Cardiomyocyte-specific Gene Expression Following Recombinant Adeno-associated Viral Vector Transduction

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Recombinant adeno-associated viral (rAAV) vectors hold promise for delivering genes for heart diseases, but cardiomyocyte-specific expression by the use of rAAV has not been demonstrated. To achieve this goal rAAV vectors were generated expressing marker or potentially therapeutic genes under the control of the cardiac muscle-specific alpha myosin heavy chain (MHC) gene promoter. The rAAV-MHC vectors expressed in primary cardiomyocytes with similar kinetics to rAAV-CMV; however, expression by the rAAV-MHC vectors was restricted to cardiomyocytes. rAAV vectors have low cytotoxicity, and it is demonstrated here that rAAV fails to induce apoptosis in cardiomyocytes compared with a recombinant adenoviral vector. rAAV-MHC or rAAV-CMV vectors were administered to mice to determine the specificity of expression in vivo. The rAAV-MHC vectors expressed specifically in cardiomyocytes, whereas the control rAAV-CMV vector expressed in heart, skeletal muscle, and brain. rAAV-MHC transduction resulted in long term (16 weeks) expression of human growth hormone following intracardiac, yet not intramuscular, injection. Finally, we defined the minimal MHC enhancer/promoter sequences required for specific and robust in vivo expression in the context of a rAAV vector. For the first time we describe a panel of rAAV vectors capable of long term cardiac specific expression of intracellular and secreted proteins.

Myocardial gene therapy represents a promising approach for the treatment of inherited heart diseases, cardiomyopathies, and congestive heart failure (1). Extensive work has demonstrated that recombinant adenoviral (rAd) vectors can efficiently transduce cardiomyocytes in vivo (2, 3) to express genes, including the potassium channel (4), sarcoplasmic calcium ATPase-2A (5, 6), and phospholamban (7). However, rAd-mediated gene transfer is limited by immune responses to viral proteins (8–12), which may cause significant myocardial inflammation (13). Designing a delivery system with low cytotoxicity and cardiomyocyte-specific gene expression has been a central goal of cardiac gene therapy.

Derived from a non-pathogenic human parvovirus (14), recombinant adeno-associated viral (rAAV) vectors are an alternative to rAd. Their small size and physical stability are advantageous for in vivo use, and transgene expression can persist in a wide range of tissues (15–17). Moreover, there is no evidence of cell damage from inflammation after rAAV administration to the liver, skeletal muscle, brain, and heart (16, 18–20), and direct heart injection can program stable transgene expression in cardiomyocytes in vivo (19, 21). rAAV vectors are being recognized as vectors for systemic and local long term delivery of gene therapy for clinical diseases (22, 23), yet their promiscuous tropism may lead to the undesirable expression of therapeutic genes in non-targeted cells. This limitation may be circumvented by the use of tissue-specific promoters. Li et al. (24) used the muscle creatine kinase (MCK) promoter to specifically express human γ-sarcoglycan in skeletal muscle using rAAV. In addition, liver-, brain-, cancer-, and rod-specific expression has been accomplished using the tissue-specific albumin, enolase, calcitonin, and rod opsin promoters, respectively (25–28).

Among the isofoms encoded by the multigene myosin heavy chain (MHC) family, only the α- and β-MHC isoforms are expressed in cardiomyocytes (29–31). In late fetal life of mice, α-MHC is expressed in the atria while β-MHC is expressed in the developing ventricles. After birth, α-MHC becomes the predominant isoform expressed in mouse ventricles (29, 30). Cell culture studies have demonstrated three different regions within the proximal α-MHC promoter that regulate cardiomyocyte-specific expression (31–33). First, deletion analysis has demonstrated that α-MHC promoter nucleotide −344 is the 5′-boundary of sequences required for high level expression in cardiomyocytes (32). Second, a 30-bp purine-rich negative regulatory (PNR) element was identified in the first intron, between +66 and +96 bp, that is important for cardiomyocyte-specific expression (33). Finally, within the α-MHC promoter a cardiac-specific enhancer spanning bases −344 to −156 was found to direct high level cardiomyocyte-specific expression with a heterologous promoter (32). These elements were characterized in vitro, which may not faithfully model expression patterns in vivo. Therefore, in addition to developing a vector for cardiomyocyte-specific gene therapy, a goal of this project was to develop the use of somatic gene transfer by rAAV as an alternative to germ-line transgenesis for characterizing long term promoter function in vivo.
Stimulation of tissues with trophic hormones may improve diverse organ-specific processes of aging and atrophy. One such protein, human growth hormone (hGH), is a candidate gene for treatment of dilated cardiomyopathy, because clinical and animal studies have indicated that long-term administration of hGH may beneficially impact weakened cardiomyocytes (34–36). In these clinical studies, hGH protein was administered systemically, but local production of therapeutic secretable proteins by rAAV may produce higher concentrations in the target organ with fewer systemic side effects and greater therapeutic benefits. As an initial step to advance this approach, we made a panel of rAAV vectors expressing hGH under the control of a cardiac-specific promoter, and here we report the pharmacokinetic properties of delivering a potentially therapeutic gene to the heart. For these studies we cloned fragments of the α-MHC promoter (−344 to +19), a larger promoter fragment containing the PNR (−344 to +119), or the α-MHC enhancer (−344 to −156) together with a heterologous promoter to control transgene expression. The strength and specificity of these α-MHC gene promoter elements in rAAV were validated in vivo, and we demonstrate rAAV-MHC-mediated long-term cardiac expression of both marker and therapeutic genes with low cytotoxicity.

EXPERIMENTAL PROCEDURES

Plasmids and Viruses—Using primers listed in Table I, promoter fragments were amplified by PCR from the murine α-MHC gene, kindly provided by Dr. Jeffrey Robbins (GenBank™ accession number U71441, University of Cincinnati, College of Medicine, Cincinnati, OH), and they replaced the CMV enhancer/promoter (−582 to +75) between the Spel and Nru1 sites of pAAV-CMV-lacz (37). For rAAV-MHC-E vectors the minimal CMV promoter (−53 to +75) was ligated to the α-MHC enhancer (−344 to −156). Each construct was verified by DNA sequencing.

rAAV Vector Production—rAAV vectors were prepared as described previously (16). Briefly, subconfluent 293 cells were co-transfected with vector plasmid and pLTAAVhelp using calcium phosphate (37). Cells were then infected with adenovirus Ad5dl312 (an E1A-deletion mutant) at a multiplicity of infection of 2, and after 72 h the cells were harvested, lysed by three freeze/thaw cycles, Ad was heat-inactivated, and the rAAV virions were purified by cesium chloride gradients. The gradient fractions containing rAAV were dialyzed against sterile PBS, and stored at −80 °C. Dot blot analysis demonstrated particle titers of 1–2 × 10^{12}/ml. The EIA-deleted rAd-lacz vector was prepared as described (38).

Infection of Tissue Culture Cells—Primary neonatal rat cardiomyocytes were prepared and maintained as described (39). HeLa, 293, C2C12, and smooth muscle cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone) and gentamicin in a humidified atmosphere at 37 °C with 5% CO2. Approximately 24 h after seeding, vector was added directly to the medium of 1 × 10^6 cells plated in each well of a six-well plate. Immunohistochemistry and Apoptosis Assay—Neonatal rat cardiomyocytes grown on coverslips were infected with rAAV. 7 days later, the cells were washed in PBS, fixed in 0.25% glutaraldehyde and 2% formaldehyde, washed with PBS, and probed with an α-troponin myocardial antibody (Sigma Chemical Co.). After washing, goat anti-mouse IgG Alexa 594 (Molecular Probes) was applied for 1 h. Nuclear staining for DNA fragmentation was performed by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method (Roche Molecular Biochemicals) according to the manufacturer’s protocol. Cells were observed using a Nikon Optiphot-2 light microscope, and images were recorded using a Spot (Diagnostic Instruments, Inc.) digital camera. 100 cardiomyocytes were counted, and the means ± S.E. percent-
age of TUNEL-positive cells was determined in four independent experiments. For cytoplasmic DNA cleavage assays, DNA preparation and agarose gel electrophoresis were performed essentially as described (39).

Direct Myocardial rAAV Injection—Animal care and surgery were performed according to Harvard Institutional Animal Care and Use Committee guidelines and approval, and mice were housed under conventional conditions. 5- to 7-week-old ICR mice (Taconic) were anesthetized by injection of ketamine/xylazine and/or inhalation of methoxyflurane (Metofane, Janssen BmbH) before direct injection of rAAV. Using a 30-gauge needle, 50 μl containing 5 × 10^{10} particles of the rAAV-lacZ vector, was injected into liver or left ventricular wall, through the diaphragm, following laparotomy. Injection with the same dose of vectors into the quadriceps femoris was performed accordingly. In addition, 10 μl containing 1 × 10^{10} particles of the rAAV-lacZ vectors were injected slowly into the forebrain. The rAAV-hGH vectors were injected into the left ventricular wall of a second group of adult mice. To inject rAAV-hGH vectors directly into mouse myocardium the respiration of anesthetized mice was controlled using a Dwyer SAR-830 small animal ventilator. Through a thoracotomy incision, the heart was exposed, and under direct visualization injected with 50 μl containing 1 × 10^{10} particles of rAAV-hGH vectors. Afterward the chest cavity was closed, and the mice were allowed to recover.

X-gal Staining and β-Galactosidase Activity—For detection of β-galactosidase activity, freshly excised tissues were fixed in O.C.T. compound (Sakura), flash-frozen, and 16-mm sections were collected on glass slides. These slides were fixed by using 0.25% glutaraldehyde and 2% formaldehyde, washed with PBS, stained overnight with 5-bromo-4-chloro-3-iodo-β-d-galactopyranoside (X-gal) as described (15). The sections were then washed in PBS and counterstained with Nuclear Fast Red.

Detection of Viral DNA by PCR—Total DNA was extracted from tissues using the Puregene DNA isolation kit (Gentra Systems). PCR was used to amplify a 268-bp fragment of the β-galactosidase gene using sense 5′-TCAATCCGCCGTTTGTTCCC-3′ and antisense 5′-TCCGATAACTGCCGTCACTCC-3′ primers.

HGH Concentration—Blood samples were taken from the retro-orbital vein of anesthetized mice, and the plasma hGH concentrations were determined by ELISA (Roche Molecular Biochemicals).

Statistical Analysis—All results were expressed as means ± S.E. For multiple treatment groups, a factorial analysis of variance was applied followed by Fisher’s least significant difference test. A p value of less than 0.05 was considered significant.

RESULTS

Effective In Vitro Transduction of Cardiomyocytes by rAAV-MHC—The structures of the recombinant vectors rAAV-MHC-PNR, rAAV-MHC-P, rAAV-MHC-E, and rAAV-CMV, used in this study, are shown in Fig. 1A. For the rAAV-MHC-PNR and rAAV-MHC-P vectors expression of the transgene is controlled by the α-MHC enhancer/promoter sequences (−344 to +119) and (−344 to +156), respectively. In the rAAV-MHC-E vector, expression of the transgene is controlled by the α-MHC enhancer/promoter (−344 to −156) coupled to the minimal CMV promoter (−156 to −41). As a control for non-tissue-restricted expression, a rAAV-CMV vector with the constitutively active CMV promoter/enhancer was used (37). First, to examine the potency and kinetics of gene expression in cardiomyocytes, 1 × 10^6 cardiomyocytes were infected with 1 × 10^{10} viral particles. β-Galactosidase expression was first detected 1 day after infection, and rapidly increased during days 3–7, peaked at day 10, and...
reached a plateau at day 14 (Fig. 1B). To determine the optimal multiplicity of infection $1 \times 10^6$ cardiomyocytes were infected with increasing amounts of rAAV, and after 7 days the cells were stained for $\beta$-galactosidase activity. The number of $\beta$-galactosidase-positive cells infected by the rAAV-MHC vectors demonstrated a dose-dependent effect in the range from $5 \times 10^3$ to $5 \times 10^4$ particles/cell (Fig. 1C). A similar threshold effect was observed using rAAV-CMV increased markedly between 1 day after infection of primary rat neonatal cardiomyocytes, the number of X-gal-positive cells was measured. The means ± S.E. from four independent experiments are shown. C, the dose-response relationship of rAAV-MHC vector-transduced cardiomyocytes. Seven days post-infection cardiomyocytes were stained with X-gal, and the number of positive cells was measured. The means ± S.E. from four independent experiments are shown.

**Cell Specificity of rAAV-MHC Vectors**—We tested the specificity of rAAV-MHC-mediated $\beta$-galactosidase expression in 293 (human embryonic kidney cell line), HeLa (human cervical carcinoma cell line), rat aortic smooth muscle cells (SMC) and C2C12 (mouse myoblast cell line). 293 cells and HeLa cells were stained 3 days after infection with $1 \times 10^5$ particles, whereas SMC, C2C12 cells, and cardiomyocytes were stained after 7 days, which for each cell type was the peak of their expression. As shown in Fig. 2A, rAAV-CMV vector-infected cells showed robust $\beta$-galactosidase staining in every cell line, whereas the rAAV-MHC vectors produced few positive cells in the non-cardiomyocyte cell lines (Fig. 2A). All three rAAV-MHC vectors produced $\beta$-galactosidase in cardiomyocytes (Fig. 2, A and B). Although rAAV-CMV produced a greater number of positive 293 and HeLa compared with cardiomyocytes, the rAAV-MHC vector produced a far greater number of positive cardiomyocytes than all other cell lines (Fig. 2A). Of the three rAAV-MHC vectors, the rAAV-MHC-PNR vector produced the lowest number of $\beta$-galactosidase-positive cardiomyocytes (Figs. 1B, 2A, and 2B). Although rAAV-CMV produced similar numbers of $\beta$-galactosidase-positive C2C12 cells as cardiomyocytes, rAAV-MHC-E, rAAV-MHC-P, or rAAV-MHC-PNR produced about 47-, 80-, and 93-fold more X-gal-positive cardiomyocytes than all other cell lines (Fig. 2A). To confirm transgene expression of rAAV-MHC vectors in cardiomyocytes, but not cardiac fibroblasts, which are co-isolated from neonatal rat hearts, we stained infected cells for both $\beta$-galactosidase activity and the muscle sarcomeric protein tropomyosin. Expression of $\beta$-galactosidase using the rAAV-MHC vectors was mostly restricted to cardiomyocytes (Fig. 2C), whereas the rAAV-CMV vector expressed $\beta$-galactosidase in both cardiomyocytes and non-myocytes (Fig. 2C, top). These results demonstrate that the rAAV-MHC vectors express preferentially in cardiomyocytes, and as expected, the PNR element conferred the most cardiomyocyte-specific expression.
Cardiac-specific rAAV Expression

Fig. 3. rAd but not rAAV vectors induce apoptosis in cardiomyocytes. A, cardiomyocytes were incubated with rAd-lacZ and rAAV-lacZ vectors, then immunostained using an α-tropomyosin antibody followed by TUNEL analysis. Following rAd-CMV infection, a purple-staining apoptotic nuclei that co-stain for tropomyosin is indicated. B, summary of the rAd and rAAV effects on apoptosis. The percentage of TUNEL-positive cells is presented as the means ± S.E. from three independent experiments. PNR, rAAV-MHC-PNR; P, rAAV-MHC-P; E, rAAV-MHC-E; and C, rAAV-CMV. * significantly more TUNEL-positive cells (p < 0.05) compared with uninfected control. C, rAd-induced DNA fragmentation in cardiomyocytes. Cytoplasmic DNA isolated from uninfected cardiomyocytes (control) and from rAd- and rAAV-infected cardiomyocytes was subjected to gel electrophoresis alongside molecular weight markers whose sizes are shown on the left in base pairs. The gel demonstrates a ladder of DNA bands following rAd transduction.

Transduction of Primary Cardiomyocytes by rAAV Is Non-cytopathic—Recombinant adeno viral vectors can have direct cytotoxic effects whereas rAAV vectors are known to be far less cytopathic. To more closely analyze cytotoxicity profile of the rAAV-MHC vectors, TUNEL analysis was performed to monitor the induction of apoptosis. Infection with $5 \times 10^4$ rAAV-CMV vector particles/cell for 8 days and 100 rAd-CMV plaque-forming units/cell for 3 days resulted in every cardiomyocyte nucleus staining positive for β-galactosidase (data not shown). Following infection, rAAV- and rAd-infected cells were subjected to TUNEL analysis at 8 and 3 days, respectively. The rAAV-CMV vector did not increase the number of TUNEL-positive cells compared with the non-infected control cells, whereas incubation with the rAd-CMV vector markedly increased the number of TUNEL-positive cells (Fig. 3A and B, p < 0.005, compared with control cells) as did treatment of cells with H$_2$O$_2$ (data not shown) (39). To confirm the occurrence of apoptosis, we examined DNA fragmentation by agarose gel electrophoresis. Although cytoplasmic DNA extracted from cardiomyocytes after rAd-CMV infection showed prominent ladder formation, incubation with the rAAV-CMV vector did not induce DNA cleavage (Fig. 3C). Infection with $5 \times 10^4$ rAAV-MHC vector particles/cell also did not induce apoptosis in primary cardiomyocytes (Fig. 3, A and C). Despite infection with more particles and for a longer time, we found that rAAV are less likely to induce apoptosis in cardiomyocytes.

In Vivo Transduction of Adult Mouse Tissues by rAAV-MHC-lacZ Vectors—To determine how efficiently the rAAV-MHC vectors could transduce cardiomyocytes in vivo, $5 \times 10^4$ particles of rAAV vectors were injected through the diaphragm into the cardiac wall of 5- to 7-week-old mice. Mice were killed 4 weeks after infection, and assayed for β-galactosidase expression in the myocardium. Positive cells were localized to the injection site. After rAAV-CMV-lacZ administration, β-galactosidase expression was observed in all tissues except liver (only two positive cells), as expected (16) (Fig. 4A). Each rAAV-MHC vector expressed β-galactosidase in the heart 4 weeks post injection, with the majority of β-galactosidase staining cells being cardiomyocytes (Fig. 4A). rAAV vectors were not only injected into the left ventricular wall but also into the liver, quadriceps femoris muscle, and brain. Interestingly, there were very few β-galactosidase-positive cells in both femoral muscle and brain tissues 4 weeks after rAAV-MHC-lacZ injection (Fig. 4A), and we did not observe positive cells following liver injection. There was no evidence of myocardial and skeletal muscle inflammation detected by hematoxylin and eosin staining in rAAV-CMV-lacZ-injected hearts (Fig. 4A). To confirm gene transfer following injection of the four recombinant viruses, total DNA was isolated from each tissue, and the presence of rAAV sequences was confirmed by PCR. Agarose gel electrophoresis demonstrated the expected 268-bp PCR product in all tissues injected with the rAAV-lacZ vectors (Fig. 4B). Thus, rAAV DNA was detected by PCR in all injected tissues, yet the rAAV-MHC-lacZ vectors expressed β-galactosidase significantly only in heart muscle.

Efficient In Vivo Transduction of Adult Mouse Heart by rAAV-MHC-hGH Vectors—To develop potentially therapeutic
The means in infection, the serum concentration of hGH was measured by ELISA. After direct left ventricular wall injection of vectors for cardiomyopathy, and to analyze the relative long term expression strength of each rAAV-MHC vector, we ex- fermed earlier studies that identified a cardiomyocyte-specific enhancer from −344 to −156 to be capable of specific high level expression in cardiomyocytes (32). Contained within this region are binding sites for myocyte-specific enhancer-binding factor, GATA, and serum response factor proteins that are important for cardiac-specific gene expression. Our studies show that the rAAV-MHC-E vector is capable of significant and specific expression in primary cardiomyocytes and that the minimal CMV promoter can be used as a heterologous pro- following IM injection of rAAV-MHC-P-hGH was not signifi- cantly elevated compared with the lacZ control (means ± S.E. 28.0 ± 12.4 versus 11.5 ± 7.5, p = 0.65); however, following IC injection, hGH levels were increased 7.5-fold (Fig. 5B). By comparison, hGH levels were significantly elevated following both IM and IC injection of rAAV-CMV-hGH. These results demonstrate that rAAV regulated by the α-MHC promoter had long term transgene expression in the heart, similar to the CMV promoter, yet with greater myocardial specificity.

**DISCUSSION**

In this report, we describe rAAV vectors regulated by ele- ments of the α-MHC promoter, which allow long term trans- gene expression predominantly in cardiomyocytes. Specific gene expression of rAAV-MHC vectors was shown both in pri- mary neonatal cardiomyocytes in vitro and in heart muscle of adult mice in vivo. The long term and tissue-specific expression pattern of these vectors presents the potential to develop cardiomyo- cyte-specific gene therapy as a treatment modality of cardiomypathy.

The expression kinetics of these constitutive and tissue-spe- cific promoters in the context of rAAV were similar in both primary cultured cardiomyocytes and heart muscle in vivo. All four vectors shared a similar profile of expression with low expression soon after infection (<3 days in culture), followed by a steep rise and then a plateau. This pattern probably reflects the dynamics of uptake, viral genome processing, and expres- sion of rAAV that has also been seen in liver (42) and skeletal muscle (43). Differences in promoter strength were reflected in the steepness of their slope and height of their plateau. The MHC (−344 to +19) promoter region is perhaps the smallest region of the α-MHC promoter that produces cardiomyocyte-specific expression. This is supported by our finding that the rAAV-MHC-P vector produced the greatest number of β-galac- tosidase staining cardiomyocytes and human growth hormone level (Figs. 1B and 5A). In addition, it should be noted that the rAAV-MHC-P-hGH vector was not significantly active in skeletal muscle compared with a control (Fig. 5B). We also con- firmation that the rAAV-MHC-E vector is capable of specific expression in primary cardiomyocytes and that the minimal CMV promoter can be used as a heterologous pro- moter with a cardiac-specific enhancer to target cardiomyocyte-specific expression in the context of rAAV. Hagstrom et al. (44) created a hybrid rAAV vector regulated by an α-skeletal muscle actin promoter and CMV enhancer/promoter that produced high level expression in all cells. The minimal CMV promoter has also been coupled to a tetracycline-responsive element and been used in rAAV for regulated transcription (41). It may be possible to use rAAV-MHC to express the tetracycline-activator for cardiac-specific and regulated transcription. The signifi- cance of our findings lies in the future use of small enhancer sequences, or minimal promoter elements rather than large promoters, to drive tissue-specific expression by rAAV.

Compared with rAAV-CMV, the rAAV-MHC vectors did not significantly express in HeLa, 293, C2C12, and smooth muscle cells, demonstrating that expression by the rAAV-MHC vectors is specific to cardiomyocytes. We have also confirmed the role of the highly conserved PNR element in the first α-MHC gene intron as being important for cardiomyocyte-specific transcrip- tion (33). Although expression by rAAV-MHC-PNR was lower in all cells tested, it was particularly low in non-cardiomyo-
cytes, which contributed to its greater specificity compared with rAAV-MHC-E and rAAV-MHC-P. Consistent with our in vitro data (Fig. 2), we found that rAAV-MHC-P and rAAV-MHC-E expressed hGH significantly stronger than rAAV-MHC-PNR in vivo, again demonstrating that the PNR negatively regulates transcription (Fig. 5A). Within the PNR a palindrome of two high affinity Ets-binding sites has been identified by DNase footprint analysis. PNR binding activity is increased in adult rat hearts subjected to pressure overload hypertrophy, a condition in which α-MHC expression is usually suppressed (33), suggesting that Ets, or an Ets-like factor (45), may be responsible for cardiac-specific α-MHC expression. Using a rAAV-based system, we have extended the in vitro results of Gupta et al. (33) that the addition of the PNR to the α-MHC promoter reduces promoter activity in vitro while improving cardiac specificity. rAAV vectors can therefore be utilized to understand long term promoter function in vivo, potentially saving the labor, time, and expense of producing multiple lines of transgenic animals.

Infection with adenovirus can cause cell damage by immune response or direct cytotoxicity (9, 46). Although it has recently been reported that rAAV selectively induces apoptosis in p53-deficient cells, rAAV is non-pathogenic in normal eukaryotic cells (16, 47). For our studies we used the first generation rAd-CMV-lacZ, which expressed strongly in all cultured cardiomyocytes and produced a similar amount of apoptosis as hydrogen peroxide treatment (data not shown) (39). We found that infection of a similar or greater number of rAAV particles did not induce apoptosis compared with the uninfected cells (Fig. 3, A and C), whereas the first generation rAd produced apoptosis. Even cardiac fibroblasts, which proliferate, did not demonstrate increased apoptosis following rAAV infection (data not shown). In the present study, we compared the cytotoxicity profile of rAAV and first generation rAd, which is known to be cytotoxic. Newer generations of adenoviral vectors demonstrate significantly less cytotoxicity than first generation adenoviral vectors (48, 49). Reduced cytotoxicity and stable maintenance of vector sequence makes rAAV more suitable for long term expression of therapeutic genes and for in vivo analysis of promoter function without interference from apoptosis.

rAAV vectors efficiently transduce a variety of cells in vivo and are being evaluated for gene therapy of myopathies (15, 17, 19, 20, 24). Transduction of muscle by rAAV results in stable expression (50–52). Using the CMV promoter, rAAV vectors have been used to transfer the human minidystrophin gene to skeletal muscle of mdx mice, resulting in long term correction of their dystrophic degeneration (24). Direct muscle injection of an rAAV vector expressing human γ-sarcoglycan under the control of the MCK promoter induced a significant numbers of muscle fibers expressing γ-sarcoglycan, and improved the histologic pattern of dystrophy in γ-sarcoglycan-deficient mice (53). Previous studies demonstrating efficient viral transduction of muscle have required either direct injection, or at least injection into the arterial supply of a target muscle (19, 21). In agreement with these findings we did not find significant cardiac expression 4 weeks after venous injection of the rAAV-MHC and rAAV-CMV vectors (data not shown). Transduction of skeletal muscle is increased by arterial infusion of rAAV with histamine-induced endothelial permeabilization (54). Future delivery of cardiac gene therapy by venous administration may require improved vector targeting together with endothelial permeabilization (55).

Growth hormone and its local effector insulin-like growth factor-I have been shown to be important for maintaining cardiac mass and performance in adult life, and it has been investigated as a treatment of cardiomyopathy (34–36). We observed long term expression of hGH following myocardial injection for 16 weeks (Fig. 5A). Interestingly, injection of the rAAV-CMV vector into the heart produced a greater amount of hGH compared with injection into skeletal muscle (Fig. 5B), suggesting that the heart may be more efficient at producing secreted proteins than skeletal muscle or that proteins secreted in the heart have greater access to the circulation. Skeletal muscle can serve as a depot for production of secretable proteins, such as erythropoietin and factor IX (15, 52), and we now demonstrate that cardiac muscle also has this capability. Because long term administration of growth hormone may be a beneficial treatment of cardiomyopathy (34, 35), the potential therapeutic use of these vectors to provide local delivery of hGH in murine cardiomyopathy disease models is now under investigation. Future improvements may depend on alternate cardiac-specific promoters, including the myosin light chain-2v promoter (MLC2v), which is a cardiac-specific promoter active in embryonic and adult ventricular myocardium, including human hearts (56, 57). Franz et al. (58) reported that rAd containing the 2100-bp MLC2v promoter expressed at high levels in heart muscle. Although a promoter of this size would limit the transgene size for rAAV, we are nonetheless developing the use of the MLC2v promoter for long term rAAV expression in cardiomyocytes.

In summary, we have demonstrated that the α-MHC promoter and enhancer regions can direct rAAV-mediated cardiomyocyte-specific expression both in vitro and in vivo. The functional importance of the development of rAAV-MHC vectors is underscored by the finding that they have tissue-specific expression and low cytotoxicity and produce long term gene expression in this tissue. Taken together, these rAAV-MHC vectors will be useful experimental tools, particularly to validate therapeutic approaches in animal disease models. Finally, we strongly consider that this approach may yield effective cardiomyocyte-specific therapies for human cardiomyopathies such as inherited heart diseases and congestive heart failure.

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