Improved muscle-derived expression of human coagulation factor IX from a skeletal actin/CMV hybrid enhancer/promoter


Hemophilia B is caused by the absence of functional coagulation factor IX (F.IX) and represents an important model for treatment of genetic diseases by gene therapy. Recent studies have shown that intramuscular injection of an adenovirus-associated viral (AAV) vector into mice and hemophilia B dogs results in vector dose–dependent, long-term expression of biologically active F.IX at therapeutic levels. In this study, we demonstrate that levels of expression of approximately 300 ng/mL (6% of normal human F.IX levels) can be reached by intramuscular injection of mice using a 2- to 4-fold lower vector dose (1 x 10^11 vector genomes/mouse, injected into 4 intramuscular sites) than previously described. This was accomplished through the use of an improved expression cassette that uses the cytomegalovirus (CMV) immediate early enhancer/promoter in combination with a 1.2-kilobase portion of human skeletal actin promoter. These results correlated with enhanced levels of F.IX transcript and secreted F.IX protein in transduced murine C2C12 myotubes. Systemic F.IX expression from constructs containing the CMV enhancer/promoter alone was 120 to 200 ng/mL in mice injected with 1 x 10^11 vector genomes. Muscle-specific promoters performed poorly for F.IX transgene expression in vitro and in vivo. However, the incorporation of a sequence from the α-skeletal actin promoter containing at least 1 muscle-specific enhancer and 1 enhancer-like element further improved muscle-derived expression of F.IX from a CMV enhancer/promoter-driven expression cassette over previously published results. These findings will allow the design of a clinical protocol for therapeutic levels of F.IX expression with lower vector doses, thus enhancing efficacy and safety of the protocol. (Blood. 2000;95:2536-2542) © 2000 by The American Society of Hematology

Introduction

The severe bleeding disorder hemophilia B is caused by an absence of functional coagulation factor IX (F.IX). The X-linked disease affects 1 in 30 000 males in the United States. Current treatment for hemophilia is based on intravenous infusion of plasma-derived or recombinant clotting factor concentrates. Treatment can be episode-based (in response to a bleeding episode) or prophylactic (2-3 infusions per week). The latter is expensive and not without complications and is therefore not widely adopted in the United States. However, 30 years of experience with prophylactic treatment as pioneered in Sweden has shown that a continuous supply of clotting factor levels above 1% of normal (greater than 50 ng F.IX/mL plasma) results in prevention of most joint damage that constitutes the major morbidity of the disease. Furthermore, life-threatening bleeding episodes, such as intracranial or retroperitoneal bleeds, are also prevented if a continuous level of factor can be maintained.1,2 These observations, as well as the availability of animal models, have contributed to the development of hemophilia into an important model for treatment of genetic diseases by gene therapy. The goal of a gene-based treatment is to achieve long-term expression of F.IX in the circulation of a severe hemophiliac in order to maintain a continuous supply of F.IX.

Progress toward gene therapy has been substantial over the past 2 years due to successful studies in a large animal model of hemophilia B using liver or muscle as a target organ for F.IX gene transfer with adenovirus-associated viral (AAV) vectors.3,4 In a recently developed approach, we have taken advantage of the observation that AAV vectors efficiently transfer genes to muscle fibers in vivo.5,7 AAV vectors are single-stranded, replication-deficient DNA viruses that can be produced in a helper virus-free system.6 Recombinant AAV contains an expression cassette flanked by viral inverted terminal repeats and is devoid of any sequences encoding viral gene products. The viral packaging limit is approximately 5 kilobases (kb). Gene transfer to nondividing cells such as muscle fibers is stable and often not associated with cellular immune responses against transduced fibers (as commonly observed with, for example, adenoviral vectors). Although F.IX is normally made in the liver, muscle cells are capable of synthesizing biologically active F.IX.9,10 In previous studies, we achieved sustained expression of therapeutic levels of F.IX in the circulation of experimental animals by intramuscular administration of AAV vectors for expression of human F.IX in immunodeficient mice or canine F.IX in hemophilia B dogs (up to 7% of normal human levels in mice and up to 1.4% in dogs).3,11 While these levels of expression are likely to have a therapeutic effect in a patient with severe...
hemophilia B, further improvements in expression are desirable. On a transcriptional level, this could be achieved by an improved expression cassette resulting in increased expression per delivered vector particle. Equally important, the ability to direct expression of a set amount of FIX using a lower total dose of vector is an important therapeutic goal, because biodistribution to sites outside muscle (eg, to brain or gonads, an undesirable outcome) is a direct function of dose (in vector genomes) administered. Thus, the use of an improved vector to achieve higher levels of FIX expression with lower total doses of vector will result in an added measure of safety for this gene therapy approach. In addition, optimization of gene expression on a transcriptional level will benefit not only a protocol for FIX, but other gene therapy strategies that might use muscle-derived expression of the transgene product (eg, growth hormone, dystrophin, lepton, erythropoietin, insulin-like growth factor-1) as well.

In our initial studies in mice and dogs, we have used the cytomegalovirus (CMV) immediate early (IE) enhancer/promoter to drive sustained high levels of FIX expression in muscle (for at least 2 years postvector administration). In this study, we demonstrate that expression of muscle-derived human FIX can be enhanced by a combination of human α-skeletal actin and CMV enhancer/promoter sequences driving transgene expression.

Materials and methods

Construction of plasmids encoding AAV vectors

Cassettes for expression of human FIX were constructed by standard cloning techniques in Escherichia coli. All cassettes were inserted between two 145-base pair (bp) AAV-2 inverted terminal repeats contained in plasmid DNA. Vector AAV-CMV-hFIX-3 contained the previously published expression cassette based on the CMV IE enhancer/promoter, human FIX (hFIX) coding region (including a 1.4-kb portion of intron I of the human FIX gene), and SV40 polyadenylation sequences. AAV-CMV-hFIX-3 was constructed by replacing the SV40 polyA sequences in AAV-CMV-hFIX-3 with the bovine growth hormone (bGH) polyadenylation signal excised from plasmid pRSV (Invitrogen, Carlsbad, CA). AAV-CMV-hFIX-8 was created by further deleting intron sequences in AAV-CMV-hFIX-3 to a 0.3-kb sequence (by removal of a 1.1-kb ScaI fragment contained in the 1.4-kb portion of intron I, deletion of the SV40 polyA sequences, and addition of the entire 3′-untranslated [UT] polyadenylation signal of the human FIX gene [up to bp position 33,071]).14,15 Vector AAV-CMV-hFIX-2 was identical to the published canine FIX vector, except that the canine FIX complementary DNA (cDNA) was substituted by a 2.5-kb portion of human FIX cDNA (up to the EcoRI site at position bp 2501 of the human FIX cDNA)16). This vector contains the CMV enhancer/promoter, a 0.4-kb chimeric CMV/b- globin intron upstream of the FIX cDNA, and a human growth hormone polyA signal. AAV-HSA-bGH-I contains a 2.2-kb region of the human α-skeletal actin (HSA) gene, which includes the promoter, first noncoding exon, and a portion of the first intron. This sequence was kindly provided by Dr. Hardeman, The Children’s Medical Research Institute, Wentworthville, New South Wales, Australia. HSA sequences in this vector replaced the CMV enhancer/promoter in AAV-CMV-hFIX-3. Intron I was further truncated to a 0.3-kb sequence as described above in order not to exceed the packaging limit of AAV. AAV-HSA-bGH-I is based on AAV-CMV-hFIX-2. In this vector, CMV enhancer/promoter sequences and the chimeric intron were replaced with the 2.2-kb HSA sequence. Subsequently, a truncated version of intron I (253 bp) of the HSA gene was introduced between the transcription start site of the HSA promoter and the FIX cDNA (HSA intron I is located 5′ to the translational start site in the HSA gene16). All of the 3′-UT of the FIX cDNA was deleted to accommodate for the additional sequences. AAV-HSA/CMV-hFIX-2 was created by adding a 1.2-kb portion of the HSA promoter region (bp position −1281 to −841)5 to the CMV enhancer/promoter in AAV-CMV-hFIX-2. Again, the entire 3′-UT of the FIX cDNA was deleted in this vector. AAV-CMV-hFIX contains a 588-bp region from the chicken cardiac troponin T (cTnT) promoter (−550 to +38), which replaces the CMV enhancer/promoter in AAV-CMV-hFIX-2. This sequence was generously provided by Dr Charles Or达尔, University of California, San Francisco, and has been demonstrated to direct expression of reporter genes in primary muscle cells.19,20

Production of AAV vectors

AAV vectors were produced in an adenovirus-free system by cotransfection of human embryonic kidney (HEK)-293 cells with the vector plasmid and 2 additional plasmids carrying adenoviral helper functions E2a, E4, and VA and AAV rep/cap functions.22 Vector was purified by 2 rounds of cesium chloride density gradient centrifugation and viral titers determined by quantitative dot blot hybridization. Titers are therefore given as vector genomes/mL. Vector was stored at −80°C in 1× phosphate-buffered saline containing an osmotic stabilizer.

Transduction of myotubes in vitro

Murine C2C12 myoblasts were grown in DMEM medium containing 10% fetal bovine serum and antibiotics. Cells were plated in 6-well plates, allowed to reach confluence, and then differentiated to myotubes by exchanging media to DMEM containing 2% horse serum (differentiation media). Cells were incubated for 5 to 6 days in differentiation medium prior to transduction with AAV vector. Immediately prior to addition of vector, fresh differentiation media was added to the cells, and vector was added at multiplicities of infection (MOIs) ranging from 104 to 105 vector genomes/cell. The media was replaced on the following day. All transduction experiments were carried out in duplicate. MOIs were calculated for number of myoblasts used per well for formation of myotubes (2×106 myoblasts per confluent 6-well). Supernatants (24-hour) were collected 7 to 12 days posttransduction, and myotubes were subsequently harvested for RNA isolation. Concentrations of human FIX in supernatants were measured by enzyme-linked immunosorbent assay (ELISA) as described.22 Human myotubes derived from primary human myoblasts were a gift from Dr Heiman-Peterson, Hahneman University, Philadelphia, PA. These cells were maintained and transduced as described for differentiated C2C12 myotubes, except that vector was added in OptiMEM medium (Gibco/BRL, Gaithersburg, MD), and the differentiation medium contained 10% horse serum.3 Transduction experiments in other cell types (HEK-293 cells and human hematopoietic [HepG2] cells) were carried out in 24-well plates as described.13 These cells were transduced at 50% confluence in DMEM/2% fetal bovine serum medium. Supernatants (24-hour) were harvested 96 hours posttransduction.

Analysis of FIX mRNA in transduced myotubes

Total RNA was isolated from transduced myotubes using the RNAzol kit (Tel Test, Friendswood, TX). Reverse transcriptase–polymerase chain reaction (RT-PCR) was carried out on 1 µg of total RNA using an RT-PCR kit purchased from Applied Biosystems (Foster City, CA) and using primers specific for messenger RNAs (mRNAs) from AAV-hFIX vectors. PCR products were separated by agarose gel electrophoresis. Duplicate Northern blots were performed by separation of 10 µg of total RNA using agarose gel electrophoresis under denaturing conditions. Following transfer to nylon membranes, 1 blot was hybridized with a radioactively labeled probe specific for the coding region of the human FIX cDNA and the other to a β-actin probe. Phosphoimage analysis of the blots was performed using a Storm 860 phosphoimager (Molecular Devices, Sunnyvale, CA) and ImageQuaNT software (Molecular Devices). The β-actin signal was used to normalize the amount of RNA loaded in each lane, and the relative amounts of human FIX transcript observed in the various RNA samples was calculated.

Animal experiments

Immunodeficient Rag-1 mice (4-6 weeks old, 20-25 g) were injected intramuscularly into 4 sites of the hind limbs with 1×1011 vector genomes
of recombinant AAV (n = 4 for each vector) as described previously.11 Rag-1 mice lack recombinease activating gene 1 and are therefore devoid of mature B and T cells and circulating immunoglobulins.23 Quadriceps and tibialis anterior muscles were exposed by a 1-cm incision through the skin while the animals were under general anesthesia from an intraperitoneal injection of ketamine/xylazine. A vector was diluted in 1 × phosphate-buffered saline and slowly injected with a 20-ga needle (50 µL of vector suspension per quadriceps and 25 µL per tibialis anterior). Incisions were closed with suture. Mice were bled retro-orbitally on a biweekly schedule for 10 to 12 weeks, and FIX levels in plasma samples were determined by ELISA specific for human FIX as described.22

Results

A series of AAV vectors for expression of the human FIX cDNA was constructed and produced in high titer (greater than or equal to 10^{12} vector genomes/mL) in HEK-293 cells using an adenovirus-free production system that is based on the triple-transfection method (see “Materials and methods”). These vectors are derived from constructs that were successfully used for expression of therapeutic levels of human or canine FIX in mice and hemophilic dogs.3-11 All expression cassettes were designed to not exceed the packaging limit of AAV (approximately 5 kb). While all vectors contain the human FIX cDNA, they differ in enhancer/promoter, intron, and polyadenylation sequences (Table 1). When tested for expression of muscle cell–derived FIX in murine C2C12 myotubes, all constructs showed detectable expression and correct splicing when analyzed by RT-PCR (data not shown) but differed substantially with regard to transcript levels and secretion of FIX into culture media (see below). For in vivo experiments, immunodeficient Rag-1 mice were chosen because intramuscular administration of vector results in antibody formation against the nonspecies-specific transgene product (human FIX) in immunocompetent mice.11 We have previously shown that FIX expression in Rag-1 mice is vector dose–dependent, and we therefore chose an intermediate dose of 1 × 10^5 to 4 × 10^11 AAV-CMV-hFIX-3 vector genomes/mouse for intramuscular administration of recombinant AAV vectors (based on experiments with 3 × 10^6 to 4 × 10^11 AAV-CMV-hFIX-3 vector genomes/mouse).24

Table 1. Components of AAV vectors for expression of muscle-derived human F IX (hFIX)

<table>
<thead>
<tr>
<th>Vector</th>
<th>Enhancer/Promoter</th>
<th>Intron</th>
<th>Human F IX 3′-UT</th>
<th>PolyA Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV-CMV-hFIX-3</td>
<td>CMV IE</td>
<td>Portion of hFIX intron I (1.4 kb)</td>
<td>0.3 kb</td>
<td>SV40 late</td>
</tr>
<tr>
<td>AAV-CMV-hFIX-3/bGH</td>
<td>CMV IE</td>
<td>Portion of hFIX intron I (1.4 kb)</td>
<td>0.3 kb</td>
<td>Bovine growth hormone (bGH)</td>
</tr>
<tr>
<td>AAV-HSA-hFIX-1</td>
<td>Human α-skeletal actin (HSA)</td>
<td>Portion of hFIX intron I (0.3 kb)</td>
<td>0.3 kb</td>
<td>SV40 late</td>
</tr>
<tr>
<td>AAV-CMV-hFIX-8</td>
<td>CMV IE</td>
<td>Portion of hFIX intron I (0.3 kb)</td>
<td>Entire 1.6 kb</td>
<td>Human FIX</td>
</tr>
<tr>
<td>AAV-CMV-hFIX-2</td>
<td>CMV IE</td>
<td>Chimeric CMV/β-globin mini-intron</td>
<td>1.1 kb</td>
<td>Human growth hormone (hGH)</td>
</tr>
<tr>
<td>AAV-HSA/CMV-hFIX-2</td>
<td>HSA/CMV IE</td>
<td>Chimeric CMV/β-globin mini-intron</td>
<td>None</td>
<td>hGH</td>
</tr>
<tr>
<td>AAV-HSA-hFIX-3</td>
<td>HSA</td>
<td>HSA intron I</td>
<td>None</td>
<td>hGH</td>
</tr>
<tr>
<td>AAV-cTnT-hFIX</td>
<td>Cardiac troponin I</td>
<td>Chimeric CMV/β-globin mini-intron</td>
<td>1.1 kb</td>
<td>hGH</td>
</tr>
</tbody>
</table>

Vector genomes were designed to approximate the size of wild-type AAV (approximately 4.5 kb) in order not to exceed the packaging limit for generation of recombinant AAV. All vectors include the entire coding region of the human F IX cDNA plus the components listed above.

Table 1) for expression of human FIX in vitro using murine C2C12 myotubes and in vivo using Rag-1 mice. Based on other investigators’ observations on transgene expression, we additionally constructed vectors AAV-CMV-hFIX-3-bGH and AAV-CMV-hFIX-8. The first vector contains the bovine growth hormone polyadenylation signal, which has been shown to improve expression in the context of a plasmid DNA vector when substituted for the SV40 polyA (which is used in AAV-CMV-hFIX-3).25 The second vector contains the entire human F IX 3′-UT region and polyadenylation signal of the human FIX message, which was found to increase expression of human FIX in mice after hepatic gene transfer with an adenoviral vector.20

Figures 1 and 2A show the results of a comparison of these vectors in vitro and in vivo. Two experiments were performed by transduction of C2C12 cells. The combined results of these experiments (Figure 1) demonstrated that vectors using the SV40 or a growth hormone polyadenylation signal (AAV-CMV-hFIX-2, -3, and -3-bGH) gave nearly identical levels of FIX expression in myotubes and in plasma samples of Rag-1 mice. Levels of systemic expression in mice (n = 4 for each vector construct) were, on average, 120 to 200 ng/mL and therefore in the therapeutic range of expression (2.4%-4% of normal human FIX levels; Figure 2A).

Expression from the construct containing the human FIX polyA sequence (CMV-8) was 2- to 4-fold lower, both in vitro and in vivo (Figure 1 and Figure 2A).

Expression from the CMV promoter is sufficient to achieve therapeutic levels

In our published experiments in mice and dogs, we have used 2 similar expression cassettes. Both use the CMV IE enhancer/promoter but differ in intron and polyadenylation sequences and in the amount of 3′-UT region of the FIX cDNA. Hence, we decided to compare these vectors (AAV-CMV-hFIX-2 and -3 as outlined in

Figure 1. Expression of human FIX in conditioned media of C2C12 murine myotubes after transduction with AAV vectors at an MOI = 10^5 vector genomes/cell. All measurements of human FIX concentrations were performed by ELISA. Transduction experiments were performed in duplicate. (A) Cells were transduced with either AAV-CMV-hFIX-2 (CMV-2), AAV-CMV-hFIX-3 (CMV-3), AAV-HSA-hFIX-1 (HSA-1), or AAV-HSA-hFIX-3 (HSA-3), FIX values (ng/mL × 24 hours) are average of 2 independent measurements performed on 24-hour supernatants at day 8 posttransduction. Vertical bars indicate standard deviation. (B) Cells were transduced with either AAV-CMV-hFIX-3 (CMV-3), AAV-CMV-hFIX-3-bGH (CMV-3-bGH), AAV-CMV-hFIX-8 (CMV-8), AAV-HSA-hFIX-3 (HSA-3), AAV-HSA-CMV-hFIX-2 (HSA-CMV), or AAV-cTnT-FIX (cTnT). FIX values (ng/mL × 24 hours) are average of a total of 4 measurements (2 independent measurements per experiment) on 24-hour supernatants at day 8 posttransduction from 2 independent transduction experiments.
Muscle-specific promoters fail to produce therapeutic levels of expression

The use of muscle-specific cellular promoters, including that of the human α-skeletal actin gene (sequence up to position $\sim -2000^\text{17}$) and the cardiac troponin I gene (cTnT, also expressed in skeletal muscle) gave low to undetectable levels of human FIX in conditioned media of myotubes (Figure 1) and in plasma samples of Rag-1 mice following intramuscular injection ($1 \times 10^{11}$ vector genomes/mouse). Data points are mean expression levels at each time point, and vertical lines indicate standard deviation. AAV-CMV-hFIX-2: open squares; AAV-CMV-hFIX-3: open diamonds; AAV-CMV-hFIX-3: open circle; AAV-CMV-hFIX-8: open triangles; AAV-HSA-hFIX-3: closed square; AAV-cTnT-hFIX: closed triangle. (B) Systemic expression of human FIX from muscle-specific promoters (HSA or troponin) following intramuscular injection of AAV vector into Rag-1 mice ($n = 4$ for each vector, $1 \times 10^{11}$ vector genomes/mouse). Data points are mean expression levels at each time point, and vertical lines indicate standard deviation. AAV-CMV-hFIX-2: open squares; AAV-CMV-hFIX-3: open diamonds; AAV-CMV-hFIX-8: open circles; AAV-CMV-hFIX-1: closed circle; AAV-HSA-hFIX-3: closed square; AAV-cTnT-hFIX: closed triangle. (C) Effect of HSA enhancer on systemic expression of human FIX following intramuscular injection of AAV vector into Rag-1 mice ($n = 4$ for each vector, $1 \times 10^{11}$ vector genomes/mouse). Note that vector AAV-HSA-CMV-hFIX-2 (CMV-2, half-closed square) was constructed by the addition of a 1.2-kb 5′-flanking sequence of the HSA promoter 5′′ to the CMV IE enhancer/promoter in vector AAV-CMV-hFIX-2 (CMV-2, open square). The 3′-UT region of the human FIX cDNA was deleted in the vector to accommodate the additional sequence. Animals injected with AAV-CMV-hFIX-2 were identical to those shown in graph A. (D) Summary of systemic human FIX expression in Rag-1 mice from AAV vectors. Columns represent average values of human FIX expression levels for each vector for time points 6 to 12 weeks postinjection, ie, the plateau of expression (average of data points shown in graphs A-C). Vertical bars are standard deviation from the mean.

Improved expression of muscle-derived FIX by addition of skeletal actin regulatory sequences to the CMV enhancer/promoter

A 1.2-kb portion of the HSA promoter region (bp position $-1281$ to $-84^\text{17}$) was added 5′′ to the CMV enhancer/promoter in construct AAV-CMV-hFIX-2 (Table 1). All of the 3′-UT of the FIX cDNA was deleted to accommodate the additional HSA sequences. The resulting vector AAV-HSA-CMV-hFIX-2 gave an approximately 2- to 3-fold increase in levels of secreted FIX in culture media of C2C12 myotubes (Figures 1 and 3) compared with expression obtained from CMV sequences alone. In agreement with these data, a 2-fold increase in expression of systemic FIX levels over the CMV constructs was measured in Rag-1 mice following intramuscular injection of the vector ($1 \times 10^{11}$ vector genomes/mouse, $n = 4$; Figure 2C), resulting in circulation of approximately 300 ng of human FIX/mL of plasma, or 6% of normal human FIX levels. Northern blot analysis showed 5-fold higher FIX transcript levels in myotubes after transduction with AAV-HSA/CMV-hFIX-2 in comparison with our previously published vector, AAV-CMV-hFIX-3 (Figure 3). When vectors AAV-CMV-hFIX-2 and AAV-HSA/CMV-hFIX-2 were tested for transduction of HepG2 and HEK-293 cells, almost identical levels of FIX expression were observed (Figure 4), indicating that expression from the CMV promoter in nonmuscle cells is not altered by inclusion of the muscle-specific HSA element. However, results in myotubes and mouse muscle demonstrate improved expression from this vector in muscle cells. Similar to expression in C2C12 murine myotubes, expression in human myotubes was enhanced 1.7-fold by addition of the 1.2-kb HSA sequence (Figure 4). Experiments in Rag-1 mice were repeated with different vector preparations of AAV-CMV-hFIX-2, AAV-CMV-hFIX-3, and AAV-HSA/CMV-hFIX-2. AAVCMV-hFIX-2.
AAV vector. After in vitro transduction of cultured human cells (24-hour supernatants) with cells were average for 24-hour supernatants at day 4 posttransduction. Vertical lines supernatants at days 11 and 18 posttransduction; values for HEK-293 and HepG2 cells were transduced in triplicate. Values for myotubes were average for 24-hour expression cassette in a clinical trial might additionally give more insight into the use of the CMV enhancer/promoter sequence for transgene expression. Moreover, the potential use of approaches. Therefore, improvement of expression levels remains an important issue. In this investigation, we have shown that addition of a muscle-specific element of the skeletal actin promoter region to our CMV-driven expression cassette improved systemic muscle-derived F.IX expression substantially. Systemic levels of 6% of the normal human F.IX concentration were achieved with a 2- to 4-fold lower vector dose per animal than described previously with a vector containing the CMV enhancer/promoter alone. These results further underline the feasibility of muscle as a target organ for systemic delivery of F.IX or other secreted proteins and give more insight into the use of the CMV enhancer/promoter sequence for transgene expression. Moreover, the potential use of this expression cassette in a clinical trial might additionally improve safety of the protocol because therapeutic levels of expression could be reached with a lower vector dose per kilogram.

Previous results suggested an approximately linear scale-up from the Rag-1 mouse model to hemophilia B dogs on a per-weight basis as a requirement to achieve therapeutic levels of expression in a large animal model. Both vectors used in these studies for expression of human and canine F.IX used the CMV enhancer/promoter, but they differed in polyadenylation and intron sequences. To confirm our interpretation of scale-up results, it was necessary to compare both vectors side by side in vitro and in vivo in Rag-1 mice. As shown in Figures 1A and 2A, vectors AAV-CMV-hF.IX-2 and -3 indeed expressed comparable F.IX levels. Minor differences in expression (less than or equal to 30%) are within the margin of error of the viral titer. Furthermore, replacement of the SV40 polyadenylation signal with a growth hormone polyA signal had no apparent effect on expression, indicating that mRNA processing is not likely the limiting factor for expression from our initially published human F.IX cassette (in vector AAV-CMV-hF.IX-3).11,13 The addition of human F.IX 3′-UT/polyA sequences slightly reduced rather than enhanced expression. It may be that these sequences contain an element that improves expression in hepatocytes where F.IX is normally synthesized and is not recognized in other cell types such as muscle fibers.

Substitution of CMV sequences with muscle-specific promoters was not advantageous to expression of F.IX. Muscle-specific skeletal actin and cardiac troponin promoters performed poorly in transgene expression when compared with the strong viral promoter element. This result may not be surprising given the experience of others that compared other muscle-specific promoters such as those from muscle creatine kinase (mck) and myosin genes with CMV or SV40 promoters for muscle-derived expression of transgenes in the context of adenoviral and plasmid vectors.28-29 However, a comparison between our and the cited studies is not straightforward because sequences of AAV, adenoviral, or plasmid vectors might influence transgene expression in different ways.

Interestingly, our results obtained in the in vitro test system (C2C12 myotubes) were predictive of results in vivo. However, this observation might not reflect a general pattern for other enhancer/promoter combinations. For example, in work with transgenic mice, the 1-kb region between the mck enhancer and the mck basal promoter has been implicated in enhancing muscle-specific expression synergistically with the 206-bp mck enhancer. This effect on expression was missed when the 1-kb region was evaluated in vitro using murine skeletal myocytes. Thus, in vivo testing of expression cassettes remains an important component for optimization studies in gene therapy.

The CMV enhancer/promoter has been successfully used for sustained muscle-derived expression of several transgenes from AAV vectors.3,5,7,11,31,32 Expression from this promoter is clearly influenced by target tissue and vector sequences. For example, efficient shutdown of expression in liver and lung has been reported for a variety of vectors, including AAV.13,33 In muscle, CMV promoter shutdown has also been observed in the context of a retroviral vector, but it could be prevented by addition of a muscle-specific enhancer element, the mck enhancer.34 In another study, addition of multiple copies of the mck enhancer to a constitutive cellular β-actin and other promoters synergistically improved expression in the context of retroviral vectors used to transduce murine muscle cells in vitro.35 Therefore, we surmised that this observation might not reflect a general pattern for other enhancer/promoter combinations.
that transcriptional activity of the CMV promoter in muscle might be improved by addition of a strong, muscle-specific element.

The HSA promoter region up to position −2000 bp is well characterized for muscle-specific gene expression. The region of bp position −153 to +239 is described as necessary and sufficient for muscle-specificity of the HSA promoter. The promoter contains at least 4 CArG boxes. The CArG box with the consensus sequence CC(A/T)6GG, also referred to as serum response element, binds to serum response factor and is found in several muscle-specific elements. A classical orientation- and position-independent enhancer is located in the region −153 to −87 of the human α-skeletal actin gene. This enhancer synergistically increases muscle-specific expression with sequences located −1300 to −628. The latter element acts in an orientation- and position-dependent fashion. Sequences upstream of the enhancer include additional CArG boxes, and sequences 3′ to −87 include transcription enhancer factor-1 (TEF-1) and Sp1 binding sites and a TATA box. The binding site for TEF-1 is known as M-CAT element and is found, for example, in the promoters of the cTnI and the α-skeletal actin gene.

The HSA sequences in vector AAV-HSA/CMV-hFIX-2 (bp position −1281 to −84 of the HSA promoter) contain at least 4 CArG boxes, a putative Sp1 binding site, and a binding site for bifunctional YY1. This portion of the HSA gene also contains the synergistic enhancer elements discussed above (bp positions −153 to −87 and −1300 to −628 of the HSA promoter, respectively). The construct containing the HSA/CMV promoter (AAV-HSA/CMV-hFIX-2) showed enhanced expression both in vitro and in vivo in muscle cells, compared with AAV-CMV-hFIX-2. A comparison of these 2 constructs reveals 2 differences: the presence of the HSA enhancer element and the absence of the FIX 3′-UT region in AAV-HSA/CMV-hFIX-2. Theoretically, the difference in expression can result from either or both of 2 reasons. First, the addition of skeletal actin enhancer sequences enhances transcriptional activity of the CMV promoter in muscle cells or, secondly, human FIX 3′-UT sequences downregulate transcript levels in a muscle cell-specific manner. The first interpretation of the data, in our opinion, is far more likely to be true, while the second possibility remains a formal one. These data would suggest that muscle-specific upregulation of the strong CMV enhancer/promoter element by HSA enhancer sequences results in promoter activity in muscle fibers that is far superior to expression from muscle-specific promoters and higher than expression from the CMV enhancer/promoter by itself. This synergistic effect on gene expression achieved by a combination of a strong viral enhancer/promoter element with strong enhancer and enhancer-like elements specific for the target tissue of gene transfer will be useful in future design of expression cassettes.

Acknowledgments

The authors thank Dr Heiman-Patterson for human myotubes, Dr Hardeman for the HSA DNA, Dr Ordahl for the troponin DNA, and J. H. Liu and N. Chung for technical assistance.

References


Improved muscle-derived expression of human coagulation factor IX from a skeletal actin/CMV hybrid enhancer/promoter