with the full-length murine muscle creatine kinase promoter (mMCK) sequence from position –3351 to +30 (GenBank accession number AF188002). CMV-mSEAP was constructed by replacing the Lux gene in CMV-Lux with the CpG-free murine secreted alkaline phosphatase coding sequence (mSEAP; InvivoGen, San Diego, CA). DCR-mSEAP, mMCK-mSEAP, and hCK-mSEAP were constructed by replacing the promoter of CMV-mSEAP. The CMV-hSEAP construct carries the human placental alkaline phosphatase gene (SEAP) from pSEAP2-Basic (Clontech, Mountain View, CA) instead of Luc in pMIR48. Empty vector pMIR266 is a kanamycin-resistant plasmid of 2257 bp containing no eukaryotic promoter. All plasmids contain the pUC-based ColE1 origin of replication. Preparations of pDNA were purchased from Aldevron (Fargo, ND) or PlasmidFactory (Bielefeld, Germany) and contained endotoxin levels of less than 100 EU/mg. The pDNA used in most studies contained <1.3% bacterial genomic DNA contamination and caused no muscle toxicity when tested as previously described (Wooddell *et al.*, 2010), but some batches of pMIR48 used in monkey studies had not been tested in this manner.

Hydrodynamic tail vein delivery of pDNA into mice

pDNA was diluted in a volume of Ringer's solution equal to 10% of the weight of the mouse. This pDNA solution was injected into the lateral tail vein through a 27-gauge needle over 5–7 sec as described previously (Zhang *et al.*, 1999).

Hydrodynamic limb vein delivery of plasmid DNA into rodents

Mice and rats were anesthetized with 1–2% isoflurane (Abbott Laboratories, North Chicago, IL) and injected via the

saphenous vein of the hind leg as previously described with a pDNA–saline solution (Hagstrom *et al.*, 2004; Zhang *et al.*, 2010). The pDNA–saline solution was injected into mice at a rate of 8 to 12 ml/min and into rats at 10 ml/min. When multiple injections were performed in the same leg, the injections were done 2 weeks apart.

Rodent euthanization and tissue preparation

Rodents were anesthetized with 3–5% isoflurane and killed. To determine luciferase or β -Gal reporter gene expression in limb muscles, the skin was removed from the hind limb to expose the muscles. All muscles of the hind leg from above the quadriceps, where the tourniquet was positioned, and down to the ankle were divided into five groups: (1) the anterior group of muscles of the upper leg (includes the quadriceps), (2) the posterior group of muscles of the upper leg (includes the biceps femoris), (3) the medial group of muscles of the upper leg (includes the gracilis and adductor muscles), (4) the posterior group of muscles of the lower leg (gastrocnemius and soleus), and (5) the anterior group of muscles of the lower leg (primarily the tibialis anterior and extensor digitorum longus). All of the hind leg muscles distal to the tourniquet were considered the “target muscle.” Weights of the muscle groups were added together to determine the total weight of the target muscle for each animal.

Hydrodynamic limb vein delivery of pDNA into primate limbs

Rhesus monkeys were given transcutaneous HLV injections of pDNA in saline via the cephalic vein of the arm and the small saphenous vein of the leg as previously described (Hegge *et al.*, 2010). A pediatric-size tourniquet cuff was applied snugly to the limb just above the elbow or knee to occlude blood flow. The tourniquet pressure varied between 450 and 1000 mmHg. It was 450 mmHg during most injections. The injection solution volume was determined by measuring limb volume (area distal to the tourniquet), using fluid displacement, and multiplying this volume by a number between 0.30 and 0.45, depending on the study. An injection solution volume of 0.4 ml/ml displaced limb volume resulted in optimal tissue swelling, which is required for efficient delivery (Vigen *et al.*, 2007). The length of time that the tourniquet remained inflated after the injection was 2 min except for two injections, after which the tourniquet was slowly released over 20 sec. The injection rate was varied from 0.5 to 4 ml/sec; it was 2 ml/sec for most injections. Only injections that resulted in at least modest swelling were compared in the analyses. Most animals had all four limbs injected during two procedures. One arm and one leg were injected on day 0 and the opposite arm and leg were injected on day 3.

Monkey euthanization and tissue preparation

For luciferase expression studies, monkeys were killed on day 7 and all limb muscles below the knee or elbow were harvested to measure expression. As previously listed, a total of 21 muscles were harvested from the arms and 15 muscles were harvested from the legs (Hagstrom *et al.*, 2004). Large muscles were divided into multiple smaller pieces and each muscle was weighed and then immediately frozen in liquid nitrogen. Limb muscles that were injected on day 0 gave “7-

day” expression and limbs that were injected on day 3 gave “4-day” expression. As previously described, luciferase expression from this plasmid with the CMV promoter increases on average 3.1-fold between day 4 and day 7 in monkey muscle (Vigen *et al.*, 2007). To allow direct comparison between both time points, therefore, the day 4 luciferase data had to be adjusted by multiplying the measured values by this factor.

For β -Gal expression studies, monkey 135-23 was killed on day 7 and monkey 135-22 was killed on day 11. Muscles were harvested as for luciferase expression studies. A piece was removed from the center of each muscle and frozen in O.C.T. compound (Sakura Finetek USA, Torrance, CA) for sectioning. The remainder of the muscle to be used for the β -Gal enzymatic assay was snap frozen in liquid nitrogen.

Luciferase and SEAP reporter gene assays

Muscle or liver pieces were homogenized and assayed for luciferase activity as previously described (Hegge *et al.*, 2010). For SEAP assays, serum was collected from rodent blood. Mice were briefly anesthetized with 1–2% isoflurane and blood samples were obtained from the retro-orbital plexus. Rats were briefly anesthetized with 2–3% isoflurane and a 23-gauge needle connected to a syringe was used to collect about 0.5 ml of blood from the jugular vein. Blood was allowed to coagulate at ambient temperature for 15 min; tubes were then centrifuged at 8000 g for 2 min to separate the serum. Serum samples were stored on ice before the assay. Alkaline phosphatase activity of the human SEAP reporter gene expressed in rat serum was measured with a Phospha-Light chemiluminescent reporter assay kit (Applied Biosystems/Life Technologies, Carlsbad, CA), according to the manufacturer’s instructions. To assay samples containing mouse SEAP, the serum (15 μ l) was diluted in dilution buffer (45 μ l; Phospha-Light chemiluminescent assay kit) and heated at 65°C for 10 min. Reaction buffer (50 μ l) was then added to 50 μ l of diluted sample, incubated at ambient temperature for 20 min, and analyzed for SEAP activity with a luminometer. Relative light units were converted to nanograms, using a standard curve with human placental alkaline phosphatase (P3895; Sigma-Aldrich, St. Louis, MO). Serum from mice injected with empty vector was used to control for background levels of alkaline phosphatase activity in the serum.

β -Galactosidase assays

For β -Gal staining, 10- μ m-thick cross-sections were cut from the middle of each rodent muscle group or from the middle of each monkey muscle. These were allowed to air dry and were then fixed in a 1.25% glutaraldehyde solution for 2 min, washed in phosphate-buffered saline (PBS), and then incubated in staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM magnesium chloride, 1 mM 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside in PBS; pH 7.6) for 2–3 hr at ambient temperature. After incubation, sections were washed in PBS and then counterstained with hematoxylin and eosin (H&E). Mouse and monkey muscles were immunostained for detection of β -Gal and dystrophin. Mouse anti- β -Gal antibody (primary antibody produced in the laboratory of J.A. Wolff) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat

anti-mouse Fab-specific IgG secondary antibody (T6528; Sigma-Aldrich) were used for β -Gal staining. Rat anti-dystrophin antibody (primary antibody; Wolff laboratory) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG secondary antibody (F6258; Sigma-Aldrich) were used for dystrophin staining. Percentages of β -Gal-positive (β -Gal⁺) cells and the total number of cells were determined in cross-sectional areas of the specimens, using a fluorescence microscope (DP71 camera and inverted microscope; Olympus American, Center Valley, PA).

To quantitate the amount of β -Gal enzymatic activity, all muscle remaining after sectioning was weighed, homogenized in lysis buffer (15 ml/g) (100 mM potassium phosphate, 0.1% Triton X-100; pH 7.8), and centrifuged at 3313 g for 10 min at 4°C. Aliquots of 10 μ l were analyzed by chemiluminescence assay (Applied Biosystems/Life Technologies) and relative light units were converted to micrograms by comparison with a standard curve (G5635; Sigma-Aldrich).

Regression analysis was used to estimate the percentage of β -Gal-expressing myofibers in each monkey muscle. Using all of the muscles for which we performed both cell counting and the enzymatic β -Gal assay, percentages of β -Gal⁺ myofibers were graphed relative to the amount of β -Gal enzymatic activity in the same muscle. Within the linear range, β -Gal expression was expected to increase in direct proportion to the dose. Higher doses result in higher expression levels as a result of increased expression within transfected myofibers as well as by transfection of more myofibers. For this reason the percentage of transfected myofibers was graphed on a linear scale and the amount of enzymatic β -Gal expression was graphed on a logarithmic scale (as in Fig. 6e). For determination of a conversion formula, day 7 and 8 postinjection muscles were analyzed separately from day 11 muscles. A trend line fitting the data provided an equation to determine the approximate percentages of transfected myofibers in groups of muscles and in the whole target area of the limb (see Table 2).

Statistical analysis

Experimental error is given as the standard error (SEM) and statistical significance was determined by two-tailed *t* tests. Prism software (GraphPad, San Diego, CA) was used for these analyses.

Results

Promoter comparison studies in rodent muscle

Promoters were compared in rats to determine which promoter would express the highest level of a plasmid-encoded gene product in skeletal muscles. The ubiquitously expressed human cytomegalovirus (CMV) promoter was compared with three promoters expressed primarily in muscle: the muscle creatine kinase promoters from mouse (mMCK) and human (hCK) and the human desmin promoter and truncated locus control region (DCR). Plasmid constructs carrying the peroxisomal luciferase gene (Lux) or the codon-optimized luciferase (Luc) gene were used (Fig. 1a). Promoters were compared from equal molar doses of plasmid constructs delivered to the hind limb muscles by HLIV injection (Hagstrom *et al.*, 2004). Animals were killed 7

days after the HLIV injection to avoid immune complications that could result from later time points. All muscles of the injected hind limb were harvested, homogenized, and assayed for luciferase expression. Luc gene constructs express approximately three times more luciferase than their Lux counterpart. Therefore, activity of the muscle gene promoters in constructs expressing Lux were compared with CMV-Lux, whereas muscle promoters in constructs expressing Luc were compared with CMV-Luc. The CMV promoter was approximately two times more active than each of the muscle promoters ($p = 0.02$), which all had similar activity (Fig. 1a).

The same promoters were then evaluated for long-term expression levels and the stability of expression. To avoid an immune response and to allow sampling from the same animals over an extended period of time, long-term comparison of these promoters was performed in mice, using mouse secreted alkaline phosphatase (mSEAP) as the reporter gene (Fig. 1b and c). Plasmid constructs were identical except for the promoter. Equal molar amounts of each construct were delivered to the hind limb muscles by HLIV injection. This study revealed that expression levels from the CMV, mMCK and hCK promoters changed considerably during the first 2 weeks after the HLIV injection (Fig. 1b). On day 4, CMV expression was 56 times greater than that of hCK, 18 times more than mMCK, and 8 times more than DCR ($p < 0.0001$). However, CMV expression of mSEAP decreased from day 4 to day 14, whereas both mMCK and hCK expression of mSEAP increased during this time. On day 7, CMV expressed 5 times more mSEAP than did mMCK and DCR and 13 times more compared with hCK ($p < 0.0001$). On day 14, however, CMV expression was 2-fold higher than mMCK expression ($p = 0.009$), 3-fold higher than hCK expression ($p = 0.0001$), and 4-fold higher than DCR expression ($p < 0.0001$). Relative expression levels of the CMV and muscle promoters changed dramatically during the first 2 weeks, but then remained more similar for the next 47 weeks (Fig. 1c).

Although the CMV promoter gave the highest expression levels at every time point, each of the promoters maintained high and nearly stable expression levels from weeks 2 to 49. Expression from the DCR promoter construct was the most stable. At week 49, the average expression levels in mice injected with DCR-mSEAP was the same as it was at week 2 (100%), whereas from the other three promoters it dropped to 67% (CMV), 64% (mMCK), and 55% (hCK).

Muscle specificity of the promoters was evaluated by delivering Lux expression plasmids driven by the CMV, DCR, hCK, or mMCK promoter to mouse liver, using hydrodynamic tail vein injection (Fig. 1d and e). Delivery of the pDNA is accomplished by rapid injection of a large volume of pDNA-containing solution into the mouse tail vein, and this results in delivery that is almost exclusively to liver hepatocytes (Liu *et al.*, 1999; Zhang *et al.*, 1999). After hydrodynamic tail vein injection of modest doses in the range typically used for tail vein injection (approximately 10–30 μ g of pDNA), the CMV promoter produced more than 100 times more luciferase than the DCR or hCK promoters (Fig. 1d). This result is not surprising for comparison of viral and muscle-specific promoters. When the pDNA doses were 10-fold higher, more similar to the doses used for HLIV injection into the skeletal muscles of the limb, CMV expression was 10-fold higher than mMCK expression in liver (data not

FIG. 1. Promoter comparisons in rodent skeletal muscle and in liver. (a) Equal molar doses of luciferase expression plasmids containing the CMV, mMCK, hCK, or DCR promoter were delivered to rat hind limb skeletal muscles by hydrodynamic limb vein (HLV) injection. All hind leg muscles were harvested on day 7, homogenized, and assayed for luciferase expression. Plasmids expressing the peroxisomal luciferase gene (Lux) were used to compare the hCK and DCR promoters with the CMV promoter. Rats were injected with 490 µg of CMV-Lux, 647 µg of hCK-Lux, or 1241 µg of DCR-Lux ($n = 5$). The mMCK promoter in mMCK-Luc was compared with the CMV promoter in CMV-Luc; these plasmids expressed the codon-optimized luciferase gene. Rats were injected with 500 µg of CMV-Luc ($n = 8$) or 735 µg of mMCK-Luc ($n = 4$). Luciferase expression for each set of plasmids is shown relative to their CMV counterpart. (b and c) Equal molar doses of mSEAP expression plasmids were injected into ICR mice: CMV-mSEAP (100 µg, solid circles, $n = 6$), mMCK-mSEAP (145 µg, open squares, $n = 8$), DCR-mSEAP (257 µg, solid triangles, $n = 8$), hCK-mSEAP (133 µg, open triangles, $n = 8$), or empty vector (98 µg, x symbols, $n = 5$). SEAP activity in the serum was measured at the indicated time points after injection. (d and e) Muscle specificity of the promoters was evaluated by hydrodynamic tail vein injection with equal molar doses of luciferase expression plasmid to deliver pDNA to mouse liver. Expression from the promoters was compared after injection of a low dose ($n = 5$) of CMV-Lux (12.3 µg), hCK-Lux (16.2 µg), or DCR-Lux (31 µg) (d) or a high dose ($n = 3$ or 4) of CMV-Lux (100 µg) or DCR-Lux (253 µg) (e). Liver was harvested 1 day later, homogenized, and assayed for luciferase activity.

shown), but only 2 times higher than expression from the DCR promoter (Fig. 1e). Thus, the DCR promoter at a high pDNA dose was not muscle specific.

For all further development and optimization of the HLV injection procedure, plasmid constructs containing the high-expressing and well-characterized CMV promoter were used to evaluate gene transfer and expression.

Linear range for dose response

For an accurate comparison of promoter strength or to evaluate changes in injection parameters, it is essential that the dose of pDNA be within the linear range for expression. To evaluate the linear range, mice were injected with various doses of CMV-mSEAP and expression was monitored weekly from serum samples (Fig. 2). mSEAP expression correlated in a linear manner with HLV-injected doses of 30 to approximately 240 µg of this plasmid (Fig. 2a). Even

though the measured amount of mSEAP was variable from assay to assay, as seen with the long-term promoter comparisons, the relative amounts of expression from each dose were the same 7 and 81 days after injection of pDNA (data not shown).

We then compared expression levels within the linear dose range, but delivered as one to four separate HLV injections of CMV-mSEAP. The stable, long-term expression from this plasmid allowed us to monitor expression levels over time in the same animals, and with injections that were given 2 weeks apart. Mice were injected with pDNA doses of 30, 60, and 120 µg (Fig. 2b and c). One injection of 60 µg of CMV-mSEAP produced an average of 2.1 times more mSEAP than one injection of 30 µg of the plasmid ($p = 0.003$; Fig. 2b). The same average amount of mSEAP was expressed from two injections of 30 µg of CMV-mSEAP as from one injection of 60 µg ($p = 0.60$). One injection of 120 µg of CMV-mSEAP produced as much mSEAP as four separate

FIG. 2. Linear range for dose response in rodents. (a) ICR mice weighing 24 to 30 g were treated once by HLX injection of 30 µg ($n=40$), or of 60, 90, 120, or 240 µg ($n=10$ mice per dose), of CMV-mSEAP. Serum was collected on day 14 and assayed for mSEAP expression. The average expression levels from each dose are shown relative to expression from 30 µg of CMV-mSEAP. (b and c) ICR mice were given one to four HLX injections of CMV-mSEAP at the indicated doses. Injections were done 2 weeks apart. Serum samples were collected 28 days (b) and 81 days (c) after the first injection and assayed for SEAP activity. (d) Wild-type C57BL/6 mice ($n=8$ per dose) weighing an average of 24 g and *mdx* mice ($n=8$ per dose) weighing an average of 28 g were injected with 100, 200, 400, or 800 µg of CMV-Luc (5.7 kb) and killed 7 days later for evaluation of luciferase

expression in muscle. For the wild-type mice (solid squares) and *mdx* mice (open circles), the total amount of luciferase per gram of target muscle in the injected leg is graphed relative to the dose of pDNA per gram of target muscle of the leg. (e) Luciferase expression in each of the five groups of hind leg muscles from the C57BL/6 mice shown in (d) is graphed relative to the HLX-injected dose of CMV-Luc.

injections of 30 µg ($p=0.97$) and an average of 4.8 times more than one injection of 30 µg of the plasmid ($p=0.004$) (Fig. 2c). Thus, multiple injections gave additive average expression levels from doses of pDNA that were in the linear range for expression.

Animals vary in size and thus the dose of pDNA per mass of muscle in the limb, the "target muscle," differs between animals injected with the same dose of pDNA if their target muscles differ in weight. Even animals with the same body weight may contain different amounts of muscle. For example, dystrophic *mdx* mice contain more muscle mass than do wild-type mice that have the same body weight (Gillis, 1999). We then compared the linear range for expression in two different strains of mice and in other species by defining the pDNA dose as micrograms of pDNA per gram of target muscle.

Escalating doses of CMV-Luc (5.7 kb) from 100 to 800 µg were administered by HLX injection into wild-type C57BL/6 and dystrophic *mdx* mice in order to determine both the linear range for expression and the maximal level of expression that could be obtained (Fig. 2d). The average weight of all muscles in the hind leg of wild-type mice was 0.6 g, and in *mdx* mice it was 1.1 g. The highest Luc-expressing group of wild-type mice had been injected with 400 µg of CMV-Luc, which was equivalent to an average of 620 µg of pDNA per gram of muscle. Luc expression was equally high in *mdx* mice injected with 400 or 800 µg of CMV-Luc, although the 400-µg dose was equivalent to an average of 360 µg/g muscle and the 800-µg dose was equivalent to an average of

826 µg/g muscle. In both strains of mice luciferase expression increased as the dose increased until maximal expression was obtained at doses of approximately 400 to 600 µg/g target muscle ($p<0.0001$ for the correlation between pDNA dose and Luc expression in both strains). Luciferase expression was notably lower when pDNA doses greater than 1000 µg/g muscle were injected into wild-type mice (Fig. 2d). None of the *mdx* mice were injected with doses this high.

Reporter gene expression in response to escalating doses of CMV-Luc (5.7 kb) was then evaluated in individual groups of muscles in wild-type mice (Fig. 2e). Maximal expression required more than 200 µg of pDNA. At doses of 200 and 400 µg of pDNA, the posterior lower leg muscles, including the gastrocnemius and soleus, expressed more luciferase than did the other groups of muscles, in which expression was more similar. Maximal expression was thus obtained from pDNA doses that were between 400 and 600 µg/g target muscle in two strains of mice (Table 1). Expression of reporter gene plasmids decreased when the dose of pDNA was high, possibly because the reporter gene was immunogenic.

Relative transfection efficiency between small and large plasmids is a function of the injected dose

With a coding sequence of 11 kb, the dystrophin gene is one of the largest mammalian genes. Plasmids carrying the full-length dystrophin gene are thus much larger than typical reporter plasmids such as the 5.7-kb CMV-Luc, the 5.6-kb

TABLE 1. DOSES OF REPORTER GENE PLASMID DNA IN RODENT AND PRIMATE STUDIES

Animal	Expression construct	pDNA size (kb)	Injection volume (ml/g target muscle)	Optimal pDNA dose (μ g pDNA/g muscle)	Concentration of pDNA in injection solution for optimal dose (mg pDNA/ml)
ICR mouse	CMV-Luc	5.7	1.6	400–600	0.25–0.38
<i>mdx</i> mouse	CMV-Luc	5.7	1.5	400–600	0.27–0.40
ICR mouse	CMV-Luc	17.1	1.6	300–600	0.19–0.38
<i>mdx</i> mouse	CMV-Luc	17.1	1.5	300–500	0.20–0.33
C57BL/6 mouse	CMV-LacZ	7.1	1.2	(120) ^a	0.1
Sprague-Dawley rat	CMV-LacZ	7.1	0.8	220 ^b	0.28
Rhesus monkey	CMV-LacZ	7.1	1.14	400	0.35

^aThe dose of CMV-LacZ was not optimized in mice.

^bNo doses of CMV-LacZ between 220 μ g/g and the toxic 540 μ g/g were tested.

CMV-mSEAP, or the 7.1-kb CMV-LacZ. We previously showed that with either intramuscular injection or hydrodynamic intraarterial injection larger plasmids were delivered into muscle fibers less efficiently than smaller plasmids (Zhang *et al.*, 2004). We determined the effect of plasmid size on HLV delivery by using the same CMV-Luc expression cassette in plasmids that were 5.7, 11.4, and 17.1 kb in size (Fig. 3). Equal molar doses of these constructs were injected into the hind limb of wild-type mice (Fig. 3a). The doses relative to muscle weight were 139 μ g/g 5.7-kb pDNA, 242 μ g/g 11.4-kb pDNA, and 337 μ g/g 17.1-kb pDNA. The highest expression levels were obtained from the smallest of the plasmids. A 2-fold increase in plasmid size, from 5.7 to 11.4 kb, resulted in $30 \pm 9\%$ less Luc expression. The plasmid that was 3-fold larger (17.1 kb) yielded $43 \pm 5\%$ lower expression than the 5.7-kb plasmid.

Experiments were then performed to determine how the size of the HLV-injected pDNA affects the linear range and maximal expression (Fig. 3b and c). Wild-type mice were injected with escalating doses of 100 to 800 μ g of the 17.1-kb CMV-Luc to compare luciferase expression levels with those from the same doses of 5.7-kb CMV-Luc. Maximal expression of the 17.1-kb plasmid was obtained from approximately the same mass of plasmid as from 5.7-kb CMV-Luc, about 400 μ g/g target muscle in the limb, and thus a lower molar dose (Fig. 3b and c). Maximal expression from the 17.1-kb plasmid was approximately 4-fold lower than that from the 5.7-kb plasmid. The same escalating doses of 17.1-kb CMV-Luc were delivered to *mdx* mice by HLV injection (Fig. 3c). Even though *mdx* mice weigh more than wild-type mice, expression levels in the two strains were similar when the dose of pDNA relative to the muscle weight was the same (Fig. 3c).

Taken together, our observations indicate that pDNA gene expression levels from larger (mass) doses of pDNA are inversely proportional to pDNA size, but that pDNA size has less effect on smaller injected doses.

Plasmid expression levels are dependent on the mass of pDNA as well as the molar quantity

Rhesus monkeys were used to optimize the HLV injection procedure for clinical application. A Luc reporter gene plasmid was used to compare injection parameters during 45 HLV injections of monkey limbs (Fig. 4a). Data from some of

these animals have been shown in prior publications regarding HLV injection optimization and safety (Vigen *et al.*, 2007; Hegge *et al.*, 2010). Here we focus on only the pDNA dose and resulting Luc expression. These studies involved several variables as described in Materials and Methods. Many different batches of pDNA were used. Monkeys varied in age, weight, and gender. The arms and legs differed in size as well. HLV injections varied in tourniquet pressure and inflation time, injection rate and injection volume. A papaverine preinjection was used in 11 of the limbs, but this variable as well as the others indicated previously had no significant effect on expression of the injected pDNA (Hegge *et al.*, 2010). These studies were not designed to be dose-response studies, but the pDNA expression data can be evaluated to better understand pDNA dose response in primate muscles.

For optimization of HLV injection parameters in monkeys, we initially injected 20 mg of pDNA per limb and then in some subsequent studies injected 5 mg of pDNA per limb to reduce study costs. Luc expression from the 5-mg dose was much more than 4-fold reduced compared with the 20-mg dose. To understand the relationship between the injected pDNA dose and resulting expression in limbs of various sizes, we compared Luc expression resulting from the injected pDNA dose per gram of target muscle (Fig. 4a and b). Despite the conditions that varied between injections, a pattern emerged. Expression was much lower with pDNA doses ranging from 40 to 70 μ g/g muscle than from doses ranging from 70 to 150 μ g/g muscle (Fig. 4b). The average amount of Luc protein expressed in the 15 limbs that were injected with doses less than 75 μ g of pDNA per gram of muscle was 0.6 ng of Luc per gram of muscle for each microgram of pDNA per gram of muscle injected (0.6 ng of Luc per microgram of pDNA; Fig. 4c). Of the 30 injections that contained at least 75 μ g of pDNA per gram of muscle, the average expression was 2.8 ng of Luc per microgram of injected pDNA. The consistent low expression from the lower pDNA doses suggested there could be a threshold effect, and doses above a threshold value of approximately 75 μ g of pDNA per gram of target muscle produced significantly more gene product relative to the injected dose than did doses below this threshold value ($p < 0.0001$, Fisher exact test for statistical significance).

Gene expression studies were performed in rats to determine whether such a threshold effect could be detected (Fig.

FIG. 3. Effect of plasmid size on dose response. (a) ICR mice weighing 25 g were given one HLIV injection of the CMV-Luc expression cassette in plasmids of various sizes: 5.7 kb (100 μ g), 11.4 kb (200 μ g), and 17.1 kb (300 μ g) ($n=8$ per group). Mice were killed 7 days later and the hind leg muscles were homogenized for evaluation of luciferase expression, shown as total luciferase in the leg normalized to the average expression from the 5.7-kb plasmid. (b) The legs of C57BL/6 mice weighing 22–25 g were each given one HLIV injection of 100, 200, 400, or 800 μ g of the 5.7-kb (solid circles) or 17.1-kb (open squares) CMV-Luc plasmid ($n=8$ per group). Average total luciferase in the leg is shown 7 days postinjection. (c) The legs of C57BL/6 mice ($n=8$ per dose) weighing 22–25 g (solid squares) and *mdx* mice ($n=8$ per dose) weighing 26–33 g (open circles) were each given one injection of 100, 200, 400, or 800 μ g of 17.1-kb CMV-Luc. The *y* axis shows total luciferase in the leg per gram of muscle of the leg 7 days postinjection. The *x* axis shows micrograms of pDNA injected per gram of muscle.

4d and e). All rats were injected with a 25- μ g dose of reporter gene pDNA (4–5 μ g/g muscle), a dose well below the putative threshold of 75 μ g of pDNA per gram of target muscle. A human SEAP-expressing plasmid (CMV-hSEAP) was used in one study and a Luc-expressing plasmid (CMV-Luc) was used in the other. Coinjected with this fixed dose of expression plasmid were escalating doses of a filler empty vector (pMIR266). To avoid interference by an immune response to these nonrat proteins, reporter gene expression was evaluated on day 4 for luciferase and on day 7 for SEAP.

Coinjection of 100, 400, or 1600 μ g of nonexpressing filler pDNA increased expression from the 25- μ g dose of either reporter pDNA (Fig. 4d and e). Rats injected with 1600 μ g of filler pDNA plus 25 μ g of CMV-hSEAP produced 3.4 times more hSEAP than rats injected with 25 μ g of CMV-hSEAP alone ($p < 0.0001$). The 425- μ g dose of pDNA (25 μ g of CMV-hSEAP plus 400 μ g of filler pDNA) was on average 72 μ g/g muscle and produced a similar amount of expression as the total dose of 280 μ g/g muscle that included 1600 μ g of filler pDNA ($p = 0.37$). Rats that were coinjected with 25 μ g of CMV-Luc plus 1600 μ g of filler pDNA expressed 8.3 times more Luc in the whole leg than did rats injected with 25 μ g of CMV-Luc alone ($p = 0.0001$). Expression from 25 μ g of CMV-Luc coinjected with either 400 μ g of filler pDNA (79 μ g/g total pDNA) or 1600 μ g of filler pDNA (323 μ g/g) yielded similar expression levels ($p = 0.14$). Therefore, maximal expression from 25 μ g of either reporter gene pDNA required 70–80 μ g of total DNA per gram of target muscle.

On the basis of these studies, the doses of pDNA used in the promoter comparison studies in Fig. 1a–c were likely in the linear range. Doses of the luciferase-expressing pDNA for promoter comparison in rats (Fig. 1a) ranged from 75 to 238 μ g/g muscle in individual animals. Doses of mSEAP-expressing pDNA (Fig. 1b and c) were an estimated 125 to 321 μ g/g, based on the average muscle weight of 25-g mice.

Percentages of muscle fibers transfected in rodent limbs

The percentage of transfected limb muscle after HLIV injection of 100 μ g of CMV-LacZ reporter plasmid into wild-type mice was evaluated by counting β -Gal⁺ muscle fibers (Fig. 5a). Transfection efficiency was between 20 and 36% in most of the large groups of muscles (9 of 12 muscle samples), even though the dose of pDNA in this experiment had not been optimized. The average percentage of β -Gal⁺ muscle fibers in the whole leg was $22 \pm 5\%$. The anterior lower leg muscles that comprise 9% of the leg muscle mass had the lowest transfection efficiency, $7 \pm 2\%$.

A β -Gal dose optimization experiment was then performed in rats with doses varying from 0.05 to 2.5 mg of CMV-LacZ (Fig. 5b–d). The percentages of β -Gal⁺ muscle fibers increased with each dose up to 1 mg of CMV-LacZ, which was equivalent to 220 μ g of pDNA per gram of target muscle (Fig. 5b). At this dose, an average of $28 \pm 6\%$ of myofibers in the entire leg were β -Gal positive. An average of $40 \pm 8\%$ of the muscle fibers were β -Gal⁺ in the large group of posterior lower leg muscles that included the gastrocnemius and soleus. An average of $34 \pm 6\%$ of the posterior thigh leg muscles and $29 \pm 9\%$ of the medial thigh muscles were β -Gal⁺. The anterior thigh and anterior lower leg muscles were less efficiently transfected, an average of

FIG. 4. Plasmid expression is dependent on the mass of injected pDNA in rhesus monkeys and rats. (a–c) The limbs of rhesus monkeys were each given one HLV injection of 5–32 mg CMV-Luc (pMIR48). Animals were killed 4 or 7 days after injection and muscles were homogenized to determine total luciferase per gram of muscle in the entire limb. Day 4 samples were adjusted with a correction factor to calculate the day 7 equivalent (see Materials and Methods). (a and b) The pDNA dose per gram of target muscle is shown on the x axis. The resulting luciferase expression is shown on the y axis. Data from all monkeys are shown in (a) and data from monkeys that received less than 150 μg of pDNA per gram of muscle are shown in (b). (c) The x axis shows the pDNA dose per gram of target muscle and the y axis shows expression per dose (total nanograms of luciferase divided by the micrograms of CMV-Luc injected into the limb). The line labeled “T” indicates the threshold pDNA dose of 75 $\mu\text{g}/\text{g}$ muscle. (d) Rats with an average weight of 157 g were given an HLV injection, via the hind limb, of a pDNA mixture containing 25 μg of CMV-hSEAP plus 0, 100, 400, or 1600 μg of empty vector ($n = 5$ or 6). Rats weighed an average of 225 g at the time of death and muscle harvest, which was 25 days later. Dose relative to muscle weight was calculated by dividing muscle weight at the end of the study by 1.43, a ratio based on the change in animal size. The average amount of hSEAP in the serum of each group is shown on the y axis. The x axis shows the average dose of added empty vector pDNA per gram of muscle of the limb. (e) Rats with an average weight of 135 g were given an HLV injection, via the hind limb, of a pDNA mixture containing 25 μg of CMV-Luc (pMIR48) plus 0, 100, 400, 1600, or 3200 μg of empty vector pDNA ($n = 4$ –6). The average total amount of luciferase in each limb is shown for each dose of added empty vector pDNA per gram of muscle injected.

12 ± 5 and $11 \pm 4\%$, respectively. The percentages of muscle fibers that were $\beta\text{-Gal}^+$ increased in a similar manner to the amount of $\beta\text{-Gal}$ enzyme from the homogenized muscles except for the lowest dose. This dose was 12 μg of pDNA per gram of target muscle and appears to have been below the threshold dose.

The highest dose of CMV-LacZ injected into rats (2.5 mg, 540 μg of pDNA per gram of muscle) appears to have caused some muscle damage (Fig. 5d). Expression of $\beta\text{-Gal}$ was lower in rats injected with this dose than in those injected with 1 mg. This was true for $\beta\text{-Gal}$ evaluated by enzymatic assay as well as by cell counting. The decline in expression at the highest dose was most pronounced in the gastrocnemius. In addition, the muscles of rats injected with the highest dose contained an infiltration of mononuclear cells. No doses between 1 and 2.5 mg were tested.

Percentages of muscle fibers transfected in rhesus monkeys

Studies were performed in rhesus monkeys to determine what percentages of muscle fibers could be transfected with a dose of pDNA per gram of target muscle that was optimal in rodents (Table 1). Maximal expression of luciferase required between 300 and 500 μg of pDNA per gram of target muscle in mice. The dose of CMV-LacZ that resulted in the highest expression in rats was 220 $\mu\text{g}/\text{g}$ muscle, but no intermediate doses were tested between this and the 540- $\mu\text{g}/\text{g}$ dose that caused an immune reaction. On the basis of these

data, we selected 400 μg of CMV-LacZ per gram of target muscle as the desired target dose in monkey limbs. The precise dose of pDNA per gram of target muscle in monkeys could not be determined until after the animals were killed and the muscles were weighed. To determine the relationship between target muscle weight and limb volume, we compared muscle weights and displaced limb volumes from 15 limbs of 4 monkeys. On average the target muscle distal to the tourniquet was 0.39 ± 0.03 g/ml displaced limb volume (data not shown). Therefore, we injected all eight limbs of the two monkeys in this $\beta\text{-Gal}$ expression study with a pDNA concentration of 0.35 mg of CMV-LacZ per milliliter of injection solution and 0.4 ml of injection solution per milliliter of displaced limb volume to result in an approximate dose of 400 μg of pDNA per gram of target muscle.

The percentages of $\beta\text{-Gal}^+$ myofibers in several muscles of three arms and three legs were determined (Table 2 and Fig. 6a). The gastrocnemius and soleus, muscles involved in standing that together comprise 38% of the lower leg muscle mass (Table 2), were the most highly transfected muscles of the leg. Between 9.1 and 22.1% of the myofibers in these muscles expressed $\beta\text{-Gal}$, an average of 15%. The majority of myofibers in the gastrocnemius are fast-twitch whereas the majority in the soleus are slow-twitch myofibers. Similar transfection efficiencies in these two muscles suggested that fast and slow myofibers were transfected equally well, which we confirmed by costaining myofibers with antibodies to $\beta\text{-Gal}$ and to specific fiber types in muscle cross-sections (data not shown). The flexor carpi radialis, which is involved in

TABLE 2. β -GALACTOSIDASE EXPRESSION IN RHESUS MONKEY MUSCLES AFTER ONE HLIV INJECTION OF CMV-LACZ PER LIMB

Arms

Muscle group	Name of muscle	Fraction of target muscle weight ^a	Muscle function	Animal identification:		135-22		135-22		135-23	
				Days between injection of limb and euthanasia:		8		11		4	
				Evaluated limb:		Left		Right		Left	
						% LacZ ^{+,b}	ng/g β -Gal ^c	% LacZ ⁺	ng/g β -Gal	% LacZ ⁺	ng/g β -Gal
Anterior superficial	Palmaris longus	0.03	Flexes hand			N/D	2,528	12.2	5,629	N/D	60
	Pronator teres	0.05	Pronates forearm and hand			N/D	249	4.8	1,437	N/D	84
	Flexor carpi radialis (FCR)	0.06	Flexes and helps abduct hand			10.7	22,391	13.0	64,387	17.3	3,755
	Flexor carpi ulnaris	0.11	Flexes hand			0.3	251	3.2	2,676	N/D	96
Anterior deep	Flexor digitorum superficialis	0.09	Flexes hand and fingers			3.4	540	4.7	737	5.8	597
	Flexor digitorum profundus	0.23	Flexes finger			2.5	952	4.6	1,173	7.9	1,035
	Pronator quadratus	0.01	Pronates hand			N/D	752	N/D	3,893	N/D	2,529
	Brachioradialis	0.10	Flexes forearm			3.7	537	4.1	1,107	4.3	667
Posterior superficial	Extensor carpi radialis longus	0.04	Extends and abducts hand			0.5	45	0.9	170	N/D	291
	Extensor carpi radialis brevis	0.06	Extends and abducts hand			N/D	219	N/D	856	N/D	262
	Extensor digitorum	0.04	Extends hand and finger			5.3	1,352	2.4	1,417	N/D	208
	Anconeus	0.00	Extends hand			N/D	0	N/D	0	N/D	240
Posterior deep	Extensor carpi ulnaris	0.04	Extends hand			0.8	313	2.5	1,400	N/D	346
	Extensor pollicis longus	0.00	Extends thumb			N/D	1,531	N/D	6,433	N/D	1,152
	Supinator	0.03	Supinates hand			1.1	3,410	3.0	4,607	N/D	2,306
	Abductor pollicis longus	0.03	Abducts thumb			N/D	3,908	6.2	5,441	N/D	1,059
Muscles of hand	Extensor digiti secund et tertii	0.01	Extends fingers			N/D	3,068	4.3	15,467	N/D	N/D
	Extensor digiti quart et minimi	0.01	Extends fingers			N/D	3,439	9.3	1,533	N/D	122
	Thumb	0.02	Abducts fingers			N/D	0	N/D	1,480	N/D	159
	Interosseus	0.02				N/D	0	15.7	9,780	N/D	51
Total ^d	Others	0.01				N/D	0	N/D	1,204	3.8	4,533
		1.00				8.4	2,392	9.4	6,178	N/D	834

(continued)

TABLE 2. (CONTINUED)

Legs

Animal identification:				135-22		135-22		135-23		
Days between injection of limb and euthanasia:				11		8		7		
Evaluated limb:				Left		Right		Left		
Muscle group	Name of muscle	Fraction of target muscle weight ^a	Muscle function	% LacZ ^{+,b}	ng/g β -Gal ^c	% LacZ ⁺	ng/g β -Gal	% LacZ ⁺	ng/g β -Gal	Muscle group average % LacZ ^{+,d}
Posterior superficial	Gastrocnemius—medial	0.14	Plantar movement of foot	10.1	11,941	9.1	9,423	15.3	400	11.8% (15% by counting)
	Gastrocnemius—lateral	0.14	(important for standing)	10.1	14,070	22.1	5,048	N/D	1401	
Posterior deep	Soleus	0.10	Plantar movement of foot	21.2	58,208	12.2	16,359	18.8	15,952	9.3
	Popliteus	0.03		N/D	10,936	N/D	3,683	N/D	217	
	Flexor digitorum longus	0.06	Flexes toes	10.4	1,810	6.0	722	11.8	19,866	
	Flexor hallucis longus	0.10	Flexes big toe	N/D	3,630	N/D	3,107	N/D	4,922	
	Tibialis posterior	0.03	Plantar flexes foot	8.5	11,953	8.1	5,807	N/D	304	
Anterior	Tibialis anterior	0.11	Dorsally flexes foot	4.1	1,938	5.2	357	N/D	1,735	6.7
	Extensor hallucis longus	0.04	Extends big toe	N/D	0	N/D	0	N/D	338	
	Extensor digitorum longus	0.04	Extends toes	15.3	11,550	10.0	2,729	N/D	713	
	Abductor hallucis longus	0.02	Abducts big toe	N/D	1,045	N/D	1,392	N/D	3,732	
	Peroneus longus	0.06	Pronates and plantar flexes foot	N/D	1,202	N/D	834	N/D	8,042	
	Peroneus brevis	0.04	Pronates and plantar flexes foot	N/D	1,019	N/D	939	N/D	648	
	Flexor digitorum brevis	0.02	Flexes toes	N/D	0	N/D	0	N/D	592	
Flexor hallucis brevis	0.03	Flexes big toe	N/D	0	N/D	0	N/D	30		
Muscles of foot	Others at bottom of foot	0.04		N/D	849	N/D	20	N/D	63	10.1
	Extensor digitorum brevis			N/D	845	N/D	317	N/D	N/D	
Total ^d		1.00		11.1	10,980	10.0	4,736	9.2	3,445	

Monkeys were given one HLIV injection per limb of CMV-LacZ (0.35 mg/ml) in saline. The injection solution volume was 0.4 ml/ml displaced limb volume. Monkey 135-22 was 4 years old and weighed 7.5 kg; monkey 135-23 was 2.5 years old and weighed 3.9 kg.

^aThe fractional weight of each muscle was determined by dividing the weight of the muscle by the total weight of all the target muscles from the same limb. Target muscles are the muscles that were located between the tourniquet and the injection site. The average fractional weights were determined from 10 arms and 12 legs.

^bThe percentage of transfected muscle fibers was determined by counting β -galactosidase⁺ muscle fibers and dividing this number by the total number of muscle fibers in a cross-section of the muscle specimen.

^cUnits for expression are nanograms of β -galactosidase protein per gram muscle.

^dThe average percentages of β -galactosidase⁺ muscle fibers in groups of muscles and in the whole target area of the limb are approximations based on the correlation between β -galactosidase (ng/g) and the percentages of expressing muscle fibers as determined in Fig. 6e. The average percentages determined by cell counting are indicated in parentheses for specified muscles. N/D, data not determined.

FIG. 5. Efficiency of pDNA delivery to muscle fibers in rodent limbs. C57BL/6 mice and rats were given a single HLV injection of β -galactosidase-expressing pDNA. Animals were killed 7 days later. The percentages of β -Gal-expressing myofibers were determined for each of the five muscle groups of the hind leg. (a) Each mouse was injected with 100 μ g of CMV-LacZ in 1 ml of injection solution. Each data point represents the average percentage of β -Gal⁺ myofibers in one group of muscles of one animal ($n = 3$). (b–d) Rats were each given one HLV injection of 0.05, 0.25, 0.5, 1.0, or 2.5 mg of CMV-LacZ ($n = 3$). (b) The average percentage of β -Gal⁺ myofibers is shown for each group of hind leg muscles and at each dose of CMV-LacZ. (c) For each dose, the total amount of β -Gal is compared with the average percentages of β -Gal⁺ myofibers in the whole leg. The x axis shows the dose as average milligrams of CMV-LacZ per gram of muscle weight of the leg. The y axis (left) shows the average amount of β -Gal in the

whole leg. The y axis (right) shows the average percentages of β -Gal⁺ myofibers in the whole leg $\pm$ SEM. (d) Representative images of β -Gal staining were taken from the gastrocnemius muscle of rats injected with the indicated dose of CMV-LacZ. Scale bar: 100 μ m.

flexing and abducting the hand, was the most highly transfected muscle of the arm, an average of 14% β -Gal⁺ myofibers.

The most highly transfected areas of the muscles were infiltrated and had a considerable number of necrotic muscle fibers where expression was highest (Fig. 6a). The monkeys appear to have mounted an immune response to the β -Gal. This was true of the arm evaluated on day 4 as well as of limbs evaluated at later time points. Even though CMV-LacZ was injected into this arm 4 days before harvest, the first two injections in the animal had occurred 7 days before harvest, sufficient time for the animal to mount an immune response. Therefore, cell-counting results likely underestimate the number of muscle fibers that were transfected.

After counting β -Gal⁺ myofibers in samples taken from each of these muscles, all the rest of the muscle within the transfected area of the limbs (distal to the tourniquet) was homogenized and assayed for β -Gal activity (Fig. 6b and c and Table 2). This assay method is much less prone to sampling variation than cell counting. It also allowed us to determine a weighted average for β -Gal expression in the entire target region of the limb and within compartments. The total amount of β -Gal expression in the arms and legs increased between days 4 and 11 postinjection (Table 2). Despite the day 4 enzymatic expression of β -Gal being so low, the percentages of expressing myofibers could easily be

counted (Fig. 6a) and the percentages of positive myofibers in the day 4 arm were similar to those in the other two arms (Table 2). Overall, results of the β -Gal enzymatic assay were consistent with the cell-counting results (Fig. 6b–d and Table 2). Enzymatic β -Gal expression in the two forearms that contributed to the data shown in the schematic (Fig. 6b), from days 8 and 11, appeared to be lower than in the legs. However, enzymatic activity from the third arm with higher percentages of β -Gal-expressing myofibers could not be included in the average values because of the greatly reduced enzymatic activity on day 4. The highest expressing muscle group of the arm, the anterior superficial muscles, comprise 33% of the total forearm muscles (Table 2). Expression in this muscle group of the arm was similar to the highest expressing group in the legs, the posterior superficial muscles (Fig. 6c and d).

Cell counting was performed in a much smaller number of muscles than the enzymatic assay because it is much more time-consuming (Table 2). We estimated the average percentages of transfected myofibers in groups of muscles and in the whole target area of each limb by regression analysis as shown in Fig. 6e (see Materials and Methods). The percentages of transfected myofibers in each group of muscles as approximated by this method are consistent with the percentages determined by cell counting in individual muscles, but cannot be applied to the day 4 evaluation. The av-

FIG. 6. Efficiency of pDNA delivery to limb muscles of rhesus monkeys. Each of the four monkey limbs was given one HLV injection with CMV-LacZ (0.35 mg/ml) in a volume of 0.4 ml of injection solution per milliliter of limb volume, injection rate of 2 ml/min, and a tourniquet pressure of 450 mmHg. One arm and one leg were injected on day 0. The other arm and other leg were injected on day 3. One monkey was killed for evaluation 7 days after the first injection (monkey 135-23) and the other was killed 11 days after the first injection (monkey 135-22). Two of the limbs from the smaller monkey killed on day 7 (monkey 135-23; Table 2) had poor swelling after the injection as a result of the tourniquet not fitting snugly. The tourniquet was designed for larger human limbs. Therefore, the data from these two limbs were omitted from the analysis. (a) Representative muscle sections from monkey 135-22 show β -Gal-expressing myofibers in the gastrocnemius of one leg (evaluated on day 8 postinjection) and the soleus of the other leg (day 11 postinjection). The flexor carpi radialis from the arm of monkey 135-23 was evaluated on day 4 after injection. Arrows point

to regions of infiltration and necrotic myofibers. Scale bar: 500 μ m. (b) The schematic shows muscles in the cross-section of the monkey forearm (A) and lower leg (B). Each muscle of the forearms and lower legs was homogenized and assayed for β -Gal activity. The amount of expression shown for arm muscles is the average from two arms (evaluated on days 8 and 11 postinjection). The palmaris longus (PL), pronator teres (PT), flexor carpi radialis (FCR), flexor carpi ulnaris (FCU), and flexor digitorum superficialis (FDS) are anterior superficial muscles of the arm. The flexor digitorum profundus (FDP) is an anterior deep muscle of the arm. The brachioradialis (BR), extensor carpi radialis longus (ECRL), extensor carpi radialis brevis (ECRB), extensor digitorum (ED), and extensor carpi ulnaris (ECU) are posterior superficial muscles. The extensor digiti secund et tertii and extensor digiti quart et minimi are shown together (Dig); they are from the posterior deep muscle group. The amount of β -Gal expression shown for the leg muscles is the average from three legs evaluated on days 7, 8, and 11 postinjection. The medial and lateral gastrocnemius [GAST (M) and GAST (L)] and the soleus (SOL) are posterior superficial muscles of the lower leg. The flexor digitorum longus (FDL), flexor hallucis longus (FHL), and tibialis posterior (TP) are posterior deep muscles. The extensor digitorum longus (EDL), peroneus longus (PL), and peroneus brevis (PB) are anterior leg muscles. (c and d) The average β -Gal expression in each group of muscles from the forearm (c) or lower leg (d) is shown. Each data point represents a group of muscles from one limb. The muscles in each group are shown in Table 2. (e) For each muscle, the percentage of β -Gal⁺ myofibers was graphed relative to the nanograms of β -Gal per gram of homogenized muscle. These muscles were from arms and legs evaluated on day 7, 8, or 11 postinjection.

erage percentages of transfected muscle thus determined were 8.4 and 9.4% in the arms and 11.1, 10.0, and 9.2% in the legs (Table 2). The percentage of transfected muscle in all limbs was 9.6%.

Discussion

These preclinical studies were undertaken to determine a suitable dose of dystrophin pDNA for gene therapy to treat

Duchenne muscular dystrophy. To scale up the dose from mice to humans, it was crucial to determine whether muscles of rodents and primates responded similarly to the pDNA dose. Subsequent to our observation that gene expression from pDNA did not appear to correspond directly to the dose, we investigated the possibility that a threshold dose requirement could be limiting gene expression from lower doses and then sought to define the range in which expression did correspond in a linear manner. By defining the dose

as the mass of pDNA relative to the mass of target tissue, we determined that muscles of rodents and nonhuman primates do show a similar dose response and, indeed, full expression of a plasmid requires that the dose be higher than a threshold of 75 μ g of pDNA per gram of muscle.

For large animal studies the dose relative to the target muscle mass is especially important because these research animals are likely to vary in size, as will human patients. Our β -Gal expression results in monkey limbs that varied in size attest to the more consistent expression that can be obtained with doses of pDNA that are tailored to the amount of target muscle. The approximate percentage of transfected myofibers was similar between all limbs, an overall average of 10% of the target muscle.

Previous studies showed that large plasmids were a little less efficiently delivered by intraarterial injection than smaller plasmids (Zhang *et al.*, 2004), and here we confirm that the same is true after intravenous delivery. Considering that the full-length dystrophin coding sequence is much larger than most reporter genes (11 kb compared with less than 2 kb), we needed to know how to adjust the dose of a large plasmid for optimal expression. We found that maximal expression was obtained from the same mass of a small or large plasmid. The consequence of this finding is that the pDNA dose per injection cannot simply be increased to obtain higher dystrophin expression from larger plasmids. One possible explanation for the observation that maximal expression from small and large plasmids occurs at an equal mass of pDNA may be related to DNase degradation of pDNA that is extracellular. If pDNA were taken up at approximately the same rate regarding the DNA mass, regardless of size, more molecules of the smaller pDNA would enter the muscle fibers and a greater proportion of the large pDNA molecules would remain in the extracellular milieu during the time of uptake. DNase degrades pDNA remaining outside the myofibers in a time-dependent manner (Satkauskas *et al.*, 2001). Thus, the limitation for transgene expression after delivery to muscle could be explained at least in part by the kinetics of muscle uptake in opposition to the kinetics of DNase degradation. The mechanisms involved may be more complex and appear to involve an intracellular hurdle that has at least a small impact (Zhang *et al.*, 2004).

To work around the limitations of delivering large plasmids, multiple injections can be used to increase expression, shown here and in the work of Zhang and colleagues (2010). Multiple HLV injections in the same animal and even in the same limb were well tolerated in nonhuman primates (Sebestyen *et al.*, 2007; Hegge *et al.*, 2010) as well as in muscular dystrophy GRMD (golden retriever muscular dystrophy) dogs (our unpublished observations). Multiple deliveries are much more feasible for pDNA than for viral vectors.

The immune response we observed in rat and monkey muscles containing high β -Gal expression is most likely immune rejection of the nonnative protein. It is unlikely to be due to contaminants in the preparation because the β -Gal expression in monkeys was obtained from a batch of pDNA that contained low levels of contaminating bacterial DNA and caused no muscle damage in mice (Wooddell *et al.*, 2010). Muscle damage from contaminants occurs earlier (days 2–3) than an immune reaction to an expressed protein. As with most other gene therapies, an immune response to the transgene must be addressed (Mendell *et al.*, 2010).

CMV and muscle gene promoters were compared for short-term and long-term expression in mouse muscle. The much more stable CMV-driven gene expression in mouse muscle contrasts dramatically with the precipitous 1000-fold drop-off of CMV-driven expression in mouse liver (Liu *et al.*, 1999; Wooddell *et al.*, 2008). Expression of mSEAP in muscle decreased 3-fold from day 4 to day 14, but CMV-driven expression of luciferase increases from day 3 to day 7 (Vigen *et al.*, 2007) and β -Gal expression in monkeys also increased between days 4 and 11. The contrasting results in muscle compared with liver correlate with the tissue types that are naturally infected by cytomegalovirus (Baskar *et al.*, 1996). Muscle would, therefore, be expected to express the transcriptional activators that allow for continued expression from the CMV promoter in the presence of chromatin. Persistent expression of genes even in pDNA was shown to be accompanied by euchromatin modifications to the pDNA (Riu *et al.*, 2007). Modification and rearrangement of nucleosomes in response to activated transcription are highly dynamic processes, resulting in chromatin modifications that help maintain an active state (Workman, 2006; Li *et al.*, 2007). Therefore, it is not surprising that the kinetics of expression change during a period of time in which chromatin is forming and epigenetic modifications are occurring. In contrast to CMV, the MCK promoters become activated more gradually. This could be explained by a requirement to first form chromatin on the naked pDNA before the chromatin-interacting factors for these promoters can participate in the activation process (Guasconi and Puri, 2009). All the tested promoters appeared to have reached a steady state for expression in muscle after approximately 2 weeks.

These studies significantly advance our understanding of dose effects with pDNA delivered to muscle and the information will be of help for all further preclinical and clinical work using HLV injection or other procedures for delivery of pDNA to muscle. On the basis of the broad range of muscles transfected by HLV injection, delivery of the dystrophin gene to patients with muscular dystrophy would likely preserve their limb function. High levels of gene delivery to the gastrocnemius and soleus make these muscles especially good candidates for therapeutic treatment. Miyoshi dysferlinopathy is also an indication for which HLV delivery of dysferlin-expressing pDNA would be suitable. Gastrocnemius muscle weakness presents in these patients when they are, on average, 19 years old (Miyoshi *et al.*, 1986). Efficient delivery to the gastrocnemius is promising for maintenance of the mobility of these patients.

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Author Disclosure Statement

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Address correspondence to:
Dr. Christine I. Wooddell
Roche Madison
465 Science Drive
Madison, WI 53711

E-mail: chris.wooddell@roche.com

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