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Correction of Glycogen Storage Disease Type II by an Adeno-associated Virus Vector Containing a Muscle-Specific Promoter

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Glycogen storage disease type II (Pompe disease) causes death in infancy from cardiorespiratory failure due to acid α-glucosidase (GAA; acid maltase) deficiency. An AAV2 vector pseudotyped as AAV6 (AAV2/6 vector) transiently expressed high-level human GAA in GAA-knockout (GAA-KO) mice without reducing glycogen storage; however, in immunodeficient GAA-KO/SCID mice the AAV2/6 vector expressed high-level GAA and reduced the glycogen content of the injected muscle for 24 weeks. A $CD4^+/CD8^+$ lymphocytic infiltrate was observed in response to the AAV2/6 vector in immunocompetent GAA-KO mice. When a muscle-specific creatine kinase promoter was substituted for the CB promoter (AAV-MCKhGAApA), that AAV2/6 vector expressed high-level GAA and reduced glycogen content in immunocompetent GAA-KO mice. Muscle-restricted expression of hGAA provoked only a humoral (not cellular) immune response. Intravenous administration of a high number of particles of AAV-MCKhGAApA as AAV2/7 reduced the glycogen content of the heart and skeletal muscle and corrected individual myofibers in immunocompetent GAA-KO mice 24 weeks postinjection. In summary, persistent correction of muscle glycogen content was achieved with an AAV vector containing a muscle-specific promoter in GAA-KO mice, and this approach should be considered for muscle-targeted gene therapy in Pompe disease.

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

INTRODUCTION

Glycogen storage disease type II (GSD-II; Pompe disease; MIM 232300) causes death in infancy from cardiomyopathy and cardiorespiratory failure. The underlying deficiency of acid α-glucosidase (GAA; acid maltase; EC 3.2.1.20) has been corrected by intravenous administration of adenovirus vectors encoding GAA, which previously demonstrated generalized correction of glycogen storage in the GAA-knockout (GAA-KO) mouse model [1,2], although glycogen gradually reaccumulated in the months following vector administration [3]. The appearance of anti-GAA antibodies correlated with the disappearance of secreted hGAA precursor from plasma [3]. Another obstacle to therapy in GSD-II is the downregulation of the mannose 6-phosphate receptor and decreased uptake of circulating hGAA by skeletal muscle, a critical target tissue [4]. An earlier experiment demonstrated supranormal expression of hGAA in transduced muscle following intracardiac or intramuscular injection of an [E1$^-$]Ad vector encoding hGAA in neonatal rats [5].

An AAV vector packaged as AAV1 (AAV2/1) corrected glycogen storage for 2 weeks when injected intramuscularly into immunocompetent GAA-KO mice; however, the effect was observed only in the injected muscle [6]. Similarly, hGAA was expressed at high levels in skeletal muscle following intramuscular injection of an Ad–AAV vector encoding hGAA in neonatal GAA-KO mice and transiently detected in plasma following intravenous injection of the Ad–AAV vector in adult GAA-KO mice; however, anti-GAA antibodies were elicited by hGAA expression and secretion of hGAA was transient with that vector in immunocompetent GAA-KO mice [7,8]. We previously demonstrated the secretion of hGAA and

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uptake in heart and diaphragm at low levels with an AAV2/2 or AAV2/6 vector following portal vein injection in immunodeficient GAA-KO/severe combined immunodeficiency (SCID) mice [7].

Newly characterized serotypes of AAV offer specific advantages in terms of tissue tropism. Traditional vectors utilizing AAV2 terminal repeat sequences can be efficiently packaged as other serotypes [7,9,10]. AAV2/1, AAV2/5, and AAV2/8 vectors transduced cells in the liver more efficiently than AAV2/2 vectors [9,10]. AAV2/1 and AAV2/7 vectors demonstrated increased transduction of muscle fibers compared to AAV2/2 vectors [9–11]. The capsid proteins of AAV6 are very homologous to AAV1 capsids [12], and administration of very high particle numbers of AAV2/6 vectors has been shown to transduce skeletal muscle with high efficiency [13].

Gene therapy in the muscular dystrophies represents a unique challenge, since dystrophic muscle is subject to humoral and cytotoxic immune responses to the replacement of deficient, therapeutic proteins [14,15]. Approaches to preventing the immune response to gene therapy in dystrophic muscle include the use of a muscle-specific promoter, because an adenovirus vector containing a muscle creatine kinase (MCK) promoter persisted in the muscle of *mdx* mice [16,17]. Similarly, an AAV vector containing the MCK promoter driving β-galactosidase expression in *mdx* mouse muscle evoked an attenuated immune response compared to an analogous AAV vector containing the CMV promoter [18].

As an extension of these earlier studies, AAV vectors encoding hGAA driven by the MCK promoter were evaluated in GAA-KO mice. We investigated the benefit of intramuscular injection of the AAV vector, because skeletal muscle is a primary target for correction of GSD-II. GAA expression and glycogen content in skeletal muscle and the heart were monitored following AAV vector administration. The immune response to introduced hGAA was investigated, and the MCK promoter was substituted for the constitutive CMV immediate-early enhancer/chicken β-actin (CB) promoter to reduce the immune response against hGAA. The AAV vector was pseudotyped as AAV2/6 and AAV2/7 to deliver the introduced gene more efficiently to striated muscle in GSD-II mice.

RESULTS

An AAV2/6 Vector Encoding hGAA Driven by the Hybrid CB Promoter Provoked an Immune Response That Attenuated hGAA Expression

We packaged an AAV vector containing the hybrid CB promoter to drive hGAA expression (AAV-CBhGAApA) as AAV2 or pseudotyped it as AAV6 and administered 1×10^{11} particles intramuscularly in the gastrocnemius muscle of 6-week-old GAA-KO mice. When we analyzed the injected gastrocnemius muscle 6 weeks following vector administration, the AAV2/2 vector generated higher levels of hGAA than the AAV2/6 vector (Fig. 1A). However, neither the AAV2/2 nor the AAV2/6 vector reduced the glycogen content in GAA-KO mouse muscle (Fig. 1B), indicating that hGAA expression was inadequate with either vector to reduce glycogen storage generally in the injected gastrocnemius.

To establish further the role of an immune response to introduced hGAA in GAA-KO mice, we injected 1×10^{11} particles of the AAV2/2 or AAV2/6 vector intramuscularly into immunodeficient GAA-KO/SCID mice. In contrast to the result for GAA-KO mice, both vectors produced high hGAA levels in the injected gastrocnemius 6 weeks following vector administration for GAA-KO/SCID mice (Fig. 1A). Indeed the GAA activity with the AAV2/6 vector was higher than that with the AAV2/2 vector and exceeded the GAA level for skeletal muscle in normal mice [19] by approximately 20-fold in the injected gastrocnemius. Glycogen content was reduced with both the AAV2/2 and the AAV2/6 vector in GAA-KO/SCID mice (Fig. 1B).

Tissue Distribution of hGAA and Vector DNA for an AAV2/6 Vector Following Intramuscular Administration

We analyzed the potential cross-correction of GAA deficiency by secretion of hGAA from the transduced muscle, followed by receptor-mediated uptake by other tissues, following intramuscular administration of AAV-CBhGAApA pseudotype as AAV2/6. We analyzed the GAA activity and glycogen content in both the injected gastrocnemius and the contralateral gastrocnemius at 6, 12, and 24 weeks following AAV2/6 vector administration in GAA-KO/SCID mice. GAA activity was elevated (mean 470 nmol/mg protein/h) for all time points only in the injected gastrocnemius, indicating a lack of cross-correction of the contralateral gastrocnemius (Fig. 1C). As expected, glycogen content was reduced only in the injected gastrocnemius and remained low at 24 weeks following vector administration (Fig. 1D). We confirmed GAA expression by Western blot analysis of the injected gastrocnemius muscle and it was absent in the contralateral gastrocnemius by that method (Fig. 2A). GAA activity and protein were similarly absent in other tissues, including liver, heart, and diaphragm, indicating a lack of significant cross-correction despite the presence of high GAA activity in the gastrocnemius muscle (data not shown). Semiquantitative PCR demonstrated approximately 1 vector genome/cell for vector DNA in the injected gastrocnemius, and vector DNA was not detected in the liver and heart (Fig. 2B). While hGAA was efficiently expressed intramuscularly with the AAV2/6 vector, transduction was localized to the injected muscle and was not secreted to allow receptor-mediated uptake in other tissues.

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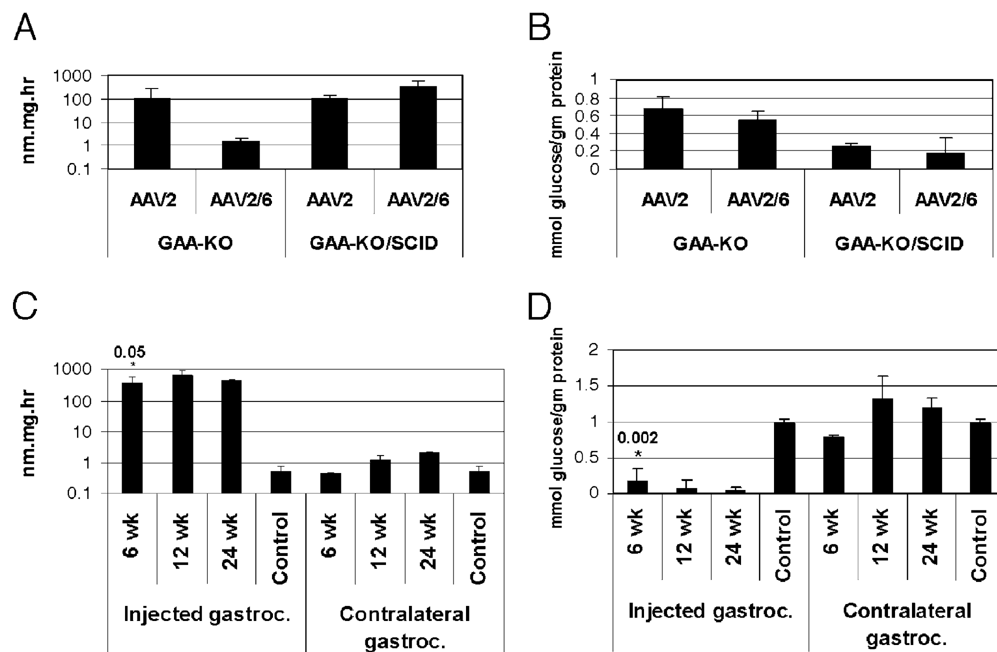

FIG. 1. GAA activity and glycogen content in GAA-KO and GAA-KO/SCID mice following AAV-CBhGAApA administration. The averages and standard deviations are shown. (A) The hGAA activity in the injected gastrocnemius at 6 weeks following AAV2/2 (GAA-KO mice, $n = 3$; GAA-KO/SCID mice, $n = 3$) or AAV2/6 (GAA-KO mice, $n = 5$; GAA-KO/SCID mice, $n = 3$) vector administration. (B) The glycogen content for the injected gastrocnemius of mice in (A). (C) The hGAA activity in the injected gastrocnemius at 6 ($n = 3$), 12 ($n = 2$), and 24 weeks ($n = 2$) following AAV2/6 vector administration. (D) The glycogen content for the injected gastrocnemius of mice in (C).

Muscle-Restricted Expression with an MCK Promoter/Enhancer Prolonged hGAA Expression in Immunocompetent GAA-KO Mice

We pseudotyped an AAV vector containing a muscle-specific MCK promoter/enhancer to drive hGAA expression (AAV-MCKhGAApA) as AAV6 and injected 1×10^{11} particles into the gastrocnemius of GAA-KO mice. In contrast to AAV-CBhGAApA, AAV-MCKhGAApA expressed hGAA at high levels 6 weeks after vector administration (Fig. 3A) and approached 100-fold above the level for normal mice [19]. Glycogen content was significantly reduced in the injected gastrocnemius with AAV-MCKhGAApA, whereas it was not reduced with AAV-CBhGAApA (Fig. 3B). However, intravenous administration of 2×10^{11} particles of AAV-MCKhGAApA pseudotyped as AAV6 did not increase GAA activity above the background level seen in the heart or skeletal muscle of GAA-KO mice when analyzed 6 weeks postinjection (not shown). Either too few vector particles were administered or vascular permeability was insufficient to allow transduction of striated muscle with the AAV2/6 vector.

Muscle-Restricted hGAA Expression with the MCK Promoter/Enhancer Eliminated the Cellular, but Not the Humoral, Immune Response to hGAA in GAA-KO Mice

We investigated the basis for prolonged expression of hGAA with AAV-MCKhGAApA compared to AAV-CBhGAApA by characterization of the immune response to each vector in GSD-II mice. We injected AAV-CBhGAApA pseudotyped as AAV6 intramuscularly into the gastrocnemius of immunocompetent GAA-KO mice (1×10^{11} particles) ($n = 2$), and histology revealed multifocal small to moderate perivascular and interfiber infiltrations of lymphocytes and plasma cells in association with an occasional degenerate myofiber at 4 weeks following vector administration (Fig. 4A). In contrast, no infiltrate was present in multiple sections of the contralateral gastrocnemius (Fig. 4B). Following intramuscular injection of AAV-MCKhGAApA pseudotyped as AAV6 gastrocnemius (1×10^{11} particles) in GAA-KO mice ($n = 3$), perivascular and interfiber infiltrations of lymphocytes and plasma cells were present in the injected gastrocnemius, and glycogen vacuoles were cleared from the majority of myofibers (Fig. 4C); in contrast, the contralateral gastrocnemius lacked infiltrations of mononuclear cells and retained large lysosomes filled with glycogen (Fig. 4D).

We evaluated the immune response to introduced hGAA further by immunostaining to detect $CD4^+$ or $CD8^+$ lymphocytes in GAA-KO mice following intramuscular administration of either AAV-CBhGAApA ($n = 2$) or AAV-MCKhGAApA ($n = 3$) pseudotyped as AAV6. For gastrocnemius injected with AAV-CBhGAApA, $CD4^+$ cells were present in moderate numbers (3+) in inflammatory foci and less prevalent (2+) between

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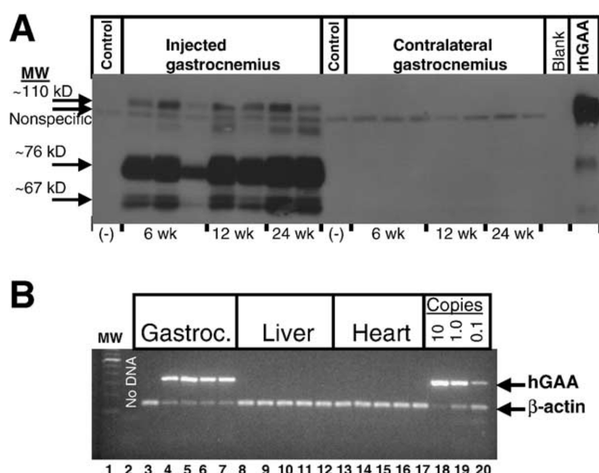

FIG. 2. Detection of hGAA following intramuscular administration of AAV-CBhGAApA pseudotyped as AAV2/6 vector in GAA-KO/SCID mice. (A) Western blot analysis of the injected and contralateral gastrocnemius at the indicated time points following vector administration. The ~67-, ~76-, and ~110-kDa hGAA species were detected [46,47]. Recombinant hGAA (rhGAA) was the standard. Each lane represents one mouse. (B) Vector DNA analysis by semiquantitative PCR following administration of AAV-CBhGAApA pseudotyped as AAV2/6. Semiquantitative PCR for the injected gastrocnemius DNA, liver DNA, and heart DNA, at 24 weeks following AAV2/6 vector administration for GAA-KO/SCID mice ($n = 4$). Control DNA was from an untreated, GAA-KO/SCID mouse (lanes 3, 8, and 13). Each lane represents an individual mouse. Lane 1 shows a 100 bp ladder molecular weight marker. The negative control consisted of no input DNA (lane 2). The control samples for quantitation consisted of added vector plasmid DNA representing from 10 to 0.1 vector genomes/cell of AAV vector plasmid DNA in muscle DNA from an untreated, GAA-KO/SCID mouse (lanes 18–20). Mouse β-actin DNA was amplified as an internal control for each sample.

muscle fibers (Fig. 4E), while lower numbers of $CD8^+$ cells were present in inflammatory foci (Fig. 4F). For gastrocnemius injected with AAV-MCKhGAApA, $CD4^+$ cells were present in moderate numbers (2+) in inflammatory foci in the injected gastrocnemius (Fig. 4G); however, $CD8^+$ staining was negative in those foci (Fig. 4H). The absence of $CD8^+$ cells and persistence of hGAA expression were consistent with a lack of a cytotoxic response in skeletal muscle transduced with AAV-MCKhGAApA.

We confirmed the presence of anti-hGAA antibodies in GAA-KO mice for each of the three vectors evaluated here by ELISA (Fig. 5). Despite the evasion of a cellular immune response directed toward transduced muscle fibers with AAV-MCKhGAApA, anti-hGAA antibodies were provoked by that vector. Similarly, anti-hGAA antibodies were clearly present following administration of AAV-CBhGAApA as either AAV2/2 or AAV2/6 and not in naive GAA-KO mice. The presence of anti-hGAA antibodies confirmed the humoral response to intramuscular injection of AAV vectors containing either the CB or the MCK promoter/enhancer.

Long-Term, Therapeutically Relevant, hGAA Expression with an AAV2/7 Vector in the Heart and Quadriceps

We analyzed the potential of a myotropic AAV vector for correction of glycogen storage in GSD-II following systemic delivery following intravenous administration of AAV-MCKhGAApA pseudotyped as AAV7 (2×10^{11} or 2×10^{12} particles). We analyzed GAA activity and glycogen content in the heart, liver, and skeletal muscle 24 weeks following vector administration (Table 1). GAA activity was significantly elevated in the heart and quadriceps following administration of a higher number of AAV-MCKhGAApA particles (2×10^{12}), compared to residual activity in untreated GAA-KO mice; moreover, glycogen content was significantly decreased in the heart and quadriceps in GAA-KO mice with high-dose AAV-MCKhGAApA (Table 1). Liver GAA activity was low, consistent with muscle-restricted expression for the MCK promoter/enhancer. Glycogen vacuolization was present throughout the quadriceps in untreated GAA-KO mice (Fig. 6A), whereas glycogen vacuolization was absent in multiple clusters of myofibers following AAV-MCKhGAApA administration (Fig. 6B). Glycogen vacuolization was uniformly absent in the quadriceps of normal mice (Fig. 6C). Thus, long-term expression of hGAA with an AAV2/7 vector partially corrected glycogen storage in

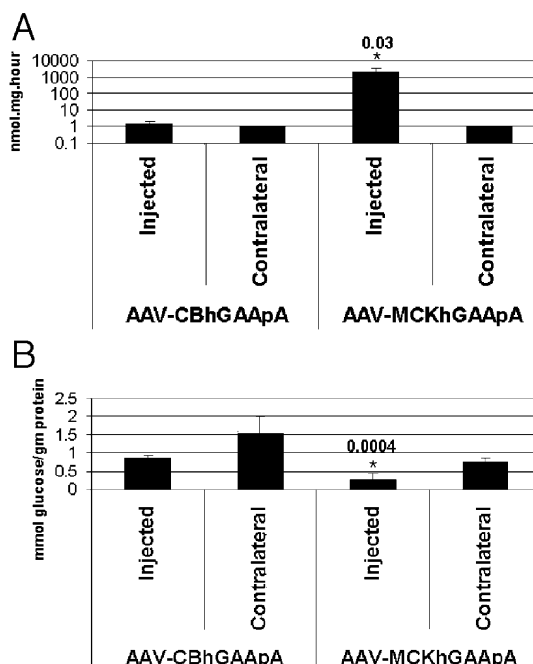

FIG. 3. GAA activity and glycogen content in GAA-KO mice following AAV2/6 vector administration. The averages and standard deviations are shown. Experimental values significantly differing from control group values are marked (*), and the P values are indicated. (A) The hGAA activity in the injected and contralateral gastrocnemius at 6 weeks following vector administration. (B) The glycogen content for the injected and contralateral gastrocnemius of mice in (A).

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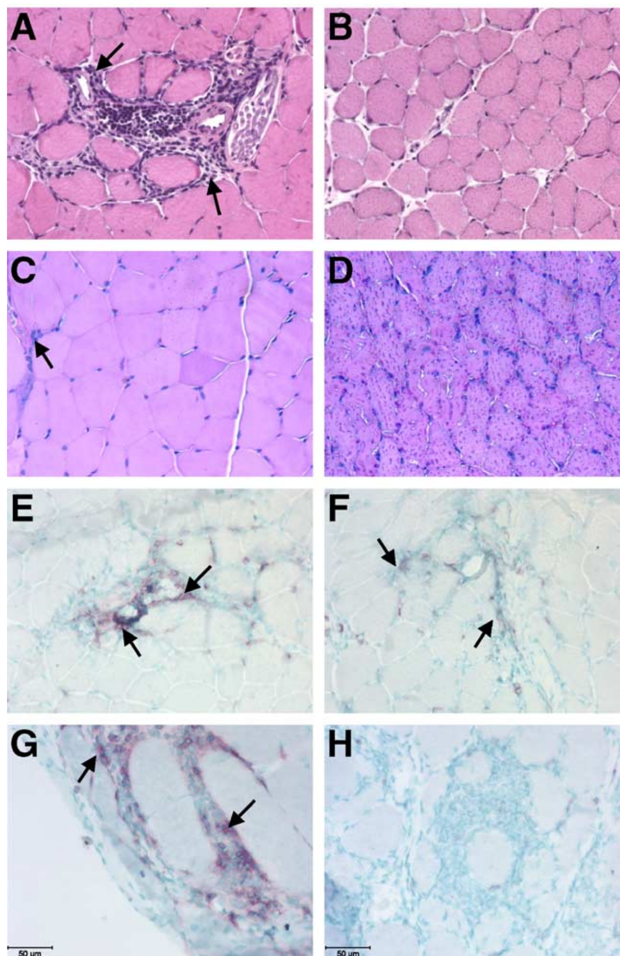

FIG. 4. Histopathology following intramuscular AAV2/6 vector administration in immunocompetent GAA-KO mice. (A) Perivascular and interfiber lymphocyte and plasma cell infiltrates in the injected gastrocnemius 4 weeks following AAV-CBhGAApA administration (hematoxylin and eosin). Mononuclear cell infiltration indicated (arrow). Original magnification ×730. (B) Uninjected contralateral gastrocnemius 4 weeks following AAV-CBhGAApA administration (hematoxylin and eosin). Original magnification ×730. (C) Glycogen staining in the gastrocnemius 6 weeks following AAV-MCKhGAApA administration (periodic acid–Schiff/alphin blue). Mononuclear cell infiltration indicated (arrow). Original magnification ×400. (D) Glycogen staining in the uninjected, contralateral gastrocnemius 6 weeks following intramuscular AAV-MCKhGAApA administration (periodic acid–Schiff/alphin blue). Original magnification ×400. (E) $CD4^+$ lymphocyte immunodetection in the injected gastrocnemius 4 weeks following AAV-CBhGAApA administration. Foci of $CD4^+$ cells indicated (arrow). Original magnification ×730. (F) $CD8^+$ lymphocyte immunodetection in the injected gastrocnemius 4 weeks following AAV-CBhGAApA administration. Foci of $CD8^+$ cells indicated (arrow). Original magnification ×730. (G) $CD4^+$ lymphocyte immunodetection in the injected gastrocnemius 6 weeks following AAV-MCKhGAApA administration. Foci of $CD4^+$ cells indicated (arrow). Original magnification ×730. (H) $CD8^+$ lymphocyte immunodetection in the injected gastrocnemius 6 weeks following AAV-MCKhGAApA administration. Original magnification ×730. All mice in each group represented had similar infiltrates in multiple sections that were analyzed (two to four per mouse).

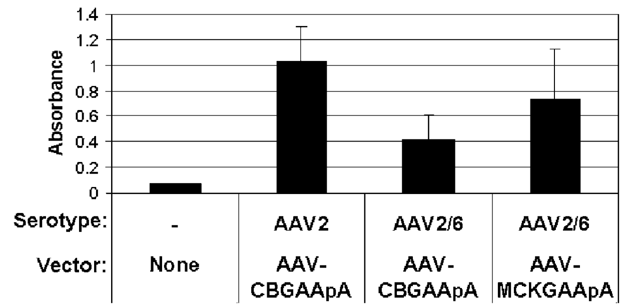

FIG. 5. ELISA of GAA-KO mouse plasma following AAV vector administration. The absorbances for anti-hGAA antibodies at 6 weeks following AAV vector administration and for untreated GAA-KO are shown. The number of mice was as follows: none, $n = 3$; AAV2/2:AAV-CBhGAApA, $n = 3$; AAV2/6:AAV-CBhGAApA, $n = 5$; and AAV2/6:AAV-MCKhGAApA, $n = 5$. Each column represents the mean and standard deviation for an experimental group.

heart and quadriceps of GAA-KO mice following intravenous injection of a high number of vector particles.

Clearance of AAV Vector DNA Containing the Constitutive CB Hybrid Promoter to Drive hGAA Expression

Semiquantitative PCR to detect vector DNA in the gastrocnemius muscle following AAV2/6 vector administration

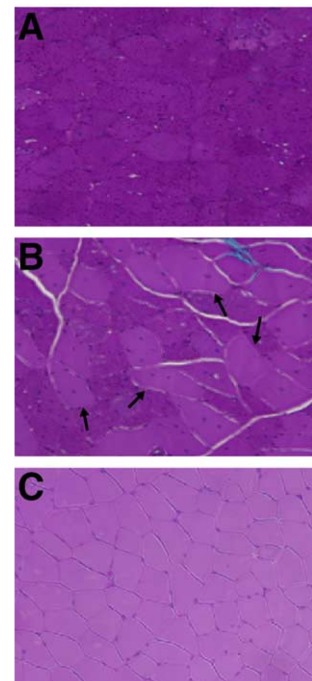

FIG. 6. Glycogen staining following intravenous AAV2/7 vector administration in immunocompetent GAA-KO mice. Periodic acid–Schiff/alphin blue. Original magnification ×400. (A) Quadriceps of an untreated, 8-month-old male GAA-KO mouse. (B) Quadriceps of a male GAA-KO mouse 24 weeks following injection of AAV2/7:AAV-MCKhGAApA. Glycogen staining reduction indicated (arrows). (C) Quadriceps of a normal (C57BL/6) mouse.

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revealed approximately 1 vector genome/cell for AAV-CBhGAApA in GAA-KO mice at 10 days and 4 weeks following vector administration, which was markedly reduced by 6 weeks to <0.01 vector genome/cell (Fig. 7A). The higher copy number for vector DNA at earlier time points corresponded to high-level hGAA expression (data not shown), while hGAA was markedly reduced by 6 weeks in GAA-KO mice (Fig. 1A). In contrast, AAV-CBhGAApA maintained approximately 1 vector genome/cell in GAA-KO/SCID mice at 6 weeks following vector administration. Presumably, the cellular immune response to constitutive hGAA expression with AAV-CBhGAApA eliminated vector DNA from skeletal muscle in immunocompetent GAA-KO mice by 6 weeks post-injection. The relatively higher copy number for AAV-MCKhGAApA, >1 vector genome/cell, at 6 weeks post-injection was associated with the persistent, high hGAA expression for that vector.

We analyzed the biodistribution of the AAV2/7 vector 24 weeks following AAV2/7 vector administration, and long-term hGAA expression was associated with persistence of vector DNA. Semiquantitation of vector DNA confirmed the presence of 0.1 to 1 vector genome/cell in the heart, quadriceps, and diaphragm, whereas up to 10 vector genomes per cell were present in the liver (Fig. 7B). The lack of transgene expression in the diaphragm with an AAV vector containing the MCK promoter/enhancer was previously reported [13] and could be related to lower activity of that promoter in transduced diaphragm. The muscle-restricted activity of the MCK promoter/enhancer was confirmed by the presence of GAA activity in the liver that was not significantly higher than in the quadriceps (Table 1), despite the higher copy number of vector genomes in the liver than in quadriceps (Fig. 7B).

DISCUSSION

Currently we describe the avoidance of a cellular immune response against muscle-targeted gene therapy in GSD-II mice by muscle-restricted expression of hGAA with a murine MCK promoter/enhancer. The presence of neutralizing antibodies did not prevent correction of glycogen content in the skeletal muscle of GSD-II mice, presumably related to the stable transduction of individual myofibers. The AAV2/6 vector containing an MCK promoter/enhancer expressed hGAA at approximately 100-fold above the normal GAA level in the injected gastrocnemius and exceeded the threshold for secretion of hGAA from muscle that was demonstrated in transgenic GAA-KO mice by approximately 8-fold [20]. Neutralizing antibodies could have obscured the secretion of hGAA from the injected muscle in these experiments, which would have prevented the cross-correction of heart and uninjected muscles. Muscle-targeted gene therapy represents an alternative approach to therapy in GSD-II, especially for nontolerant individuals in whom the formation of neutralizing antibodies prevents a response to enzyme replacement therapy.

Previously an AAV2/1 vector encoding GAA had corrected glycogen content in the muscle of GAA-KO mice for 2 weeks following vector administration [21]. If later time points had been analyzed in that study, the AAV2/1 vector containing a CMV promoter to drive GAA expression could have provoked a cellular immune response accompanied by reduced GAA expression, as observed here for an AAV2/6 vector containing the constitutive CB promoter to drive hGAA expression.

The advantages of muscle-restricted expression of a therapeutic protein with an AAV vector in mouse models of muscular dystrophy have been previously demonstrated and reproduced here in GSD-II mice. Dystrophin-deficient *mdx* mice had increased immune responses to β-galactosidase expression driven by a CMV promoter compared to nondeficient mice, and substitution of an MCK promoter/enhancer delayed the onset of the immune reaction in *mdx* mice [18]. The lymphocytic infiltration in response to the introduced protein in *mdx* mice was predominantly $CD4^+$ lymphocytes, as is the response in GSD-II mice. Similarly, substitution of an MCK promoter/enhancer for a CMV promoter/enhancer in an AAV vector encoding γ-sarcoglycan reduced the immune response and increased levels of transgene expression in γ-sarcoglycan-deficient mice [15,22].

Recently AAV2/6 vectors have been demonstrated to transduce striated muscle following intravenous administration, if accompanied by VEGF to increase vascular permeability [13]. The choice of promoter influenced the level of transduction of heart with that AAV2/6 vector, because the CMV promoter/enhancer drove expression of β-galactosidase in heart, whereas the MCK promoter/enhancer did not. We found that the MCK promoter/enhancer was active in the heart following AAV2/7 vector administration, and glycogen content was reduced significantly 24 weeks following vector administration in GSD-II mice. Interestingly, VEGF was not required to increase transduction of skeletal muscle with the AAV2/6 vector containing a CMV promoter/enhancer at higher number of vector particles (1×10^{12}) [13], consistent with this report of long-term transduction of heart and quadriceps following administration of higher particle numbers of the AAV2/7 vector (2×10^{12}). The present data confirm that higher doses of a myotropic AAV vector can persistently transduce striated muscle following intravenous administration and extend the period of observation from 6 [13] to 24 weeks.

The apparently higher hGAA expression with an AAV vector packaged as AAV2 in immunocompetent mice, compared to the same vector packaged as AAV6 vector, is attributable to a more robust cellular immune response to the AAV2/6 vector 6 weeks following administration of the vectors. AAV2/1 vectors transduced skeletal muscle more efficiently than AAV2/2 vectors [12,14], and the

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TABLE 1: GAA activity and glycogen content 24 weeks after AAV2/7 administration

	Group[a]	GAA activity		Glycogen content	
		GAA[b] (mean ± SD)	Fold[c]	Glycogen[d] (mean ± SD)	Decrease (%)[e]
Heart	AAV2/7, 2×10^{11}	2.3 ± 0.6	0.1	2.7 ± 0.5	0
	AAV2/7, 2×10^{12}	16 ± 3**	0.7	1.8 ± 0.3*	25
	Control	3.1 ± 1.1	—	2.4 ± 0.1	—
	Normal	22 ± 7.9	—	0.04 ± 0.03	98
Diaphragm	AAV2/7, 2×10^{11}	4.2 ± 2.0	0.7	1.1 ± 0.1	15
	AAV2/7, 2×10^{12}	4.8 ± 1.0	0.8	1.0 ± 0.1	23
	Control	4.0 ± 0.6	—	1.3 ± 0.1	—
	Normal	6.2 ± 2.7	—	0.01 ± 0.08	99
Quadriceps	AAV2/7, 2×10^{11}	2.9 ± 0.8*	0.2	1.0 ± 0.2*	17
	AAV2/7, 2×10^{12}	19 ± 6**	1.5	0.9 ± 0.2*	25
	Control	2.2 ± 0.4	—	1.2 ± 0.1	—
	Normal	13 ± 1.2	—	0.02 ± 0.01	98
Liver	AAV2/7, 2×10^{11}	13 ± 5*	0.09	ND[f]	—
	AAV2/7, 2×10^{12}	6.0 ± 1.0*	0.04	ND	—
	Control	3.9 ± 1.0	—	ND	—
	Normal	140 ± 30	—	ND	—

[a] Groups as follows, mice euthanized at the indicated time point following administration of the indicated number of AAV vector particles of pseudotyped AAV-MCKGAApA: AAV2/7, 2×10^{11} (24 weeks, GAA-KO, $n = 5$); AAV2/7, 2×10^{12} (24 weeks, GAA-KO, $n = 3$); Control (untreated, age-matched GAA-KO, $n = 4$); Normal (C57BL/6, $n = 5$).
[b] nmol/h/mg protein.
[c] Group mean/Normal group mean.
[d] mmol glucose/g protein.
[e] Reduction compared to Control group (%).
[f] Not done (liver glycogen is primarily cytoplasmic and not consistently elevated in GSD-II).
* $P < 0.05$.
** $P < 0.01$.

AAV6 capsid has very high homology to the AAV1 capsid [15]. The AAV2/6 vector containing an MCK promoter/enhancer expressed approximately 10-fold higher hGAA than the analogous vector containing the CMV promoter/enhancer (and the latter was evaluated in the absence of an immune response). Therefore, an AAV2/2 vector containing the MCK promoter/enhancer might also express persistent hGAA above the level needed to correct glycogen storage in transduced myofibers; however, it would be expected to have lower efficacy than if the vector were pseudotyped as AAV6.

The presence of a humoral immune response to persistent muscle-restricted hGAA expression is consistent with previous observations following AAV-mediated delivery of therapeutic proteins, especially FIX. While liver-targeted expression of FIX with AAV vectors typically failed to elicit neutralizing antibodies against FIX [23–25], muscle-targeted expression of FIX did not avoid a humoral immune response [26–28]. The mechanism for induction of tolerance to liver-targeted expression of FIX involved regulatory $CD4^+$ T cells that suppressed neutralizing antibody formation, and higher levels of FIX expression increased the likelihood of tolerance [25]. In contrast, muscle-targeted FIX expression was more likely to provoke neutralizing antibodies in response to higher FIX expression and involved a Th2-type response that could be prevented by transient immunosuppression with cyclophosphamide pretreatment [27–29]. A similar humoral response was reported following intramuscular injection of an AAV2/2 vector encoding human FIX driven by the immediate-early CMV promoter/enhancer [26]; current data for an AAV2/2 vector containing the hybrid CB promoter to drive hGAA expression were also consistent with this earlier study, because neutralizing antibodies were present, whereas hGAA expression persisted in the injected muscle. It is possible that delivery of murine GAA driven by the MCK promoter in GAA-KO mice would evade the humoral immune response to introduced GAA, as reported for a vector encoding murine dystrophin in the *mdx* mouse model [14].

Advances in AAV vector packaging including the availability of column purification have increased the efficiency and reliability of AAV vector production, such that large-scale experiments are feasible and new clinical trials can be considered. Purification utilizing a heparin-agarose column increased the quality and infectivity of AAV vector stocks and eliminated laborious, time-consuming cesium chloride gradient centrifugations [30]. AAV2 and AAV2/6 vectors have been amenable to column purification [7,30,31]. Large-scale column purification [32–34], roller bottle production [35], herpes-based hybrid vector systems [36], and an insect cell "factory" [37] have been developed as scalable production methods for AAV2 vectors. Further advances in AAV vector production will facilitate new clinical trials with AAV vectors.

Further studies to evaluate the transduction of skeletal muscle following systemic delivery of AAV vectors encoding hGAA could circumvent the complications of neu-

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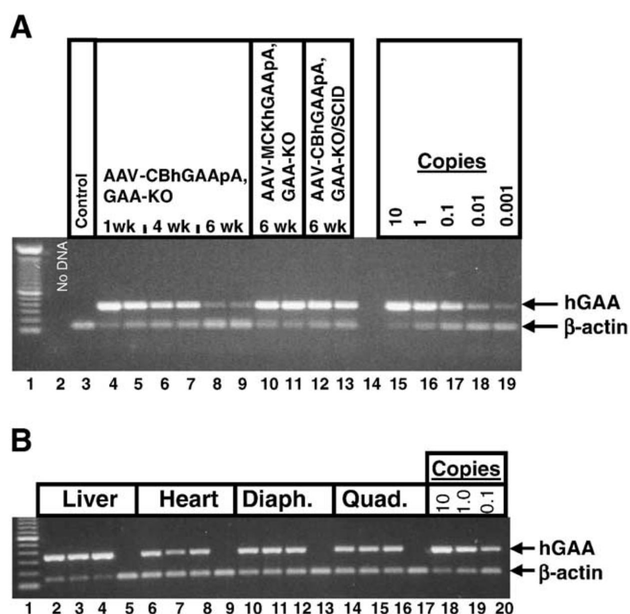

FIG. 7. AAV vector DNA analysis by semiquantitative PCR following AAV-MCKhGAApA administration. (A) Semiquantitation of vector DNA by PCR of gastrocnemius DNA following AAV2/6 vector administration and for an untreated GAA-KO mouse (lane 3). Each lane represents an individual mouse. Lane 1 shows a 100 bp ladder molecular weight marker. The negative control consisted of no input DNA (lane 2). The control samples for quantitation consisted of added vector plasmid DNA representing from 10 to 0.001 copy/cell of AAV vector plasmid DNA in muscle DNA from an untreated GAA-KO/SCID mouse (lanes 15–19). Mouse β-actin DNA was amplified as an internal control for each sample. (B) Semiquantitation of vector DNA by PCR of tissue DNA at 24 weeks following AAV2/7 vector administration and for an untreated GAA-KO mouse. Each lane represents an individual mouse. Lane 1 shows a 100 bp ladder molecular weight marker. The control samples for quantitation consisted of added vector plasmid DNA representing from 10 to 0.1 copy/cell of AAV vector plasmid DNA in muscle DNA from an untreated GAA-KO mouse (lanes 18–20). Liver (lanes 2–5), heart (lanes 6–9), diaphragm (lanes 10–13), and quadriceps (lanes 14–17) DNAs were analyzed from GAA-KO mice following AAV2/7 vector administration ($n = 3$; lanes 2–4, 6–8, 10–12, and 14–16) and from an untreated GAA-KO mouse (lanes 5, 9, 13, and 17). Mouse β-actin DNA was amplified as an internal control for each sample.

tralizing antibodies [3] and mannose 6-phosphate receptor down-regulation in mature skeletal muscle [4], which currently present obstacles to enzyme replacement therapy and liver-targeted gene therapy in Pompe disease.

MATERIALS AND METHODS

Cell culture. 293 cells and C-7 cells [38] were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 U penicillin per milliliter, and 100 μg streptomycin per milliliter at 37°C in a 5% CO_2–air atmosphere. C-7 cells were grown in the presence of hygromycin, 50 μg/ml.

Construction of AAV-MCKhGAApA vector plasmid. A 206-bp MCK enhancer sequence (−1256 to −1050 relative to the transcription start site [17], Accession No. AF188002) was amplified by PCR with primers that created an *Xba*I site upstream and a *Bam*HI site downstream (sense, 5′-CCTCTCCCTAGGGTCTAGAGCAGCCACTAC-3′, and antisense, 5′-TCAGCCTGCCCCTCACCCTGGATCCACCAG-3′). An MCK promoter fragment extending from nucleotide −358 to +7 [17] was also amplified by PCR with primers that introduced unique upstream *Bam*HI and downstream *Kpn*I sites (sense, 5′-GAAGGAGGGTGCTGGATCCAATCA-AGGCTG-3′, and antisense, 5′-CTGTGGAGGTACCGGTGACCCGGGG-GCAGC-3′). The genomic DNA isolated from wild-type mouse skeletal muscle served as template in both PCRs. The *Xba*I–*Bam*HI enhancer fragment and the *Bam*HI–*Kpn*I promoter fragment were cloned into a hGAA-containing AAV vector plasmid, pAAV-CBGAApA, at *Xba*I and *Kpn*I sites to replace the hybrid CB promoter and to generate AAV-MCKhGAApA.

Construction of a hybrid [E1$^-$, polymerase, preterminal protein$^-$] Ad-AAV vector encoding hGAA. The vector sequences from pAAV-MCKhGAApA were isolated as a 4.4-kb fragment from a partial *Bgl*II digest and ligated with the calf intestinal alkaline phosphatase-dephosphorylated *Bgl*II site of pShuttle [39]. The recombinant hybrid Ad-AAV vector was packaged, purified, and analyzed as described [7].

Preparation of AAV vectors. AAV2/2 vector stocks were prepared as described with modifications [7]. For purification of vectors pseudotyped as AAV6, heparin-affinity column purification was modified as described [31]. AAV2/7 vector stocks were prepared as described [10]. AAV stocks were dialyzed against three changes of Hanks' buffer, and aliquots were stored at −80°C. AAV vector DNA-containing particles were quantitated by DNase I digestion, DNA extraction, and Southern blot analysis. Contaminating Ad-AAV hybrid vector was absent by a sensitive plaque-forming unit assay of serial dilutions of the AAV vector preparations on C7 cells [7]. All viral vector stocks were handled according to Biohazard Safety Level 2 guidelines published by the NIH.

In vivo administration of AAV vector stocks. AAV vector stocks were administered by injection of the gastrocnemius muscle in 6-week-old GAA-KO or GAA-KO/SCID mice [40]; the latter strain was generated by crossing SCID and GAA-KO mice [41]. The immunodeficient SCID mice have a spontaneous point mutation in the catalytic subunit of the DNA-dependent protein kinase [42] gene and lack both B- and T-cell-mediated immunity [43,44]. Intravenous injection of AAV vector stocks was administered by retro-orbital injection in 12-week-old GAA-KO and GAA-KO/SCID mice. At the indicated time points postinjection, tissue samples were obtained and processed as described below. All animal procedures were done in accordance with Duke University Institutional Animal Care and Use Committee-approved guidelines.

Determination of hGAA activity. hGAA activity was measured following removal of tissues from control or treated mice, flash-freezing on dry ice, homogenization and sonication in distilled water, and pelleting of insoluble membranes/proteins by centrifugation. The protein concentrations of the clarified suspensions were quantified via the Bradford assay. hGAA activity tissues were determined as described [1].

Glycogen content of tissues was measured using the *Aspergillus niger* assay system, as described [45]. A two-tailed homoscedastic Student *t* test was used to determine significant differences in hGAA levels, glycogen content, and other measurements between GAA-KO mice with or without administration of the vector encoding hGAA.

Western blotting analysis of hGAA. For direct detection of hGAA in tissues, homogenate samples were electrophoresed overnight in a 6% polyacrylamide gel to separate proteins and transferred to a nitrocellulose membrane. The blots were blocked with 5% nonfat milk solution, incubated with primary and secondary antibodies, and visualized via the enhanced chemiluminescence detection system (Amersham Pharmacia, Piscataway, NJ, USA).

Semiquantitation of hGAA vector DNA by PCR. Genomic DNA was extracted from mouse tissues, and PCR was performed in a 50-μl reaction containing 500 ng of mouse DNA as described [7].

ELISA detection of plasma anti-hGAA. An ELISA was performed as described [19]. Briefly, recombinant hGAA (5 μg) in carbonate buffer was coated onto each well of a 96-well plate at 4°C overnight. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween 20, 1:200 dilutions of plasma samples were added to the wells and incubated for 1 h

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at room temperature. The wells were washed with 0.05% Tween 20 + PBS, incubated with a 1:2500 dilution of alkaline phosphatase-conjugated sheep anti-mouse IgG (H + L) at room temperature for 1 h, and washed, and alkaline phosphatase substrate (*p*-nitrophenyl phosphate) was added. The absorbance at 405 nm was measured with a Bio-Rad microplate reader. All samples yielded absorbance values that were within the linear range of the assay at this dilution.

$CD4^+$ and $CD8^+$ lymphocyte immunodetection. Flash-frozen sections of gastrocnemius were fixed in cold acetone for 20 min and air dried prior to being washed in PBS (pH 7.4–7.6) and incubated with normal goat serum for 20 min at room temperature. Subsequently, slides were incubated with an anti-CD4 or anti-CD8 primary antibody (Pharmingen; San Diego, CA, USA) for 1 h at room temperature, followed by three washes with PBS, and incubated with secondary biotinylated goat anti-rat antibody (Pharmingen) for 30 min at room temperature followed by three washes with PBS. Incubation with streptavidin-conjugated alkaline phosphatase (Biogenix; Bogota, DC, Colombia) for 20 min at room temperature followed by three washes with PBS preceded developing with fresh fuchsin substrate (Biogenix) for 4–5 min to effect. Sections were counterstained with methyl green for 1 min and allowed to air dry to preserve substrate, prior to being coated with xylene and covered with a coverslip with permanent mounting medium.

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