doi:10.1016/j.ymthe.2004.01.006

A New Method for Recombinant Adeno-associated Virus Vector Delivery to Murine Diaphragm

Cathryn Mah,[1,2,*,†] Thomas J. Fraites, Jr.,[1,3,*] Kerry O. Cresawn,[1,3] Irene Zolotukhin,[1,2] Melissa A. Lewis,[1,2] and Barry J. Byrne[1,2,3]

[1] *Powell Gene Therapy Center;* [2] *Division of Cell and Molecular Therapies and Division of Pediatric Cardiology, Department of Pediatrics;* [3] *Interdisciplinary Program in Biomedical Sciences, Genetics Advanced Concentration, University of Florida, Gainesville, FL 32610, USA*

**These authors contributed equally to this work.*

[†] *To whom correspondence and reprint requests should be addressed at the Powell Gene Therapy Center, University of Florida College of Medicine, P.O. Box 100296, Gainesville, FL 32610-0296, USA. Fax: (352) 392-4853. E-mail: cmah@ufl.edu.*

Genetically modified mice are important models for evaluation of potential gene therapies for human diseases. However, their small size often precludes the use of clinically feasible methods for vector delivery, therefore, alternative methods must be used. We have developed a gel-based method for delivery of recombinant adeno-associated virus vectors to the mouse diaphragm, an important target organ for many myopathic diseases. We hypothesized that delivery of vectors in a viscous solution would increase transduction by providing a longer exposure time to target cells. We demonstrate that gel-mediated delivery of rAAV serotypes 1, 2, and 5 results in higher transduction efficiencies than free vectors alone when administered *in vivo* to mouse diaphragms. We further establish greater tropism of rAAV1 vectors for the diaphragm compared to serotypes 2 and 5. This report describes a novel method for efficient delivery of rAAV vectors to the mouse diaphragm and is the first demonstration of gene transfer to the diaphragm using recombinant adeno-associated virus vectors.

Key Words: adeno-associated virus, diaphragm, gene delivery, acid α-glucosidase, glycogen storage disease type II, Pompe

INTRODUCTION

Mouse models of human disease provide invaluable opportunities to evaluate the potential efficacy of candidate therapies. Gene therapy strategies in particular have benefited enormously from the profusion of knockout and transgenic mice that recapitulate the genetic and pathophysiologic features of human diseases. Congenital myopathies, including the muscular dystrophies, have been widely investigated as targets for gene therapy interventions, and the diaphragm is often cited as one of the important target organs for functional correction [1].

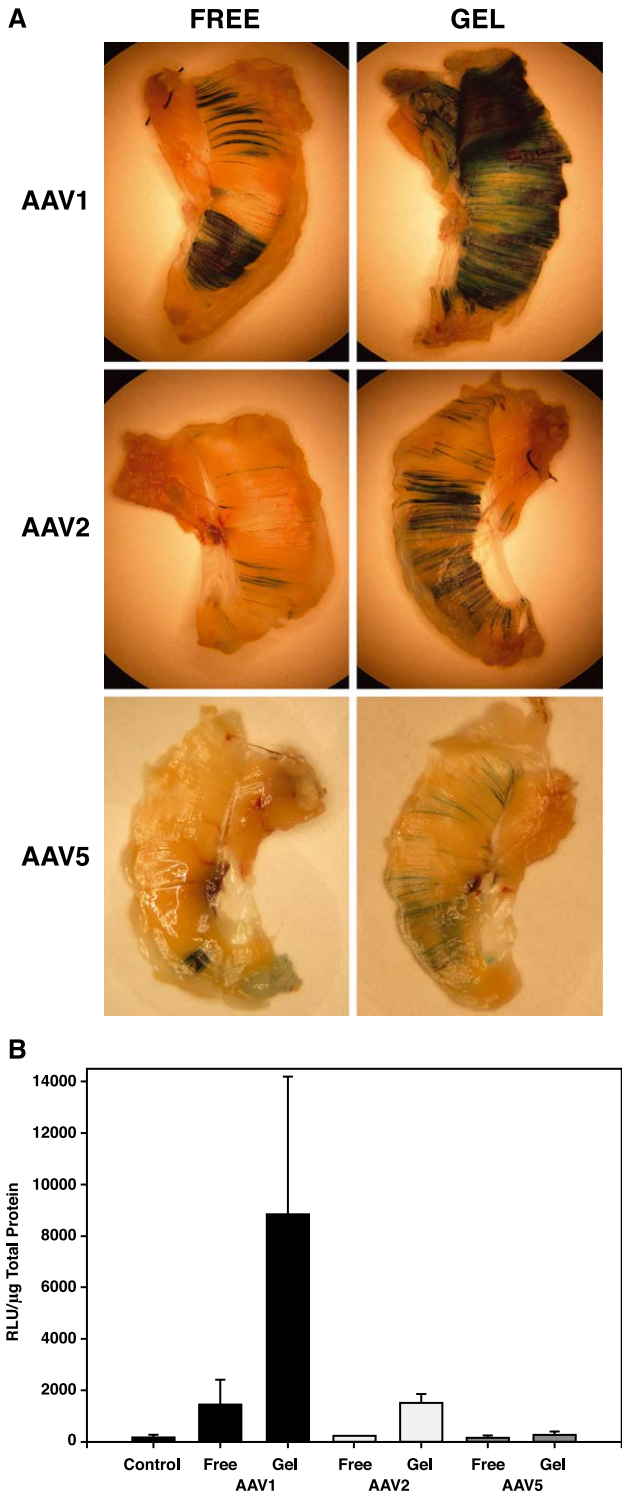
The mouse diaphragm presents unique challenges in terms of delivery of therapeutic agents due to its small size and thickness, which preclude direct injection into the muscle. Intravenous or intra-arterial delivery of vectors may be effective alternatives and are currently under investigation [2,3]; however, isolation of blood vessels that specifically perfuse the diaphragm is also difficult in the mouse. Systemic delivery of vectors may eventually require the application of capsid-based targeting methods that have recently been reported [4–10].

Adeno-associated virus is a single-stranded DNA-containing, nonpathogenic human parvovirus that is being widely investigated as a therapeutic vector for a host of muscle disorders [11–14]. Six serotypes of the virus (AAV1–6) were originally described, and two more have recently been identified in rhesus macaques [15]. Recombinant adeno-associated virus (rAAV) vectors have been developed in which the *rep* and *cap* open reading frames of the wild-type virus have been completely replaced by a therapeutic or reporter gene, retaining only the characteristic inverted terminal repeats, the sole *cis*-acting elements required for virus packaging. Using helper plasmids expressing various combinations of the AAV2 *rep* and AAV1, 2, and 5 *cap* genes, our group and others

1525-0016/$30.00

have demonstrated efficient cross-packaging of AAV2 genomes into particles containing the AAV1, 2, or 5 capsid protein [16–19]. The various serotype vectors have demonstrated distinct tropisms for different tissue types *in vivo*, due in part to their putative cell surface receptors. Although several reports have indicated that rAAV1 vectors efficiently transduce skeletal muscle in general [20–23], no study to date has reported which of the serotypes, if any, might transduce the diaphragm in particular.

The aim of this study is to provide a safe, effective, and uniform method for delivery of recombinant adeno-associated virus vectors to the mouse diaphragm to evaluate their potential as therapeutic agents. We evaluate the ability of rAAV serotypes 1, 2, and 5 to transduce the mouse diaphragm. We further describe the application of a gel-based delivery method and demonstrate its utility for delivery of rAAV1, 2, and 5 to the mouse diaphragm. Our results are the first to demonstrate efficient, uniform expression of a transgene in the murine diaphragm using rAAV vectors. Finally, we assess the utility of this method using a mouse model ($Gaa^{-/-}$) of glycogen storage disease type II (GSDII) [24], an autosomal recessive disorder that is characterized by respiratory insufficiency secondary to diaphragmatic weakness in affected juveniles [25]. We believe the method we describe may have broad applicability for delivery of gene therapy vectors not only to the diaphragm but perhaps to other tissues as well.



RESULTS

Efficiency of Transduction Using Gel-Based Delivery of rAAV *in Vivo*

We investigated the efficiency of rAAV delivery using the gel-based method compared to free virus delivery using β-galactosidase as a reporter gene (Fig. 1A). Direct particle-to-particle comparisons of histochemistry from free-virus-treated animals (left column) versus gel-based delivery (right column) indicate an increased efficiency of transduction for all serotypes using the latter method. Quantitative analysis of

FIG. 1. Free virus and gel-based delivery of rAAV-βgal vectors based on AAV serotypes 1, 2, and 5. Adult wild-type mice (129X1×C57BL/6) were treated with 1×10^{11} particles of rAAV-βgal, with virus either applied directly to the diaphragm or applied using the gel-based method. The animals were sacrificed 6 weeks later and tissues were collected and assayed for β-galactosidase activity. (A) Representative histochemical (X-gal) stained diaphragm segments from treated animals. Each row corresponds to the respective serotype into which the recombinant vector genome was packaged (AAV1, 2, or 5). The columns represent application of free virus (left) or virus-gel suspension (right) to the abdominal surface of the diaphragms. Note the intense blue staining in both columns for vector virions packaged using the rAAV1 capsid (top row), with increased intensity using the gel-based method (top row, right). (B) Quantitative assay of β-galactosidase activity from the same animals. The bars represent mean ± SEM GAA activity for three mice in each group.

doi:10.1016/j.ymthe.2004.01.006

tissue lysates from these animals using the Galacto-Star enzymatic assay for β-galactosidase confirms these results (Fig. 1B). Activities for subjects treated with gel–vector suspensions had higher activities for all three serotypes.

Varying Tropisms of rAAV Serotypes 1, 2, and 5 for Diaphragm Muscle *in Vivo*

The results from Figs. 1A and 1B also indicate a distinct gradient of tropism for mouse diaphragm among the three tested serotypes. Qualitatively, rAAV1 vectors led to the most intense staining under both the free virus and the gel-based conditions. Differences between rAAV2 and rAAV5 were hard to distinguish in the free virus case due to the low levels of transduction for both vectors, but the gel-mediated subjects demonstrated a clear preference for rAAV2 compared to rAAV5. These results are further verified in Fig. 1B, which indicates higher levels of enzyme activity for rAAV2 gel suspensions compared to rAAV5. Taken together, the results of histochemical staining and enzymatic activity indicate (1) a substantial increase in viral transduction using a physical delivery system and (2) a clearly enhanced mouse diaphragm tropism for rAAV1 and a potentially important difference between rAAV2 and rAAV5.

Gel-Based Delivery of rAAV1-*GAA* Results in Biochemical Correction of Diaphragms in *Gaa*$^{-/-}$ Mice

Having demonstrated increased transduction of the mouse diaphragm using the gel-based method, we assessed the ability of this method to restore enzymatic activity in a mouse model of glycogen storage disorder type II (GSDII; MIM 232300), a lysosomal glycogen storage disease caused by a lack or deficiency of the lysosomal enzyme acid α-glucosidase (GAA; EC 3.2.1.20). The mouse model of this disease stores glycogen in all tissues, with significant pathologies in the heart and skeletal muscle [24]. We have previously characterized the use of rAAV vectors to restore enzymatic and functional activity in skeletal and cardiac muscle in these mice [20]. Coupled with our new findings using a gel-based delivery method, we hypothesized that gel-based delivery of rAAV1-*GAA* would be able to restore GAA activity in *Gaa*$^{-/-}$ diaphragms and, in turn, reverse lysosomal glycogen accumulation.

Using rAAV1-*GAA* vectors, we found increases in diaphragmatic transduction in *Gaa*$^{-/-}$ mice similar to those seen in control mice with β-galactosidase vector. GAA enzymatic activities were restored to 50% of wild type with free vector and were further increased to 120% of normal levels using a vector–gel suspension (Fig. 2A). These activities had a profound effect on glycogen storage, as assessed by periodic acid–Schiff's reagent (PAS) staining (Fig. 2B). Dark pink vacuoles, indicative of stored glycogen, are observed in free-vector-treated diaphragms from *Gaa*$^{-/-}$ mice, whereas a near-complete reversal of glycogen accumulation from diaphragms is seen in gel-treated mice.

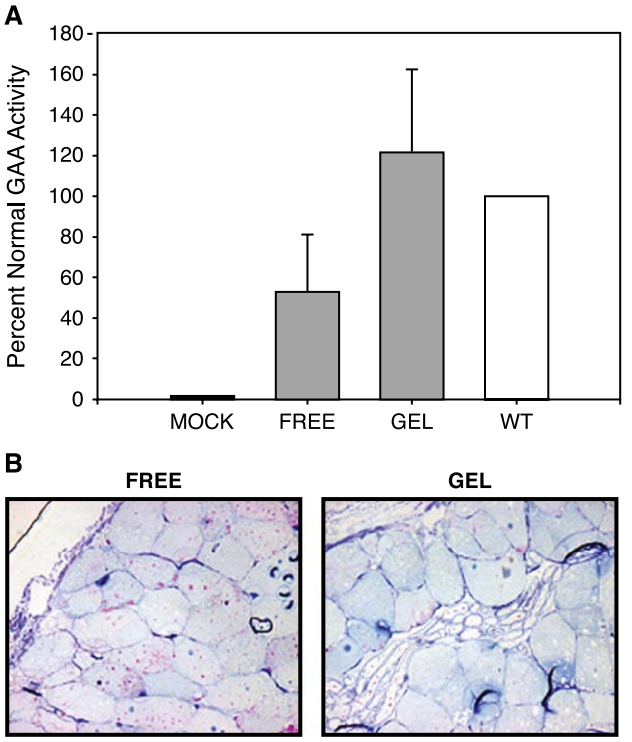


FIG. 2. rAAV1-*GAA*-mediated transduction of diaphragms of *Gaa*$^{-/-}$ mice. (A) Adult *Gaa*$^{-/-}$ mice were treated with 1×10^{11} particles of rAAV1-*GAA* in the quadriceps muscle. Wild-type (WT) and untreated *Gaa*$^{-/-}$ (MOCK) mice were used as controls. Muscle tissues were isolated at 6 weeks after treatment and assayed for GAA activity. The bars represent mean ± SEM GAA activity for three mice in each group. (B) Representative sections from free vector- (left) and gel-based vector-treated (right) *Gaa*$^{-/-}$ mouse diaphragms, stained for glycogen using periodic acid–Schiff's reagent. Glycogen-containing vacuoles and regions acquire a pink stain using this technique.

Biodistribution of rAAV Genomes after Gel-Based Delivery

Since a secondary advantage of physical delivery systems may be the ability to restrict viral spread, we also sought to determine which tissues endocytosed our viral vectors after gel-based delivery. To this end, we harvested various tissues from rAAV1-βgal gel-treated mice and extracted total cellular DNA. Using a nested PCR technique, we amplified a portion of the β-galactosidase gene from vector genomes (Fig. 3). As expected, vector genomes could be detected in treated diaphragms. We could not detect vector genomes in any other tissue examined (including sections of the peritoneal wall and liver adjacent to the diaphragm); however, it is possible that more sensitive detection methods

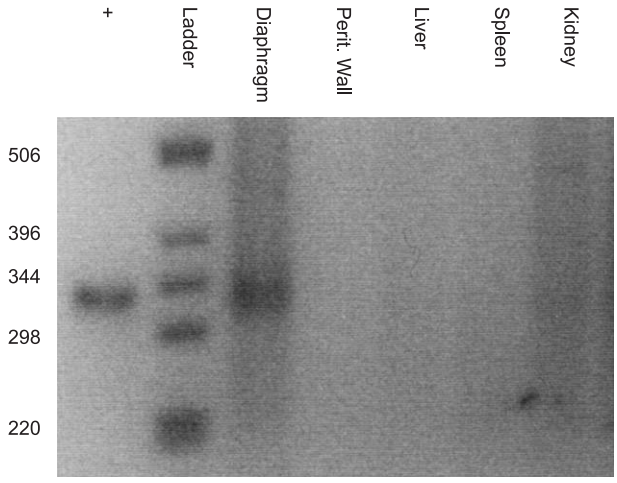


FIG. 3. Biodistribution of rAAV1 vector genomes after gel-based delivery. We used nested PCR to amplify AAV genomes carrying the β-galactosidase gene after isolating tissues from gel-based rAAV1-βgal-treated mice. Total cellular DNA was extracted and AAV genomes were amplified using primers specific for the βgal transgene. The expected product is 333 bp, and the positive control is the vector plasmid that was used to package the rAAV particles.

(such as real-time PCR) would detect trace amounts of vector genomes.

DISCUSSION

Transduction events for recombinant adeno-associated viruses can be separated into five general stages: (1) binding and entry (endocytosis), (2) endosomal processing and escape, (3) transcytosis, (4) nuclear import and uncoating, and (5) genome conversion, including second-strand synthesis (or alternatively self-complementation), followed by genome concatemerization and/or integration into the host chromosome. We have sought in this report to enhance the first step of this process using a physical method to prolong viral dwell time—we expect to increase the efficiency of transduction by providing longer viral particle exposure times to receptors on target tissues.

Carrier molecules and delivery agents have been used extensively for gene therapy applications, particularly for nonviral gene delivery. With regard to viral vectors, recombinant adenoviruses have been used in concert with a variety of agents to increase or prolong bioavailability, thereby enhancing the efficiency of delivery. March and colleagues [26] reported the use of poloxamer 407, a hydrogel that exhibits potentially useful, thermoreversible gelation, enabling formulation at low temperature with subsequent hardening to a robust gel at room and physiologic temperatures. They demonstrated increased transduction of vascular smooth muscle cells *in vitro*, with similar findings reported *in vivo* by Van Belle *et al.* [27]. Unfortunately, poloxamers have recently been shown to have adverse effects on adeno-associated virus stability [28]. Likewise, thixotropic solutions have also shown promise for enhancing adenovirus-mediated transduction of airway epithelia [29]. Several other promising agents have also been effectively used with adenovirus vectors, including β-cyclodextrins, surfactants, and collagen- or gelatin-based matrices.

While extensive testing of potential adenovirus formulations has been reported, few similar studies are extant for adeno-associated viruses. Most of the available literature describes formulations that increase stability for storage or purification, but few reports address the need for augmented physical delivery of viral particles *in vivo*. We have previously described the use of microsphere-conjugated rAAV for systemic delivery of viral vectors, in which we were able to increase significantly the transduction efficiency in target tissue beds *in vivo* by increasing vector dwell time [30]. Similarly, a number of groups are currently developing capsid-modified rAAV vectors to target specific vascular beds upon systemic delivery. To date, however, we are unaware of other examples of physical delivery agents or methods to improve rAAV delivery to tissue surfaces, such as skin, blood vessel adventitia, or diaphragm. The presented strategy for diaphragmatic delivery of rAAV relies on retention of vector on the peritoneal surface of the diaphragm. Local delivery using this strategy is clinically achievable by endoscopic delivery and has the added benefit of reduced risk associated with systemic vascular delivery. While the method that we describe has been specifically applied to the murine diaphragm, we anticipate that this formulation or similar ones will have broad utility for a variety of tissues, both for preclinical proof-of-principle studies in animal models and for eventual clinical applications (e.g., topical applications for wound healing).

Previous studies of gene transfer to the diaphragm in rodents have been attempted *via* delivery of nonviral or adenoviral gene transfer vectors. Liu *et al.* recently described a method for systemic delivery of plasmid DNA carrying the full-length dystrophin gene with subsequent targeting to the diaphragm in *mdx* mice [3], a mouse strain with X-linked muscular dystrophy that mimics the diaphragmatic degeneration observed in Duchenne muscular dystrophy [31]. In that study, which used no carrier molecules, plasmid DNA was delivered intravenously *via* tail vein followed by transient (8-s) occlusion of the vena cava at the level of the diaphragm. High levels of gene expression were measured in diaphragm homogenates the next day and for 180 days [3], implicating dwell time as potentially the most significant determinant of successful gene transfer to the diaphragm with naked DNA. Two reports [32,33], by Petrof, Yang, and colleagues, also indicated successful direct injection of recombinant adenoviruses carrying a mini-dystrophin gene to the diaphragms of *mdx* mice. Both studies demonstrated high levels of expression focally, presumably due to the delivery method. Transient gene expression, due to vector-

doi:10.1016/j.ymthe.2004.01.006

related, dose-dependent inflammation, made assessment of the uniformity of gene expression difficult, but even with focal expression the authors observed measurable improvements in contractile function. More recently, Sakamoto *et al.* have developed an *mdx* strain that is transgenic for a micro-dystrophin construct [34], which is within the packaging capacity of rAAV. We are currently investigating whether the combination of the dystrophin-associated complex transgenes and gel-based delivery of rAAV will lead to uniform phenotypic correction in diaphragms of dystrophic mice.

Comparisons of rAAV serotype tropisms for skeletal muscle have already been reported [20–23]. Several recombinant AAV vectors based on alternative serotypes have demonstrated greater transduction efficiencies in skeletal muscle than serotype 2. In particular, several reports have shown nearly 1 log greater expression of a variety of transgenes when packaged in rAAV1 capsids compared to rAAV2. Similar findings have been reported with rAAV6, although this serotype has not been as widely studied [23,35]. Clear differences in serotype tropism were observed between rAAV1 and the other two serotypes in the context of gel-based delivery and free virus administration, with significant differences observed between rAAV1 and rAAV5 ($P<0.1$). We note that the eightfold overexpression of GAA in Gaa-deficient diaphragms after delivery of free rAAV1-*GAA* compared to serotype 2 (Fig. 1B, AAV1 Free vs AAV2 Free) is nearly identical to our prior observations after direct intramuscular administration of the same two vectors in *tibialis anterior* muscles of $Gaa^{-/-}$ mice [20], indicating a conserved rAAV1 tropism for skeletal muscle.

MATERIALS AND METHODS

Packaging and purification of recombinant AAV1, 2, and 5 vectors. The recombinant AAV2 plasmids pAAV-*lacZ* [12] and p43.2-*GAA* [20] have been described previously. Recombinant AAV vectors were generated, purified, and titered at the University of Florida Powell Gene Therapy Center Vector Core Lab as previously described [18]. Recombinant AAV particles based on serotypes 1, 2, and 5 were produced using pAAV-*lacZ*, whereas only rAAV1 particles (rAAV1-*GAA*) were packaged with p43.2-*GAA*.

Vector/vehicle preparation. A sterile, bacteriostatic, water-soluble, glycerin-based gel was used as a vehicle for vector application to the diaphragm (K-Y Sterile; Johnson & Johnson Medical, Arlington, TX, USA). Individual doses of virus were diluted in sterile phosphate buffered saline (PBS) for a total volume of 10 μl and then added to 150 μl of gel in a 2-ml microcentrifuge tube. The virus–vehicle suspension was vortexed for 1 min and then centrifuged for one minute at maximum speed. Free virus was diluted in sterile PBS for a total volume of 50 μl.

In vivo delivery. All animal studies were performed in accordance with the guidelines of the University of Florida Institutional Animal Care and Use Committee. Adult 129X1×C57BL/6 (wild type) or $Gaa^{-/-}$ mice [24] were anesthetized using 2% isoflurane and restrained supine on a warmed operating surface. In a sterile field, after reaching a surgical plane of anesthesia, a midline incision was made through the skin extending from the xyphoid process to the suprapubic region. An incision was made through the abdominal wall following the *linea alba*. The abdominal walls were retracted laterally, the gall bladder was carefully separated from the rib cage, and the liver was carefully retracted from the diaphragm using sterile cotton swabs.

While the xyphoid was lifted, free virus or virus mixed with vehicle was applied directly to the abdominal surface of the diaphragm. Free virus was applied using a pipet. To facilitate application of the gel to the diaphragm, a 22-gauge needle was used to puncture the bottom of the microcentrifuge tube and a plunger from a 3-cc syringe was used to force the gel through the hole and onto the diaphragm surface (Fig. 4). In some cases, a cotton-tipped applicator was used to ensure even spread over the entire diaphragm. After 5 min the abdominal muscles were sutured and the skin was closed. Subcutaneous ampicillin (20–100 mg/kg) and buprenorphine (0.1 mg/kg) were administered prior to removing the animal from anesthesia.

Assays of β-galactosidase and GAA enzymatic activity. Six weeks after the surgical procedure and gene delivery, tissue lysates were assayed for enzyme activity using the Galacto-Star chemiluminescence reporter gene assay system (Tropix, Inc., Bedford, MA, USA). Protein concentrations for tissue lysates were determined using the Bio-Rad *DC* protein assay kit (Bio-Rad, Hercules, CA, USA). For rAAV1-*GAA*-treated animals, enzymatic activity assays for GAA were performed 6 weeks after vector delivery as described previously [20]. Tissue homogenates were assayed for GAA activity by measuring the cleavage of the synthetic substrate 4-methylumbelliferyl-α-D-glucoside (Sigma M9766; Sigma–Aldrich, St. Louis, MO, USA) after incubation for 1 h at 37°C. Successful cleavage yielded a fluorescent product that emits at 448 nm, as measured with an FLx800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA). Protein concentration was measured as described above. Data are represented as nanomoles of substrate cleaved in 1 h per milligram of total protein in the lysate.

Histological assessment of glycogen clearance. Segments of treated and untreated diaphragm were fixed overnight in 2% glutaraldehyde in PBS, embedded in Epon, sectioned, and stained with PAS by standard methods [24].

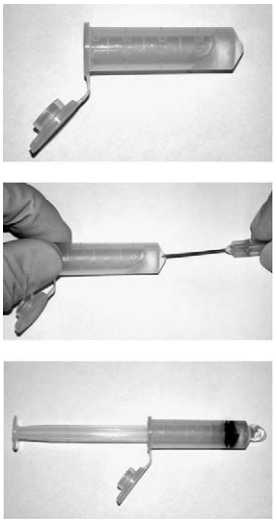


FIG. 4. Gel-based delivery preparation. (Top) rAAV vectors are mixed in a 2-mL microcentrifuge tube and then centrifuged briefly. (Middle) The tube is punctured using a 22-gauge needle, creating an aperture through which the virus–gel suspension can be propelled. (Bottom) A plunger from a standard 3-cc syringe is used to push the vector from the tube, enabling its application to the diaphragmatic surface. The oblique, bottom surface of the microcentrifuge tube is used to distribute the vector–gel suspension evenly on the surface.

Biodistribution of vector genomes. Tissues were removed using sterile instruments and snap-frozen in liquid nitrogen. Total cellular DNA was extracted from tissue homogenates using a Qiagen DNeasy kit per the manufacturer's instructions (Qiagen, Valencia, CA, USA). Nested PCR was performed as follows: 1.5 μg total DNA was used as a template for the initial PCR amplification using the sense primer 5′-AGCTGGCGTAATAGCGAAGA-3′ and reverse primer 5′-CGCGTCTCTCCAGGTAGCGAA-3′, yielding a 1486-bp product. The PCR product was purified using the Qiagen MinElute PCR purification kit per the manufacturer's instructions, followed by PCR amplification using the sense primer 5′-CGGTGATGGTGCTGCGTTGGAG-3′ and reverse primer 5′-TCGACGTTCAGACGTAGTGT-3′, resulting in a final product of 333 bp. All reactions were performed under the following conditions: hot start denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 2 min. Products were electrophoresed and analyzed using a 2% agarose gel.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the University of Florida Powell Gene Therapy Center Vector Core Laboratory, which produced some of the rAAV vectors used in this study. This work was supported by grants from the National Institutes of Health (NHLBI P01 HL59412-06, NIDDK P01 DK58327-03) and the American Heart Association, Florida and Puerto Rico Affiliate (T.J.F.). B.J.B., The Johns Hopkins University, and the University of Florida could be entitled to patent royalties for inventions described in this article.

RECEIVED FOR PUBLICATION NOVEMBER 5, 2003; ACCEPTED JANUARY 5, 2004.

REFERENCES

1. Petrof, B. J. (1998). Respiratory muscles as a target for adenovirus-mediated gene therapy. *Eur. Respir. J.* **11:** 492–497.
2. Baranov, A., *et al.* (1999). Local and distant transfection of mdx muscle fibers with dystrophin and LacZ genes delivered in vivo by synthetic microspheres. *Gene Ther.* **6:** 1406–1414.
3. Liu, F., Nishikawa, M., Clemens, P. R., and Huang, L. (2001). Transfer of full-length Dmd to the diaphragm muscle of Dmd(mdx/mdx) mice through systemic administration of plasmid DNA. *Mol. Ther.* **4:** 45–51.
4. Buning, H., *et al.* (2003). Receptor targeting of adeno-associated virus vectors. *Gene Ther.* **10:** 1142–1151.
5. Muller, O. J., *et al.* (2003). Random peptide libraries displayed on adeno-associated virus to select for targeted gene therapy vectors. *Nat. Biotechnol.* **21:** 1040–1046.
6. Perabo, L., *et al.* (2003). In vitro selection of viral vectors with modified tropism: the adeno-associated virus display. *Mol. Ther.* **8:** 151–157.
7. Ponnazhagan, S., Mahendra, G., Kumar, S., Thompson, J. A., and Castillas, M., Jr. (2002). Conjugate-based targeting of recombinant adeno-associated virus type 2 vectors by using avidin-linked ligands. *J. Virol.* **76:** 12900–12907.
8. Shi, W., Arnold, G. S., and Bartlett, J. S. (2001). Insertional mutagenesis of the adeno-associated virus type 2 (AAV2) capsid gene and generation of AAV2 vectors targeted to alternative cell-surface receptors. *Hum. Gene Ther.* **12:** 1697–1711.
9. Shi, W., and Bartlett, J. S. (2003). RGD inclusion in VP3 provides adeno-associated virus type 2 (AAV2)-based vectors with a heparan sulfate-independent cell entry mechanism. *Mol. Ther.* **7:** 515–525.
10. Wu, P., *et al.* (2000). Mutational analysis of the adeno-associated virus type 2 (AAV2) capsid gene and construction of AAV2 vectors with altered tropism. *J. Virol.* **74:** 8635–8647.
11. Muzyczka, N. (1992). Use of adeno-associated virus as a general transduction vector for mammalian cells. *Curr. Top. Microbiol. Immunol.* **158:** 97–129.
12. Kessler, P. D., *et al.* (1996). Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. *Proc. Natl. Acad. Sci. USA* **93:** 14082–14087.
13. Clark, K. R., Sferra, T. J., and Johnson, P. R. (1997). Recombinant adeno-associated viral vectors mediate long-term transgene expression in muscle. *Hum. Gene Ther.* **8:** 659–669.
14. Fisher, K. J., *et al.* (1997). Recombinant adeno-associated virus for muscle directed gene therapy. *Nat. Med.* **3:** 306–312.
15. Gao, G. P., *et al.* (2002). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc. Natl. Acad. Sci. USA* **99:** 11854–11859.
16. Grimm, D., Kay, M. A., and Kleinschmidt, J. A. (2003). Helper virus-free, optically controllable, and two-plasmid-based production of adeno-associated virus vectors of serotypes 1 to 6. *Mol. Ther.* **7:** 839–850.
17. Xiao, W., *et al.* (1999). Gene therapy vectors based on adeno-associated virus type 1. *J. Virol.* **73:** 3994–4003.
18. Zolotukhin, S., *et al.* (2002). Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. *Methods* **28:** 158–167.
19. Rabinowitz, J. E., *et al.* (2002). Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. *J. Virol.* **76:** 791–801.
20. Fraites, T. J., Jr., *et al.* (2002). Correction of the enzymatic and functional deficits in a model of Pompe disease using adeno-associated virus vectors. *Mol. Ther.* **5:** 571–578.
21. Chao, H., Monahan, P. E., Liu, Y., Samulski, R. J., and Walsh, C. E. (2001). Sustained and complete phenotype correction of hemophilia B mice following intramuscular injection of AAV1 serotype vectors. *Mol. Ther.* **4:** 217–222.
22. Chao, H., *et al.* (2000). Several log increase in therapeutic transgene delivery by distinct adeno-associated viral serotype vectors. *Mol. Ther.* **2:** 619–623.
23. Hauck, B., and Xiao, W. (2003). Characterization of tissue tropism determinants of adeno-associated virus type 1. *J. Virol.* **77:** 2768–2774.
24. Raben, N., *et al.* (1998). Targeted disruption of the acid alpha-glucosidase gene in mice causes an illness with critical features of both infantile and adult human glycogen storage disease type II. *J. Biol. Chem.* **273:** 19086–19092.
25. Moufarrej, N. A., and Bertorini, T. E. (1993). Respiratory insufficiency in adult-type acid maltase deficiency. *South. Med. J.* **86:** 560–567.
26. March, K. L., Madison, J. E., and Trapnell, B. C. (1995). Pharmacokinetics of adenoviral vector-mediated gene delivery to vascular smooth muscle cells: modulation by poloxamer 407 and implications for cardiovascular gene therapy. *Hum. Gene Ther.* **6:** 41–53.
27. Van Belle, E., *et al.* (1998). Effects of poloxamer 407 on transfection time and percutaneous adenovirus-mediated gene transfer in native and stented vessels. *Hum. Gene Ther.* **9:** 1013–1024.
28. Croyle, M. A., Cheng, X., and Wilson, J. M. (2001). Development of formulations that enhance physical stability of viral vectors for gene therapy. *Gene Ther.* **8:** 1281–1290.
29. Seiler, M. P., *et al.* (2002). Thixotropic solutions enhance viral-mediated gene transfer to airway epithelia. *Am. J. Respir. Cell Mol. Biol.* **27:** 133–140.
30. Mah, C., *et al.* (2002). Improved method of recombinant AAV2 delivery for systemic targeted gene therapy. *Mol. Ther.* **6:** 106–112.
31. Stedman, H. H., *et al.* (1991). The mdx mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy. *Nature* **352:** 536–539.
32. Petrof, B. J., *et al.* (1995). Efficiency and functional consequences of adenovirus-mediated in vivo gene transfer to normal and dystrophic (mdx) mouse diaphragm. *Am. J. Respir. Cell Mol. Biol.* **13:** 508–517.
33. Yang, L., *et al.* (1998). Adenovirus-mediated dystrophin minigene transfer improves muscle strength in adult dystrophic (MDX) mice. *Gene Ther.* **5:** 369–379.
34. Sakamoto, M., *et al.* (2002). Micro-dystrophin cDNA ameliorates dystrophic phenotypes when introduced into mdx mice as a transgene. *Biochem. Biophys. Res. Commun.* **293:** 1265–1272.
35. Kawamoto, S., *et al.* (2003). AAV6 vectors promote efficient transduction and gene dissemination through myocardium *in vivo*. *Mol. Ther.* **7:** S228.