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Efficient Long-Term Gene Transfer into Muscle Tissue of Immunocompetent Mice by Adeno-Associated Virus Vector

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Muscle-directed gene transfer is being considered for the treatment of several metabolic diseases, including hemophilia and Duchene's muscular dystrophy. Previous efforts to target this tissue for somatic delivery with various vector systems have resulted in transient expression due to silencing of the transgene or to an immune response against the vector-transduced cells. We introduced recombinant adeno-associated virus vector (rAAV) carrying a *lacZ* reporter into muscle tissue of immunocompetent mice. The *lacZ* reporter gene was efficiently transduced and expressed with no evidence of a cellular immune response. Moreover, gene expression persisted for more than 1.5 years. Molecular characterization of rAAV vector DNA suggests a mechanism for persistence, since vector episomes convert to high-molecular-weight genomic DNA. These data provide the first report for establishing long-term gene transduction into mammalian muscle cells in vivo without the need for immune modulation of the organism.

Muscle tissue has recently become an attractive target for ex vivo and in vivo gene delivery due to the identification of genes responsible for various diseases of this tissue and because muscle cells can be genetically modified to secrete nonmuscular therapeutic factors. Despite success with ex vivo gene delivery via implanted myoblasts, efficient long-term gene delivery directly into muscle tissue has been unsuccessful (8, 36). Previously, a variety of nonviral (naked DNA [50]) and viral (retrovirus [14] and adenovirus [41, 48]) mechanisms of introduction have been tested. Uptake and expression can be achieved upon injection of naked plasmids. However, gene transfer is limited to a small percentage of the cells near the injection site, restricting the usefulness of this method. Moreover, the plasmid DNA remains episomal and is present only transiently, currently precluding long-term correction (32).

Viral vectors provide an alternative efficient mechanism of delivery. Although used widely for ex vivo gene transduction, retrovirus delivery into mature muscle tissue has been hampered due to the nondividing status of these cells. Even in the Duchene's muscular dystrophy animal model, in which numerous cells are dividing due to spontaneous regeneration, retroviral delivery appears inefficient when compared with DNA transfer (11). Transgene silencing has also been observed in this system, which has resulted in the need for further vector modification (39). Recent reports of human immunodeficiency virus-based vectors capable of transducing nondividing cells may provide an alternative strategy (38). However, the safety and efficiency of these promising vectors remain to be tested (38). Contrary to retroviruses, recombinant adenovirus (rAd)-based vectors are able to infect both dividing and nondividing cells (41, 48). While transduction of nondividing cells has met with great success, expression has been short-lived primarily due to elimination of the rAd-transduced cells by the host immune system (10, 51, 53, 55). Modulation of the immune system extends transgene expression, but the episomal nature of this vector results in eventual loss of the therapeutic gene. Furthermore, rAd vectors seem to transduce mature muscle cells much less efficiently than they do neonatal muscle, possibly due to limiting receptor levels (1, 2, 24, 27).

Adeno-associated virus (AAV) vectors represent a promising alternative to the above-mentioned vector systems (22, 37). The parental wild-type AAV is nonpathogenic (6, 7). The vector consists only of AAV inverted terminal repeats necessary for replication, packaging, and integration (45). Removal of all viral coding sequences (96% of the genome) prevents the generation of wild-type helper virus and eliminates the possibility of immune responses to residual viral gene expression (4, 19). In place of the 4.4-kb coding region, the virus can carry and express nonviral genes of up to 5 kb (23, 37). Like the rAd vector, rAAV can infect both dividing and nondividing cells (20, 23, 28, 34, 37, 43). Importantly, the rAAV genome will integrate into the host chromosome, facilitating long-term transduction (7, 22, 37, 44). Recombinant AAV preparations are stable and can be produced at high titers of more than 10^{12} particles per ml (21, 50a). An rAAV vector carrying the *CFTR* gene has been tested in rabbit lung with vector transduction for up to 6 months, providing preclinical data that has facilitated a phase I toxicity trial in humans (13). In cultured cells, rAAV integrates stably into host chromosomes, although recent reports suggest that rAAV vectors can persist as free episomal DNA in nondividing cells (4, 20, 43). Persistence of rAAV in vivo has only begun to be studied in detail (4).

Here we test the ability of rAAV to transduce genes into somatic muscle tissue of the mouse. We report the first long-term gene transduction into this tissue, using direct virus introduction. Moreover, a cellular immune response to the transduced cells was not observed. Characterization of the reporter gene which has sustained expression for more than 1.5 years suggests that the input viral episomes are being stably maintained as a high-molecular-weight species, providing a viable mechanism for establishing long-term gene transduction in nondividing muscle cells.

MATERIALS AND METHODS

Preparation of viral vectors. Preparations of rAAV-LacZ viral vector were made by cotransfection methods according to published protocols (34, 45) with modifications. Briefly, at 1 to 2 h before transfection, 20 15-cm dishes of human 293 cells of 80% confluency were fed with 25 ml of fresh Iscove modified Eagle medium (Gibco) containing 10% fetal bovine serum (Hyclone) without antibi-

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otics. Fifty micrograms of plasmid DNA (25 μg AAV-LacZ vector plasmid [25] plus 25 μg of helper plasmid [45]) was dissolved in 2 ml of 0.25 M $CaCl_2$ and then quickly mixed with 2 ml of HBS buffer (50 mM HEPES, 280 mM NaCl, and 1.5 mM Na_2HPO_4, pH 7.12) and added to the cells. After 8 to 12 h of transfection, the medium was replaced with fresh Dulbecco modified Eagle medium (DMEM) (Gibco) containing 10% fetal bovine serum and antibiotics. Adenovirus type 5 (*dl*309) was added to the cells at a multiplicity of infection of 2. At 2.5 days post-adenovirus infection, the cells were harvested by low-speed centrifugation, resuspended in lysis buffer (10 mM Tris-Cl, pH 8.5, 150 mM NaCl), frozen, and thawed four times. Cell debris was removed by low-speed centrifugation.

To the supernatant, an equal volume of ice-cold saturated $(NH_4)_2SO_4$ (pH 7.0) was added and placed on ice for 20 min. The sample was then centrifuged at 15,000 × *g* for 10 min. The pellet was redissolved in CsCl-phosphate-buffered saline (PBS) (pH 7.5) solution (density, 1.38 g/ml) and centrifuged in an SW41 rotor (Beckman) at 40,000 rpm for 48 h with a 0.5-ml CsCl-PBS cushion (density, 1.5 g/ml). The rAAV band was collected and recentrifuged as described above for 48 h. Finally, the rAAV band was collected and dialyzed against DMEM and heated at 56°C for 15 to 30 min. The rAAV-LacZ virus titers were determined by infecting 293 cells at various dilutions with 1 multiplicity of infection of adenovirus type 5. The cells were fixed 24 h after infection and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (46). Each blue cell was accounted as transduced by one infectious rAAV-LacZ particle.

The rAd-LacZ vector was constructed and prepared as described by Acsadi et al. (2). The titering was performed similarly to that for rAAV-LacZ by counting the number of blue cells transduced by rAd-LacZ.

In vivo viral delivery and detection of transgene expression. Swiss Webster mice were purchased from Taconic (German Town, N.Y.) and handled in accordance with the institutional guidelines of the University of North Carolina. Before virus injection, 3-week- to 4-month-old mice were anesthetized with 2.5% Avertin intraperitoneally. Thirty microliters of rAAV-LacZ (3×10^6 infectious units) or rAd-LacZ (3×10^6 infectious units) individually, or a 30-μl mix of rAAV-LacZ plus rAd-LacZ (3×10^6 infectious units each) was injected into the hind leg tibialis anterior muscles percutaneously. Secondary in vivo delivery was carried out at different intervals by injecting the same amount of rAAV-LacZ into the foreleg of the mice which had previously received the vector in the hind legs.

At various time points, the mice were euthanized and the muscle tissues were harvested and rapidly frozen in liquid nitrogen. Cryostat sectioning of the tissue was performed at 20-μm thickness with a Leica microtone. The sections were then fixed and stained according to previously published methods (46). The samples were lightly stained with hematoxylin-eosin (H&E), dehydrated with ethanol, and mounted for photography.

Southern analysis of the DNA from muscle tissues. Muscle tissues were harvested, minced, and suspended in lysis buffer (10 mM Tris-HCl, 100 mM EDTA, 0.5% sodium dodecyl sulfate, and 100 μg of proteinase K per ml, pH 8.0). The samples were digested at 37°C overnight and incubated for an additional 2 h after adding 20 μg of RNase A per ml. After extraction with phenol, phenol-chloroform, and chloroform, the total DNA was precipitated with 0.3 M sodium acetate and 2.5 vol of ethanol, washed twice with 70% ethanol, and redissolved in TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). Restriction enzyme digestion with *Eco*RV (New England BioLabs) was carried out overnight. DNA samples were separated on a 1% agarose gel, and Southern hybridization was performed with GeneScreen Plus membrane according to the manufacturer's standard protocol (NEN-DuPont). The *lacZ* DNA probe was a 2.1-kb *Cla*I and *Nde*I fragment labeled with [α-^{32}P]dATP and Random Primer kit (Boehringer Mannheim).

RESULTS

Efficient transduction of muscle cells in vivo with rAAV. To test the feasibility of gene transduction by rAAV, we inserted a *lacZ* reporter gene that harbors a nuclear localization signal under the regulation of the cytomegalovirus immediate-early promoter into an AAV vector (25). For comparison, an adenovirus vector was designed to express a cytoplasmic β-galactosidase (β-gal) also under CMV control. Hence, transgene expression by the two vectors could be distinguished simultaneously on the basis of cellular localization of β-gal activity. Mouse muscle tissue displays no detectable β-gal activity under the assay conditions used (data not shown). Virus preparations of rAAV and/or rAd were injected into the hind leg muscle of 3-week-old mice. Nine mice received rAAV-LacZ in the left hind leg and an equal dose of rAd-LacZ in the right hind leg. Eight additional mice received rAAV-LacZ in the left hind leg and a mixture containing equal doses of both vectors in the right hind leg. We then assessed (i) the transduction efficiency of rAAV vector alone in muscle, (ii) the difference in transduction efficiency between rAd and rAAV vectors in the same animal at different injection sites, and (iii) the relative transduction efficiencies when rAd and rAAV vectors were coinjected into the same site.

At various times post-vector delivery, mice were sacrificed and muscle tissue was cryosectioned and stained for β-gal activity, using X-Gal as a substrate. The experimental results from animals 3 weeks post-vector delivery are illustrated in Fig. 1. Muscle tissue from mice injected with rAAV-LacZ vector, either alone (left hind leg) or with rAd-LacZ (right hind leg of same animal), showed β-gal nuclear staining within hundreds of myotubes, indicating efficient gene transduction by the rAAV vector (Fig. 1a, panels B, C, E, and F). In contrast, the rAd-LacZ vector injected alone (left hind leg) or coinjected with rAAV-LacZ (right hind leg of the same animal) transduced inefficiently (less than 20 positive fibers per sections; Fig. 1a, panels A, D, B, and E). Inefficient rAd vector transduction of mature muscle has also been observed by others (1–3, 27). These experiments indicate that rAAV transduction, unlike that of rAd, is efficient in mature muscle and that direct injection of viral vector is a viable approach for gene delivery.

rAAV does not elicit significant cellular immune response. In previous experiments using adenovirus in immunocompetent animals, cellular immune response was substantial, resulting in inflammation, tissue destruction, and eventual removal of the transduced cells (2, 3, 9, 33, 51, 53, 55). To determine whether similar responses were induced upon introduction of rAAV into the muscle, we examined muscle tissue for evidence of lymphocyte infiltration. Examination of H&E-stained tissue samples 4 days postinjection showed marked infiltrates of all muscles injected with rAd-LacZ (Fig. 2A). In rAd-LacZ-injected samples, such infiltration was undetectable after 3 weeks when rAd-LacZ-positive myotubes were no longer present (Fig. 3A and B) (2, 3, 9, 33, 51, 53, 55). However, in samples injected with rAAV vector, only mild and transient inflammation reactions were observed (Fig. 2B). Figure 2B shows some regional infiltration reaction in rAAV-transduced tissues 4 days after injection, noticeably with many transduced cells free of infiltration. More importantly, this inflammation was self-limiting and disappeared at later time points without apparent loss of transduction efficiency (Fig. 1a, panels B, C, E, and F; Fig. 3C, D, E, and F; and Fig. 4). Interestingly, when rAAV and rAd vectors were coinjected in the same animal at different sites (Fig. 1a, panels C and F, and Fig. 3E and F) or identical sites (Fig. 1a, panels B and E, and Fig. 3C and D), rAAV nuclear β-gal expression persisted despite the strong initial infiltration and the disappearance of rAd-transduced cells. This result indicates that the rAAV vector does not induce a strong cellular immune response to the transduced cell, allowing for the persistence of gene expression and the preservation of healthy tissue (3, 52, 53, 55).

Although we found no indication of a cellular (cytotoxic T-lymphocyte [CTL]) immune response to rAAV, secondary administration of rAAV vectors into the foreleg muscle of mice that had been injected 9 months earlier with rAAV-LacZ in the hind leg resulted in no additional transduction when assayed 2 weeks after the second administration. Since control naive adult mice were successfully transduced at later times (Fig. 1b), initial rAAV introduction may have raised a humoral immune response. Indeed, serum collected from rAAV-injected animals inhibited rAAV transduction in cell culture. We observed neutralizing titers of 250 to 1,250 in animals that received a primary injection of rAAV, which after secondary administration increased more than 25-fold to titers of greater than 30,000 (data not shown). Since naive animals had no neutralizing activity, these data suggest that the primary injection was sufficient to elicit a humoral response which resulted

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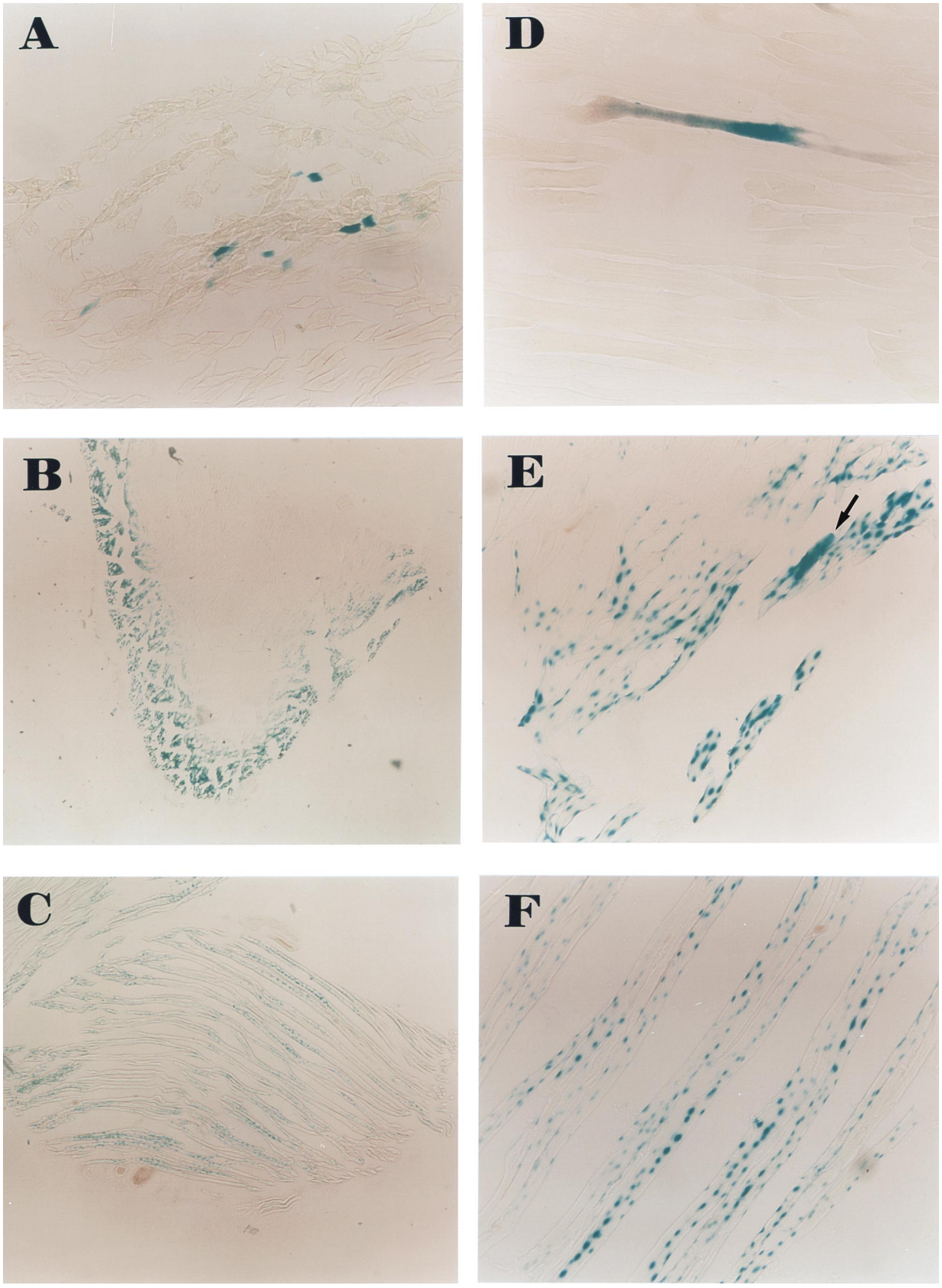

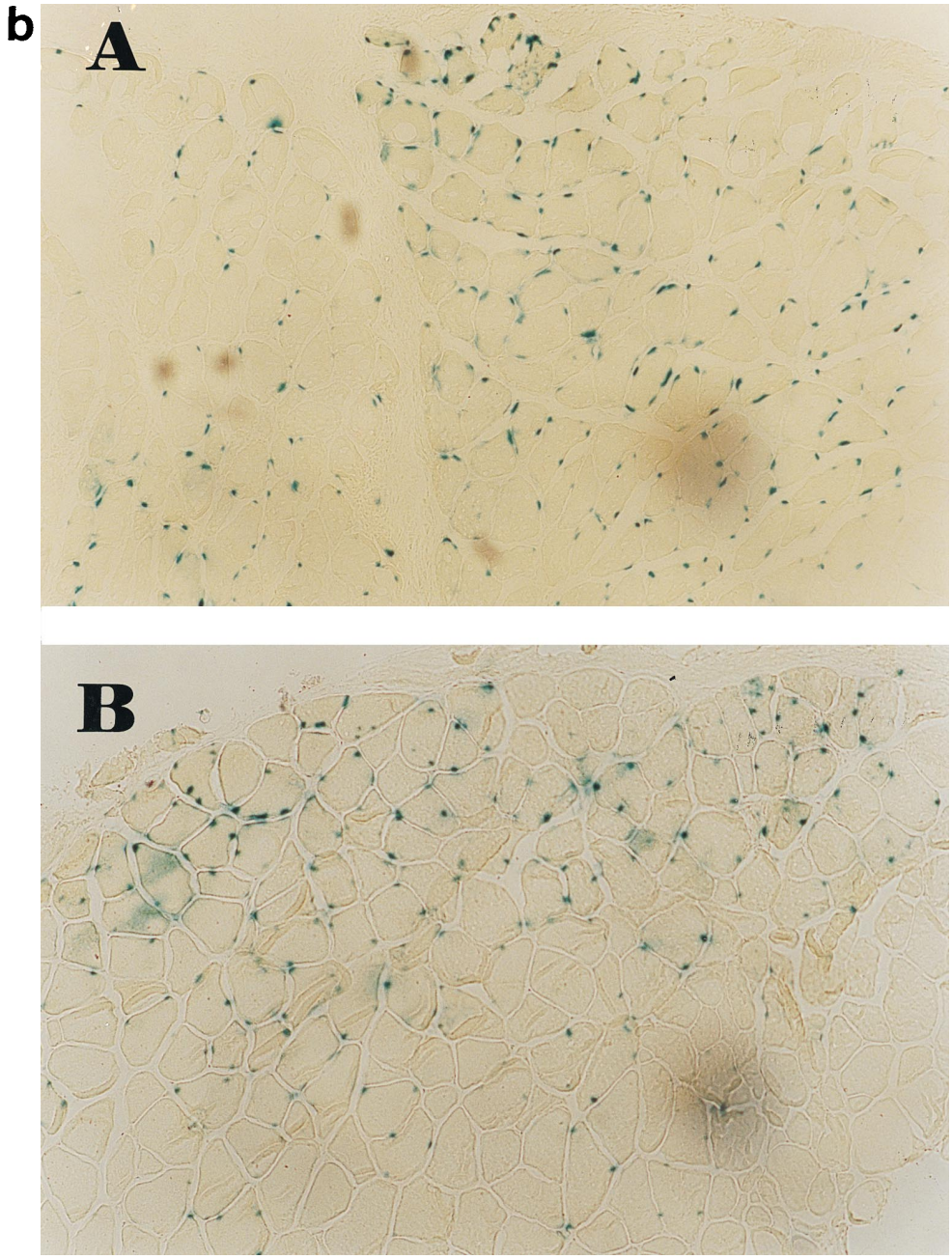

FIG. 1. X-Gal staining of the cryostat sections of the tibialis anterior muscle injected with an rAd-LacZ vector and/or an rAAV-LacZ vector. (a) Three-week-old mice were injected with rAd-LacZ (3×10^6 infectious units) (A and D), rAAV-LacZ (3×10^6 infectious units) (C and F), or rAAV-LacZ plus rAd-LacZ (3×10^6 infectious units each, in a mixture) (B and E). The arrow shows a myotube transduced by both vectors. The histochemical staining was performed at 3 weeks post-vector injections. Panels B and C were photographed at ×6.25, while panels A, D, E, and F were photographed at ×25 magnifications. (b) Four-month-old mice were injected with rAAV-LacZ (3×10^6 infectious units), and the tissues were dissected and stained at 7 days (A) and 8 months (B) post-vector injection. Magnification, ×50.

in significant neutralizing activity after the second administration. Nevertheless, transduced cells at the primary injection sites in the hind leg were not affected by a second injection of rAAV in the foreleg and no lymphocyte infiltration was found at the primary injection site (data not shown), supporting the conclusion that rAAV vector does not elicit a detrimental CTL response.

Long-term gene expression in rAAV-transduced muscle cells. Encouraged by the short-term transduction results, we assayed the remaining mice for long-term rAAV transgene expression at various time points from 2 months (Fig. 3C, D, E, and F) to 19 months (Fig. 4). The rAd-LacZ expression had diminished to undetectable levels when examined 2 months postinjection (Fig. 3A, B, C, and D), consistent with previously published results in immunocompetent adult animals (2, 3, 9, 33). However, rAAV-transduced animals sustained LacZ expression throughout the period tested with only limited animal-to-animal variation (Fig. 3C, D, E, and F, and Fig. 4). The persistent transduction was not unique to animals transduced at 3 weeks, since rAAV transduction of 4-month-old animals

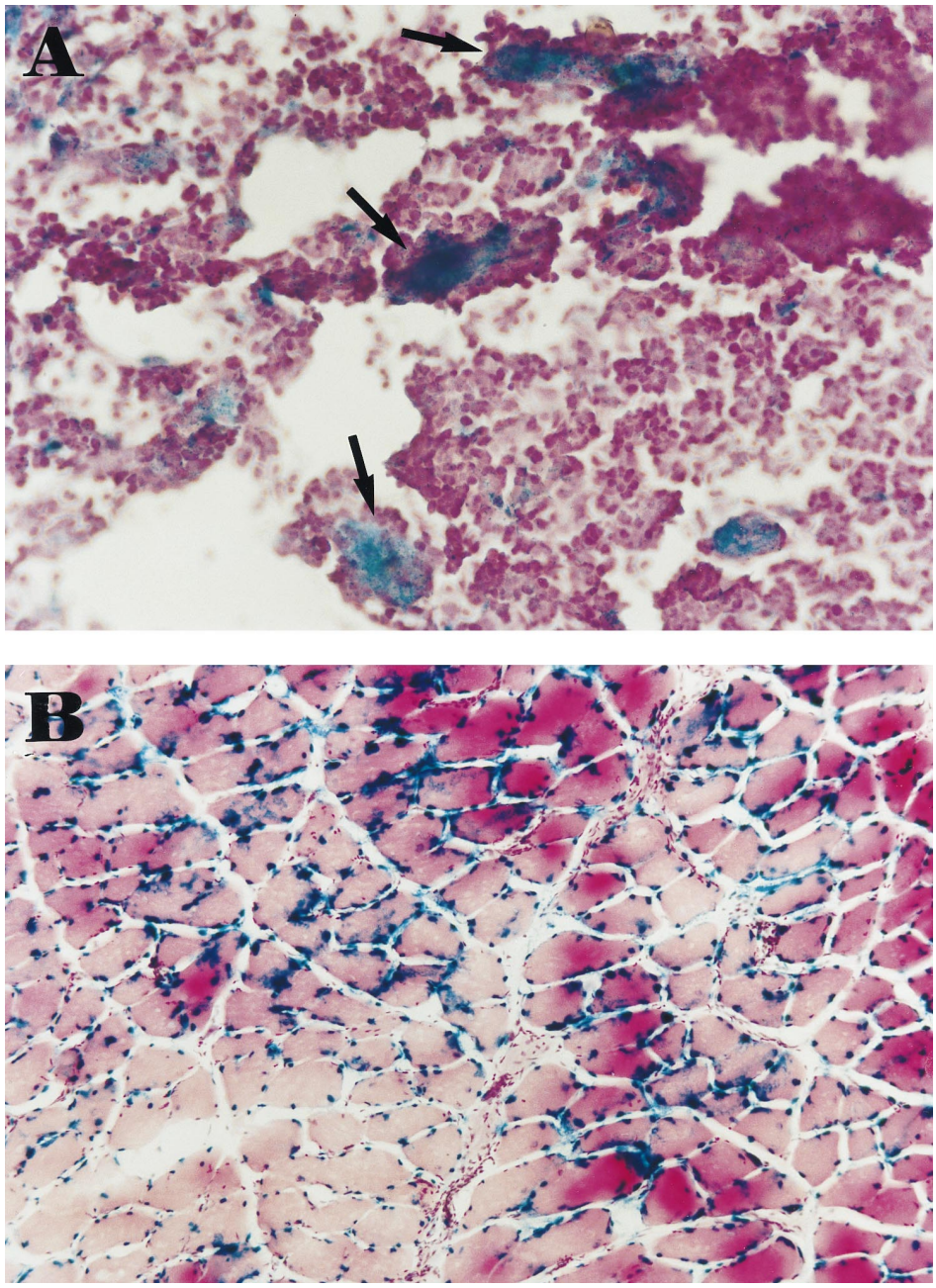

FIG. 2. X-Gal and H&E staining of the cryostat sections of the tibialis anterior muscle 4 days postinjection with an rAd-LacZ vector (A) or an rAAV-LacZ vector (B). Note the severe lymphocyte infiltration of the rAd vector-transduced myotubes (A) and milder and regional infiltration in AAV-LacZ-transduced tissue (B). The arrows show some myotubes transduced by rAd-LacZ vector and invaded by the lymphocytes. Magnifications, ×100 (panel A) and ×50 (panel B).

also sustained expression for at least 8 months (the period tested) (Fig. 1b, panel B). The results of the above-mentioned experiments are summarized in Table 1.

Molecular characterization of rAAV vector in vivo. rAAV vectors as well as wild-type AAV have been shown to efficiently integrate into the genomes of proliferating cells in culture (35, 37, 45). This feature of AAV provides an obvious advantage for long-term gene transfer. Nonetheless, recent reports suggest that rAAV is maintained episomally in nonproliferating cultured cells when tested for a relatively short period of time (1 to 14 days) (20, 43). The fact that muscle cells could be efficiently transduced in vivo and that expression persisted for more than 1.5 years led us to determine the molecular structure of the rAAV genomes. Total cellular DNA (both high and low molecular weight) was isolated from muscle tissues at various intervals 2 to 12 months post-rAAV injection. The DNA samples were characterized by Southern analysis either with no treatment or after digestion with *Eco*RV which cuts once within the vector LacZ coding region (Fig. 5a). Using a probe specific only for the *lacZ* sequences, all undigested DNA samples from rAAV-treated tissue showed the vector DNA migrating with high-molecular-size genomic DNA (Fig. 5b, lanes 4, 6, 8, and 10). This high-molecular-size signal is specific, based on its absence in control samples (Fig. 5b, lanes 12 and

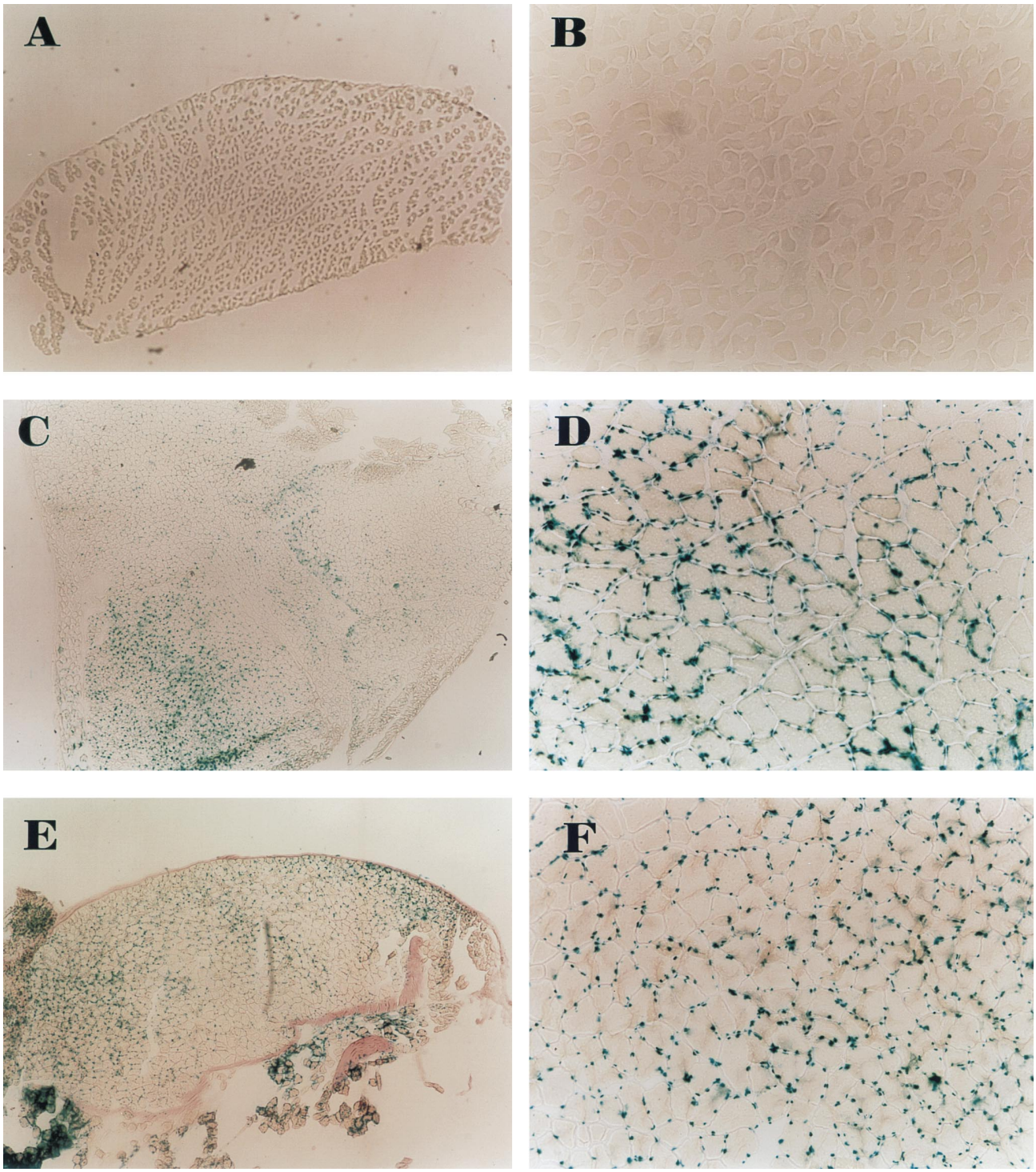

FIG. 3. X-Gal staining of hind leg muscle sections 2 months post-viral vector injections into 3-week-old mice. (A and B) rAd-LacZ (3×10^6 infectious units). (C and D) AAV-LacZ plus rAd-LacZ (3×10^6 infectious units each, in a mixture). (E and F) rAAV-LacZ (3×10^6 infectious units). Panels A, C, and E were photographed at ×6.25, while panels B, D, and F were photographed at ×25 magnifications. Note that no cytoplasmic staining of rAd-LacZ-transduced cells was detected at this time point (A, B, C, and D), while the nuclear staining from rAAV-LacZ vector persisted (C, D, E, and F).

13). None of the untreated samples showed evidence of free episomal forms of less than 12 kb (lanes 4, 6, 8, and 10). Digestion with *Eco*RV converted the high-molecular-size vector-specific signal to a 5.0-kb unit length fragment with an additional diffuse signal ranging from about 3 kb to more than 12 kb (lanes 5, 7, 9, and 11). Although a 5-kb fragment could either be derived from concatemeric integration or from circular episomes (Fig. 5a), it is likely to represent a concatemeric genomic insertion since no free low-molecular-size DNA was detectable in the undigested samples (lanes 4, 6, 8, and 10).

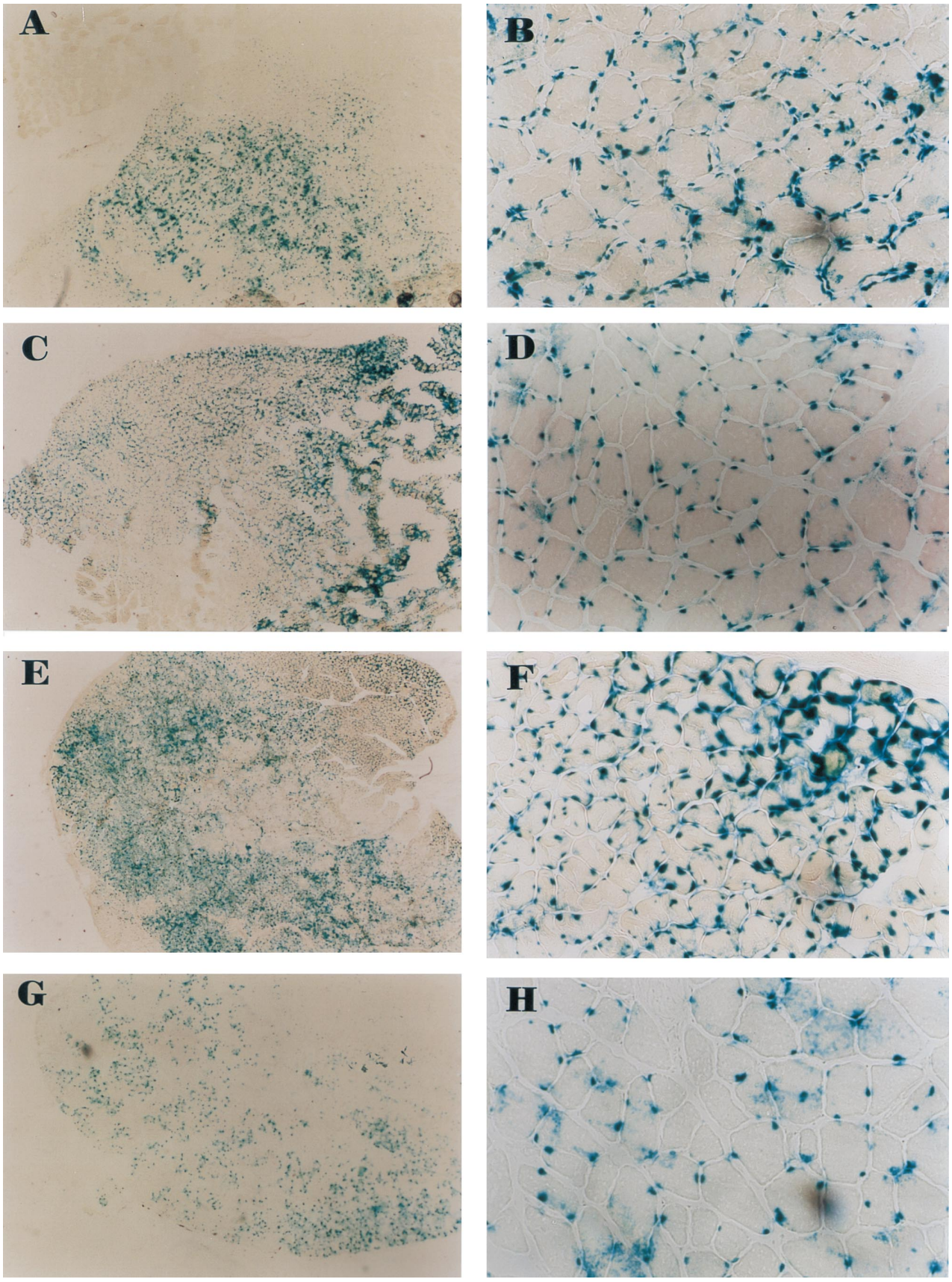

FIG. 4. Long-term gene expression of rAAV-LacZ vector-transduced tibialis anterior muscle tissues. Three-week-old mice were injected with rAAV-LacZ (3×10^6 infectious units). X-Gal staining of the cryostat sections was performed at 5 months (A and B), 8 months (C and D), 12 months (E and F), and 19 months (G and H) post-AAV vector injections. Panels A, C, E, and G are photographed at ×6.25, and panels B, D, F, and H are photographed at ×50 magnifications.

TABLE 1. In vivo transduction by rAd-LacZ and rAAV-LacZ in mouse muscle

Age at injection	No. of animals	Time postinjection	Avg no. of positive fibers[a]	
			Ad-LacZ	AAV-LacZ
3 wk	1	4 days	17	490
	2	1 wk		580
	2	3 wk	8	630
	2	2 mo	0	1,250
	2	4 mo	0	510
	1	5 mo		760
	2	8 mo		1,050
	1	10 mo		700
	1	12 mo		1,420
	1	19 mo		450
3 mo	1	3 days		120
	1	2 wk		570
4 mo	1	2 wk		430
	2	8 mo		220

[a] The numbers of LacZ-positive fibers were determined by arbitrarily dividing the tissue sections into four to six grid areas (four for samples with low transduction and six for samples with high transduction) and counting the blue cells or cells with blue nuclei in one or two represented areas. The total positive fiber number in each section was then obtained by multiplying the positive cell number in one grid or the average number from two grids by the total number of grids.

Moreover, the diffuse signal generated after *Eco*RV digestion, while unusually strong, is characteristic of junction fragments of randomly sized genomic insertion sites. Finally, the presence of a 5.6-kb fragment in the *Eco*RV-digested vector-treated samples (lanes 5, 7, 9, and 11) is indicative of a tail-to-tail dimeric integration also seen with wild-type AAV (31). A corresponding head-to-head fragment would not be detected by this assay because of the specificity of the probe. This analysis shows no evidence of the presence of linear episomal forms which would be indicated by the presence of 2.8- and 2.2-kb fragments after *Eco*RV digestion (see lanes 5, 7, 9, and 11). While the rAAV vector sequences appear to have stably associated with genomic DNA, we cannot rule out a high-molecular-size, undefined episomal concatemer.

DISCUSSION

We have characterized somatic delivery into muscle tissues of immunocompetent mice, using rAAV-LacZ vector as a model system to examine the efficiency of vector transduction, host immune response to transduce cells, persistence of gene expression, and the molecular fate of the vector genome. Our results clearly demonstrate that rAAV can transduce muscle cells efficiently in vivo without significant cellular immune response. In addition, we provide evidence for conversion of monomer input rAAV genomes to high-molecular-weight species, suggestive of integration as a mechanism for prolonged gene transduction. These data provide the first report for establishing long-term gene transduction in mammalian muscle cells in vivo without the need for immune modulation of the organism.

rAAV persistence via viral integration. Recently an rAAV vector carrying cystic fibrosis transmembrane conductance regulator (*CFTR*) cDNA has been reported capable of long-term transduction in normal rabbit lungs for up to 6 months (19). In addition, rAAV vectors have been shown to transduce neuronal cells in vivo in rat brain, with prolonged expression of up to 3 months (28, 34). Moreover, rAAV vectors harboring the human tyrosine hydroxylase gene rendered significant therapeutic efficacy in the Parkinsonian rat model for 4 months (28). Although in situ PCR confirmed the presence of vector DNA in rabbit lung (19) and rat brain (28) tissues, a detailed characterization of the molecular fate of the vector sequence was not reported. In the present study, Southern analysis supports rAAV integration into host chromosomes as the mechanism for persistence in mammalian muscle. Signals characteristic of tandem head-to-tail and tail-to-tail integration were observed after single-cut restriction endonuclease digestion similar to in vitro integration analysis (35). Previous studies suggest that unlike wild-type AAV, recombinants do not target integration to a specific location (7, 44, 49). Signals characteristic of junction sequences migrating at various sizes after single-cut restriction endonuclease digestion support this observation. Re-

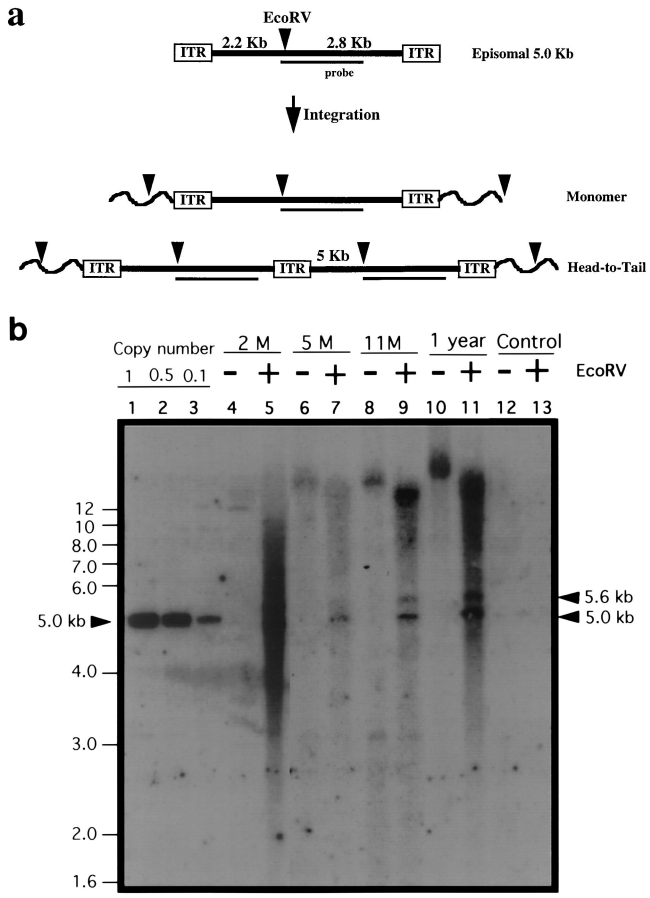

FIG. 5. Southern analysis of total DNA from rAAV-LacZ-transduced muscle tissues collected at various time points post-vector injection. (a) Schematic diagram of rAAV-LacZ vector as episome, single integrated copy (monomer), or tandem (head-to-tail) and expected fragment sizes generated after *Eco*RV digestion. ITR represents AAV inverted terminal repeats. Arrows are the *Eco*RV cleavage sites. The wavy lines represent the genomic DNA junctions. The line labeled "probe" is the vector fragment radiolabeled for Southern blot. (b) Autoradiograph of Southern blot of total DNA from vector-injected or negative control tissues. The DNA (20 μg per lane) was untreated (lanes 4, 6, 8, 10, and 12) or digested with *Eco*RV (lanes 5, 7, 9, 11, and 13). Copy number standards are *Pst*I-digested rAAV-LacZ plasmid coloaded with 20 μg of *Nde*I-digested negative control muscle DNA per lane. The 1-kb molecular size marker is depicted on the side of the autoradiograph. The time points were 2 months (lanes 4 and 5), 5 months (lanes 6 and 7), 11 months (lanes 8 and 9), and 1 year (lanes 10 and 11) post-rAAV injection. The reduction of vector signal in undigested lane 4 was seen multiple times and presumed to be due to masking by high-molecular-size genomic DNA, since signal was readily detectable after enzyme digestion (lane 5).

cent analysis of rAAV in monkey airways 3 months after administration demonstrated the presence of an episomal (putative dimer) and an uncharacterized high-molecular-weight species in genomic DNA (4). In the present study, we did not find evidence of a simple concatemeric (dimer) structure independent of chromosome integration. However, our analysis could not rule out a high-molecular-weight species composed of vector and nonvector sequences existing as a separate minichromosome. Nor could we rule out completely the presence of both episomal and integrated copies of the vector in these cells. In any case, our data suggest the conversion of input vector DNA from low- to high-molecular-weight species, with restriction digestion patterns suggestive of association with nonvector sequences. The intensity of the vector-specific signal after 1 year indicates an average of one to two copies per transduced cell (Fig. 5b). Although the kinetics of rAAV integration into muscle tissue have not been addressed, by 2 months postadministration, the input vector genome had associated with high-molecular-weight DNA (Fig. 5b).

Nondividing cells are transduced by rAAV. Studies in cultured cells revealed that rAAV vector can transduce resting cells and persist either as single- or double-stranded free episomal DNA (20, 40, 43). In one report, rAAV persisted as single-stranded DNA in nondividing cells for up to 12 days (43). When cell cycle inhibitors were removed, characterization of the rAAV template demonstrated integration into the chromosome (20, 40, 43). These results show that episomal rAAV DNA in resting cells remains competent for integration for extended periods after initial vector administration. While indirect data such as gene expression have implied that integration in nondividing cells is possible (28, 34, 43), this issue has remained unresolved because of technical difficulties in analyzing episomal and integrated rAAV vectors.

In our in vivo studies, we took advantage of the natural postmitotic nature of mature muscle cells which have an extremely slow turnover rate. In adult muscle, the majority of the cells are mature myotubes. Histochemical staining and Southern analysis illustrated that the rAAV vector transduced and persisted in these nondividing cells for more than 18 months. This is likely the result of de novo rAAV transduction rather than proliferation of a few transduced myoblasts. By postnatal day 8, muscle cells are predominantly mature myotubes. Injection of vectors was carried out on postnatal day 21. In addition, the high percentage of transduced cells (approaching 100% in some areas) is difficult to explain by transducing a few progenitor cells, since massive elimination and regeneration of mature cells would be required by the earliest tested time point at 4 and 7 days postinjection (Fig. 2B and Fig. 1b, panel A). The central nucleated morphology indicative of regenerating cells was not observed at any significant level during our analysis (Fig. 1 to 4). Rather, the vast majority of rAAV-LacZ-transduced muscle cells possessed peripheral nucleated morphology indicative of mature myotubes (Fig. 1 to 4).

Previous reports suggested that rAAV vectors transduce primary cells and nondividing cells less efficiently than transformed and dividing cells (26, 43). Our results are in general agreement with these observations. We have used 3×10^6 infectious units (3×10^8 viral particles) of AAV vector per injection site in mouse muscle. If the total number of myotubes in each injection site (around 1,000 per cross section) is considered, the transduction efficiency was about 3,000 infectious units or 3×10^5 particles per myotube. The particle number may be even higher at the injection site. Since myotubes are multinucleated, a single nucleus in a fiber transduced by rAAV vector may render the entire fiber positive after gene expression. Currently it is unclear how many of the nuclei actually contain the vector DNA in individual myotubes. In our in vivo experiments, the transduction efficiency was calculated to be 30 infectious units or 3,000 particles per genome. This efficiency appears better than that of either adenovirus or plasmid DNA, for which transducing a single myotube requires 10^4 to 10^6 adenoviruses or 10^{11} to 10^{13} DNA molecules (2, 3, 50). Although rAd is excellent in transducing newborn muscles, the same wild-type-free rAd-LacZ stock is inefficient in transducing mature muscle (2, 3, 27), suggesting an obvious reason for the difference in efficiency we observed. As with rAd, we also observed a decrease in transduction when using rAAV in 4-month-old compared with 3-week-old animals, although the reduction was not as great (Fig. 1b and Table 1). These results suggest that other variables besides vector dose may influence optimum rAAV transduction in vivo.

Several groups have recently reported that transduction efficiency of rAAV vectors can be significantly augmented by simultaneous treatment with genotoxic chemicals such as hydroxyurea or with physical agents such as UV and X-ray irradiation and by wild-type adenovirus or its early gene E4 ORF6 (5, 17, 18, 42). While it remains to be seen if all these methods hold true for rAAV in vivo, preliminary data suggest that transduction after wild-type adenovirus infection and X-ray irradiation increases rAAV transduction (18, 50b). Although previous work has demonstrated that wild-type adenovirus can facilitate rAAV transduction in vitro and in vivo (17, 18), it is unlikely that the transduction efficiency in the present experiments was enhanced due to residual contaminating wild-type adenovirus, since the rAAV vectors are gradient purified and heat treated (see Materials and Methods). Moreover, contamination of an rAd-LacZ stock with wild-type adenovirus triggered a severe immune response even when delivered into newborn rat muscle (33).

Lack of immune response supports rAAV persistence. The fact that rAAV did not induce substantial cell-mediated immunogenicity is highly significant. Afione et al. and Flotte et al. have also shown that a cellular immune response was not mounted when using rAAV in vivo for airway-specific delivery (4, 19). The cellular immunogenicity induced by rAd vectors is attributed to the undesirable expression of the viral genes in the adenovirus vectors (51–53, 55). Various efforts have been made to either lower the extent of viral gene expression by introducing additional viral gene mutations or to repress the host immune system by administrating immunosuppressive drugs (9, 15, 16, 30, 54). While most of these modifications have improved the longevity of transduction by adenovirus vectors and enabled secondary administration, these strategies are still not optimum. Recent work with such modified rAd vectors demonstrated less of an immune response to the viral gene products, but immune response to the expressed transgene persisted (47). In contrast to the adenovirus vectors, all viral genes have been removed from rAAV, accounting for the negligible immune response. Moreover, a cellular immune response to the *lacZ* gene was not observed after rAAV transduction. It is possible that the adenovirus virion itself serves as an adjuvant owing to the complex virus structure composed of 14 distinct proteins. In contrast, the AAV virion consists primarily of one major polypeptide (Vp3) (6). Our experiments demonstrated that rAAV-LacZ vector causes only transient and minor lymphocyte infiltration and does not elicit a CTL response to the vector-transduced cells. Silver staining of our vector preparations demonstrated residual foreign proteins that may have contributed to the lymphocyte infiltration observed. Since no significant CTL response was induced in the experiments reported here using rAAV, this also supports the absence of wild-type adenovirus contamination. The transient

inflammatory reaction observed in rAAV-injected sites could have been induced by physical damage to the muscle tissue during the injection or due to impurities in the virus preparation. Indeed, when similar rAAV-LacZ stocks were delivered through the coronary artery to the heart muscle, a noninvasive means to the muscle tissue, efficient and long-term transduction was achieved without any sign of lymphocyte infiltration (29). In addition, direct injection of a retroviral vector devoid of any viral genes also induced inflammatory reactions in the muscle tissue (11). Regardless of the transient immune response we observed, when rAAV-LacZ and rAd-LacZ were coinjected into the same sites, rAAV-transduced cells prevailed while rAd-transduced cells were diminished. Furthermore, upon secondary delivery of rAAV vector, the numerous myotubes transduced by rAAV at the primary sites were unaffected even though a significant neutralizing response could be detected (data not shown).

Even if immune problems associated with rAd vectors are eventually overcome, the fact that transduced genomes remain episomal suggests that repeated dosing will be inevitable. Although repeated administration of rAAV-LacZ was unsuccessful due to a humoral response, repeated dosing appears not to be necessary given that the originally transduced cells escaped a CTL response and persisted long term. Since greater than 80% of the public is seropositive for AAV, it raises the question of viable vector delivery in general. However, the percentage of seropositive individuals that are also positive for neutralizing antibody remains to be determined. Moreover, already-established strategies for manipulating the host immune system for rAd-repeated dosing (9, 13, 30, 54) could also be modified and adopted for the rAAV vector system. In the case of rAAV, transient suppression of the humoral responses should be sufficient. Recent success with different serotypes of rAAV should also be valuable if repeated dosing is necessary (33a).

In summary, we have demonstrated that rAAV can be used as an efficient, safe, and practical in vivo gene therapy vector. By means of direct injection, rAAV vectors transduced muscle tissue of immunocompetent mice without significant detrimental immune consequences, resulting in sustained long-term expression. Stable persistence of the vector genome with the host indicates that rAAV vector-mediated gene therapy may be of general use for numerous metabolic disorders.

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