A muscle hybrid promoter as a novel tool for gene therapy

Katarzyna Piekarowicz, Anne Bertrand, Feriel Azibani, Maud Beuvin, Laura Julien, Magdalena Machowska, Gisèle Bonne, Ryszard Rzepecki

► To cite this version:
Katarzyna Piekarowicz, Anne Bertrand, Feriel Azibani, Maud Beuvin, Laura Julien, et al.. A muscle hybrid promoter as a novel tool for gene therapy. Molecular Therapy - Methods and Clinical Development, Nature Publishing Group, 2019, 15 (8), pp.157 - 169. 10.1016/j.omtm.2019.09.001 . hal-03269927

HAL Id: hal-03269927
https://hal.archives-ouvertes.fr/hal-03269927
Submitted on 24 Jun 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives| 4.0 International License
A Muscle Hybrid Promoter as a Novel Tool for Gene Therapy

Katarzyna Piekarowicz,1 Anne T. Bertrand,2 Feriel Azibani,2,3 Maud Beuvin,2 Laura Julien,2 Magdalena Machowska,1 Gisèle Bonne,2 and Ryszard Rzepecki1

1Laboratory of Nuclear Proteins, Faculty of Biotechnology, University of Wroclaw, Wroclaw 50-383, Poland; 2Sorbonne Université, INSERM UMR974, Center of Research in Myology, Institute of Myology, Paris 75 651, France

INTRODUCTION

Gene therapy is a promising strategy to treat rare diseases. The lack of regulatory sequences ensuring specific and robust expression in skeletal and cardiac muscle is a substantial limitation of gene therapy efficiency targeting the muscle tissue. Here we describe a novel muscle hybrid (MH) promoter that is highly active in both skeletal and cardiac muscle cells. It has an easily exchangeable modular structure, including an intronic module that highly enhances the expression of the gene driven by it. In cultured myoblasts, myotubes, and cardiomyocytes, the MH promoter gives relatively stable expression as well as higher activity and protein levels than the standard CMV and desmin gene promoters or the previously developed synthetic or CKM-based promoters. Combined with AAV2/9, the MH promoter also provides a high in vivo expression level in skeletal muscle and the heart after both intramuscular and systemic delivery. It is much more efficient than the desmin-encoding gene promoter, and it maintains the same specificity. This novel promoter has potential for gene therapy in muscle cells. It can provide stable transgene expression, ensuring high levels of therapeutic protein, and limited side effects because of its specificity. This constitutes an improvement in the efficiency of genetic disease therapy.

Gene therapy is a promising strategy to treat rare genetic diseases. Muscle tissue is an important target for such therapy because of the wide range of muscle-related genetic disorders (prevalence, 20–25 per 100,000 births per year1) that can potentially be treated with gene replacement or correction. Moreover, muscle tissue is an optimal platform for the production of secretory proteins, such as clotting factors, hormones, and specific enzymes.2-6

Clinical trials have proven that muscle tissue-focused gene therapy procedures can be safe. However, in many cases, the efficiency of therapy was low, with only short-term effects.7,8 On the other hand, to obtain a satisfying therapeutic effect, the vector doses were increased, and toxicity was observed. In large animals studies (including humans), strong adverse effects were observed for adeno-associated virus (AAV) doses exceeding $5 \times 10^{13}$ vg/kg (vector genome number per kilogram), whereas, in currently ongoing trials for Duchenne muscular dystrophy (DMD), higher doses were chosen (reviewed in Duan9). Therefore, despite first optimistic results in the field of muscle gene therapy, there is still a need for vector improvements.

Many molecular mechanisms affect the efficiency of current gene therapy for muscle-based disorders. A potential solution is to enhance the efficiency and specificity of the vectors in one of three ways: with better cell recognition, with efficient entry to the desired cell, or with tissue-specific expression at sufficient levels. This should decrease the vector copy number needed to obtain a therapeutic effect and, by avoiding expression in antigen-presenting cells, also decrease the immune response to therapy.10-12 The lack of tissue-specific and sufficiently efficient promoters is a major issue.

A high expression level in muscle cells is a crucial feature for the optimal expression cassette. Although muscle tissue volume makes it hard to target efficiently, muscle cell fusion allows a bystander therapeutic effect to be achieved even when only some of the nuclei in the syncytium are modified. However, the expression of the therapeutic molecules must be high enough to correct the whole myotube. Some therapeutic effects may be observed with a low expression level (3%–5% of wild-type dystrophin expression for DMD treatment13,14), but for some disorders, or to obtain more satisfying results, a higher level is needed (for DMD treatment, an estimated 20%–30% dystrophin expression level in 50% of fibers15,16).

Previously used regulatory sequences were based on promoters derived from muscle-specific genes, such as those encoding for desmin, skeletal actin, heart α-actin, muscle creatine kinase (CKM), myosin heavy and light chains, and troponin T/I.17-22 Although these promoters show muscle-specific activity, their expression levels are not sufficient to achieve a therapeutic effect.

Strong and constitutive promoters, such as respiratory syncytial virus (RSV), cytomegalovirus (CMV), and elongation factor 1a (EF1a), can...
be used to reach high expression levels, but their application is limited because of their activity in non-muscle tissues. Additionally, viral promoters are quickly silenced in transduced cells, leading to a short period of expression.  

Previously developed synthetic, modified, or hybrid promoters such as C5-12, 24 CK6–CK9, 25,26 and mCK2 27 show moderate to high expression levels, similar to the CMV or RSV promoters, but suffer from being active in other tissues (liver, kidney) or having limited activity in the heart.

A regulatory sequence that ensures stable, high-level expression in skeletal muscle and the heart and limited activity in other cell types is needed. Therefore, we have undertaken the challenge to design and build a highly active, muscle-specific promoter for efficient gene expression, targeting muscle tissue. Our goal was to design a promoter that would fit into a wide variety of vectors and strategies that are potentially useful for gene therapy. For reference, we used CMV and the desmin promoter (DES), most commonly used in past and ongoing preclinical and clinical trials.

Here, we report the development of our modular muscle hybrid (MH) promoter, which shows high activity in myoblasts, myotubes, and cardiomyocytes and low activity in non-muscle cell types in vivo. Furthermore, when combined with AAV2/9 delivery, MH promoter-driven expression ensures a specific and high level of expression in skeletal muscle and the heart in vivo.

RESULTS
Development of the MH Promoter
We hypothesized that, when all the necessary functional elements are preserved, a combination of sequences efficiently clustering muscle-specific transcription factors (TFs) should give a highly active transcriptional regulatory sequence. Various muscle-specific murine genes were analyzed in silico, and 4 clusters obtained from the desmin-encoding gene (Des) and CKM-encoding gene (Ckm) were chosen as functional modules for the hybrid promoter.

An enhancer of the DES promoter has been reported previously from −976 to −798 bp upstream of the transcription start site (TSS). 38 Based on in silico analysis of the Des gene, a 144-bp sequence from −970 to −826 bp relative to the TSS (Figure 1A) was chosen as the first module of the hybrid promoter and called enhancer 1 (enh1). It was predicted to bind TFs such as MyoD, Myf6, Sp1 and SRF. The presence of binding sequences for MyoD, MEF2, E12 (E2A gene), and myogenin in this region had been experimentally confirmed previously. 29

Other modules were obtained from the Ckm gene that encodes one of the most abundant mRNAs in skeletal muscle. 30 It has 3 independent clusters binding TFs, corresponding to the enhancer, core promoter, and first intron. The Ckm enhancer has been reported previously to be located from −1,256 to −1,051 bp relative to the TSS. 31 Based on the in silico analysis of the Ckm gene, a 202-bp sequence from −1,060 bp upstream of the TSS (Figure 1B) was chosen as the second module of a hybrid promoter and called enhancer 2 (enh2). It was predicted to bind TFs such as MyoD, Myf6, SRF, Sp1, USF1, and E47 (E2A gene).

The core promoter of the Ckm gene was mapped to a sequence from −358 to +7 bp relative to the TSS (Figure 1B). 32 This sequence was predicted to bind TFs such as MyoD, USF, Sp1, and SRF. Moreover, a sequence around −80 bp, called MPEX (MCK Promoter Element X), was confirmed to be necessary for expression in skeletal and cardiac myocytes. 33 The core promoter was further modified to introduce an initiator (Inr) sequence, AGCTTC(+1)A, to create a third module called the proximal promoter (pp).

To increase mRNA stability, a minimal intron was inserted into our construct as the fourth module. It also contains a conservative sequence from the first intron of the Ckm gene: the small intronic enhancer (SIE), localized from +901 to 995 bp relative to the TSS, which able to bind TFs such as MyoD, MEF2, and AP-1. 34 SIE is flanked by a splicing donor site, branching and acceptor sites, and the 5′ UTR from the Ckm gene, which is responsible for mRNA binding to ribosomes.

Each module was flanked by short sequences that introduce restriction sites to make exchange easily, allowing further modifications such as module inversion, deletion, or multiplication. In summary, our MH promoter sequence consists of 4 joint modules (Figure 1C) and is predicted to bind a cluster of muscle-specific TFs with high probability (Figure 1D).

Analysis of MH Promoter Activity in Different Cell Culture Models
The MH promoter activity was first analyzed via transient transfection of various striated muscle cell lines (H9C2 and C2C12) with comparison of the results for transfection with the DES, CMV, and EF1α gene promoters.

MH promoter activity was, respectively, 60- and 3-fold higher than that of the DES and CMV promoters in proliferating C2C12 myoblasts (Figure 2A). Differentiating C2C12 cells showed increases in the expression levels driven by the MH and DES promoters and decreases in those driven by the CMV promoter. In C2C12 myotubes, the MH promoter was, respectively, 8 and 67 times more active than the DES and CMV promoters (Figure 2B). The MH promoter was also more active in H9C2 cardiomyocytes, showing, respectively, 66-fold and 2-fold more activity than the DES and CMV promoters (Figure 2C).

The highest activity was observed for the EF1α gene promoter, which is a strong constitutive promoter. Because it was several fold stronger than the other promoters in all analyzed cell types (a comparison with the MH promoter is shown in Figure S1), its strong signal disrupted the comparison. It provided too high of an expression level in non-muscle cells for use in therapy, so it was not considered for further experiments. CMV was chosen as the main reference promoter.
because of the larger amount of available clinical data that support promoter comparison.

To determine the contribution of the different modules of the MH promoter to MH activity, a set of constructs lacking single or multiple modules was prepared, and their activity was measured in myoblasts, myotubes, cardiomyocytes, HEK293 cells, and hepatocellular carcinoma (HepG2) cells. The sequences removed were (Figure 3A) (1) the \textit{Des} gene-based enhancer (enh1); (2) the \textit{Ckm} gene-based enhancer (enh2); (3) both the \textit{Des} and \textit{Ckm} enhancers (2enh); (4) the core promoter without the TATA box (pp); (5) the core promoter with the TATA box (TATA); and (6) the minimal intron (intron).

All designed elements were proven to influence muscle-specific expression. The removal of any module decreased MH promoter activity by at least half in muscle cells but not in HEK293 or HepG2 cells. Interestingly, the module with the most significant effect was the intron (Figure 3). Its removal leads to a decrease in MH promoter activity in all cell types, with the greatest reduction in muscle cells, where a 240-fold and 340-fold decrease were observed in myoblasts (Figure 3B) and myotubes (Figure 3C), respectively. The MH

**Figure 1. The MH Promoter Is Composed of 4 Main Modules that Together Are Predicted to Efficiently Bind Transcription Factors**

(A) Analysis of the \textit{Des} gene (encoding desmin) promoter, from 2,500 bp before the TSS to the first intron inclusive, revealed predicted cluster-binding TFs (enhancer) in positions \(-970\) bp to \(-826\) bp according to the TSS and the second cluster in the final part of intron 1. (B) Analysis of the \textit{Ckm} promoter from 2,500 bp before the TSS to the start codon (including the first intron) revealed 3 predicted main cluster-binding TFs. The strongest cluster corresponded to an enhancer identified from \(-1,256\) bp to \(-1,050\) bp, the second one was identified within intron 1, and the third cluster corresponded to the core promoter (from \(-358\) bp to \(+7\) bp). (C) Functional elements of the promoter were chosen using \textit{in silico} analysis and then optimized and combined to give a modular structure. The MH promoter is composed of the following linked modules: (1) the \textit{Des} gene enhancer (enh1); (2) the \textit{Ckm} gene enhancer (enh2); (3) the \textit{Ckm} gene core promoter (with modifications within the proximal promoter [pp]); and (4) a designed intron consisting of a SIE derived from the \textit{Ckm} gene. Moreover, the 5' UTR derived from the \textit{Ckm} gene, including potential sequences enhancing translation, was inserted after the designed intron. Around the TSS, the following sequences occur: TATA box, Inr, and DPE. (D) Analysis of the MH promoter revealed predicted, very strong cluster-binding TFs localized through almost the entire 1,030-bp sequence. (A), (B), and (D) were prepared using Cister software.
promoter without its intron had activity comparable with that of the Des gene promoter. The intron module can influence expression by enhancing expression and by increasing the stability of mRNA. There was no observed significant influence of the TATA box sequence. Although many promoters lack a TATA box and can nevertheless form a transcription initiation complex, it was still expected to increase expression efficiency. However, the two MH promoter variants lacking the core promoter (MH-TATA and MH-pp) had comparably decreased activity (4-fold in myoblasts and 6-fold in myotubes) regardless of the presence or absence of the TATA box. This suggests that, in the presence of Inr and downstream promoter element (DPE) sequences, the TATA box was irrelevant for promoter strength. Moreover, Inr or DPE sequences can substitute a full-length core promoter and play the role of a minimal promoter together with enhancers (Figure 3).

The removal of enh1 lowered the expression level in myotubes more than the removal of enh2 (to 16% versus 23% of MH). The opposite effect was observed in myoblasts and cardiomyocytes, where removal of enh2 reduced the expression level more (by 5%–7% in comparison with enh1 removal). However, only the removal of both enhancers significantly decreased MH promoter activity in the C2C12 and H9C2 cell lines (Figure 3).

Figure 2. The MH Promoter Is Highly Active in Muscle Cells
(A–C) Proliferating C2C12 cells (A), C2C12 cells differentiated to myotubes (B), and H9C2 cells (C) were transfected with constructs encoding secretory luciferase under the control of different promoters. Luciferase and enzyme activity was measured based on luminescence 48 h after transfection. The MH promoter was more active than the DES or CMV promoters but less active than the constitutive promoter of EF1a. During C2C12 differentiation, the activity of the DES promoter increased, whereas the activity of the CMV promoter decreased. However, the activity of the MH promoter was higher than that of these two promoters. 4 biological replicates were performed, with 4 technical replicates each. Boxes represent 25/75 percentiles, with the median value and whiskers representing minimum and maximum values. RLU, relative luminescence units normalized to transfection efficiency and total protein amount. Values are presented using a log10 scale.

Analysis of MH Promoter Activity and Specificity

There was no observed significant influence of the TATA box sequence. Although many promoters lack a TATA box and can nevertheless form a transcription initiation complex, it was still expected to increase expression efficiency. However, the two MH promoter variants lacking the core promoter (MH-TATA and MH-pp) had comparably decreased activity (4-fold in myoblasts and 6-fold in myotubes) regardless of the presence or absence of the TATA box. This suggests that, in the presence of Inr and downstream promoter element (DPE) sequences, the TATA box was irrelevant for promoter strength. Moreover, Inr or DPE sequences can substitute a full-length core promoter and play the role of a minimal promoter together with enhancers (Figure 3).

The removal of enh1 lowered the expression level in myotubes more than the removal of enh2 (to 16% versus 23% of MH). The opposite effect was observed in myoblasts and cardiomyocytes, where removal of enh2 reduced the expression level more (by 5%–7% in comparison with enh1 removal). However, only the removal of both enhancers significantly decreased MH promoter activity in the C2C12 and H9C2 cell lines (Figure 3).

Analysis of MH Promoter Specificity and Kinetics

The activities of the MH, CMV, and DES promoters were compared in a set of muscle and non-muscle cell lines 2 days post-transfection. In each cell line, the activity of the DES promoter was the lowest (data not shown). In myoblasts, myotubes, and cardiomyocytes, the MH promoter was more active than the CMV promoter. The opposite was observed in non-muscle cell lines such as HEK293, HepG2 and HeLa cells; NHDFs (normal human dermal fibroblasts); and HDMECs (human dermal microvascular endothelial cells). This shows the muscle specificity of the MH promoter in vitro (Figure 4A).

Measurement of the reporter protein for several days following C2C12 transfection allowed comparison of the promoter activity kinetics in myoblasts (Figure 4B). During the first 24 h, the highest level of luciferase production was observed for the CMV construct. Only slightly lower efficiency was observed for the MH promoter. The DES promoter reached only a small fraction of the maximal value during the first 24 h.

However, in the following days, the expression level driven by the CMV promoter progressively decreased, probably because of promoter silencing or faster transcript turnover. For the DES, MH, and MH variant promoters, the luciferase levels reached a plateau 2 days after transfection before a slight decrease on the fourth day. From day 2 post-transfection, luciferase activity was higher with the MH than with the CMV promoter. Similar trends were observed for HEK293 and H9C2 cells (Figure S2).

Analysis of MH Promoter Activity and Specificity In Vivo

The MH promoter activity was also analyzed in vivo. AAV2/9 vectors encoding EGFP under control of the MH, CMV, or Des gene promoter were prepared. Wild-type newborn mice were injected intramuscularly in the tibialis anterior (TA) muscle. The newborn mice were used for the widest vector spreading to analyze the promoter’s activity in different tissues. After 6 weeks, the TA, quadriceps, and gastrocnemius muscles of the injected leg and the heart, lungs, and liver were harvested and analyzed using light or fluorescence microscopy and qPCR.

No significant muscle damage or fibrosis was observed with any of the constructs (Figure S3). The distribution of EGFP in the muscle fibers...
was homogeneous for all promoters, both in the skeletal muscles (Figure 5C) and the heart (Figure S4).

Although we observed variability in the Egfp mRNA levels among the various mice, the mean levels of Egfp mRNA were always higher for the CMV promoter in all analyzed skeletal and heart muscles and lower in other tissues compared with the CMV promoter. In the heart, Egfp mRNA expression was 1.8 times higher with the MH promoter than with the CMV promoter and 11 times higher than with DES promoter. In skeletal muscle, the MH promoter was 1.2- to 3-fold more active than the CMV promoter and 70- to 160-fold more active than DES promoter (depending on muscle type; Figure 5A). Similar results were obtained for the EGFP fluorescence signal in muscle tissues, with the highest levels found for the MH promoter, intermediate levels for the CMV promoter, and the lowest levels for the DES promoter (Figure 5C).

Because the expression levels differed between mice, an additional quantification of vector copy number was performed for the gastrocnemius muscle for data normalization (Figure 5B). The analysis showed that the Egfp transcript level for the MH promoter was 2.7 times higher than for the CMV promoter and 150 times higher than for the DES promoter.

In the liver, the MH promoter showed 4-fold higher expression than the DES promoter but 2-fold lower expression than the CMV promoter. In the lungs, the expression with the MH promoter was comparable with that with the DES promoter and 6-fold lower than with the CMV promoter (Figure 5A).

To compare promoters in more detail, systemic injections were performed at $10^{11}$ vg/mouse. The mice were euthanized after 8 weeks. To investigate promoter specificity, additional non-muscular tissues were collected: liver, lung, brain, kidney and small intestine. In this experimental setup, in the heart, MH promoter-driven Egfp expression was comparable with that obtained with the CMV promoter and 3-fold higher than that obtained with DES promoter (Figure 6A). However, the protein level was higher for the MH promoter than for...
At the protein level, EGFP was only detectable in liver, with the highest level observed for the CMV promoter (Figures 6C and 6D). These results suggest that the MH cassette contains elements that enhance transcript stability or translation specifically in muscle tissue.

**DISCUSSION**

In this study, we designed a novel modular muscle-specific promoter and demonstrated its high activity in muscle cells and low activity in other cell types. Attempts to obtain such muscle-specific promoters based on optimal combinations of binding sites for muscle-specific TFs have already been made. For example, Li et al.37 based their attempt on the synthetic C5-12 promoter. Despite optimistic initial results in vitro, further studies performed in vivo showed relatively low activity in combination with genetic drugs; i.e., 2-fold lower than with the CMV promoter.34

The importance of specific TFs to enhance gene expression in muscle tissue is still under investigation.33,38–40 Therefore, we decided to use another strategy based on the combination and optimization of functional gene elements. We also decided to avoid viral genome-derived sequences, which have been proven to be prone to methylation and silencing.41,42 Our approach successfully led to development of the MH promoter, which drives gene expression at a high level only in striated muscle both in vitro and in vivo.

Our MH promoter is composed of enhancers, a core promoter, and an intron that increases mRNA stability and ensures pre-mRNA splicing. It possesses a modular structure that allows further modifications, including size reduction if necessary. However, the intron module must be preserved. It has been shown that the presence of an intronic sequence enhances gene expression and translation efficiency33–45 and improves the therapeutic effect of a vector.46 In this study, we used a novel approach that combines the SIE sequence identified in intron 1 of the Ckm gene, which may itself act as an enhancer,34 with splice sites (donor, branching, and acceptor) to develop an artificial intron. The designed intron of the MH promoter emerged as the crucial element because its removal greatly decreased MH promoter activity to a level similar to that obtained with the DES promoter (Figure 3).

The MH promoter has been shown to be highly active in muscle cells in vitro (myoblasts, myotubes, and cardiomyocytes; Figure 2) with limited activity in non-muscular cells, in comparison with the CMV (Figure 4A), suggesting high muscle specificity. Additionally, it seems that enh1 enhances an expression level in myotubes and enh2 in myoblasts and cardiomyocytes that may be used in disease-specific vector optimization. When combined with the AAV2/9
carrier for *in vivo* delivery, the MH promoter also showed the same muscle-specific activity (Figures 5 and 6).

We observed some differences between expression levels obtained after intramuscular and intravenous delivery. This may arise from varied vector distribution, time, and dose resulting in a different immune response, potential promoter silencing, or viral DNA stability. It was observed that a decrease in CMV promoter-driven expression is greater after intramuscular injection, so for intravenous injection, the difference between MH and CMV promoter expression might be greater for a longer analysis time. However, both experimental setups lead to the conclusion that the MH promoter is a better choice for the expression in muscle tissue.

Our data also suggest muscle-specific enhancement of protein production *in vivo*. After systemic injection, we observed similar transcript levels in the heart for the MH and CMV promoters but a higher protein level for the MH promoter. In skeletal muscle, lower transcript levels were obtained for the MH promoter than for the CMV promoter, but comparable or higher protein levels were given by the MH promoter. The opposite effect was observed in the liver, where the slightly lower transcript levels observed for the MH promoter led to an 8-fold lower protein level than with the CMV promoter (Figure 6). This may result from muscle-specific enhancement of translation provided by the MH promoter’s 5’ UTR region, which has a ribosome-binding sequence derived from the *Ckm* gene.

The previously described combinations of various muscle-specific promoters with different virus vectors were not active or specific enough in muscle cells. For instance, a CK6 cassette based on the *Ckm* gene promoter showed only 12% activity of the CMV promoter in skeletal muscle. Its variants, CK7, CK8, and CK9, and even its strongest variant, MHCK7, gave efficiency comparable with the CMV or RSV promoters *in vitro*. However, after systemic injection of AAV6, the reporter protein level obtained with the MHCK7 cassette was comparable with that obtained with CMV or RSV promoters in skeletal and heart muscles but also in the liver, spleen, and lung. Although CK8, MHCK7, or minimal *CKM* promoters are currently used in DMD gene therapy clinical trials, the necessary AAV vector...
doses are high (at least $5 \times 10^{13}$ vg/kg) and side effects are observed, even resulting in temporary hold of one trial. Our MH promoter seems to be similarly active in the skeletal muscles as the CMV promoter but more active in the heart and less active in non-muscle tissues. Moreover, using our MH promoter, the protein level in muscle tissue was highest.

Another approach aimed to reduce $CKM$ promoter length while increasing its activity by addition of 2 (dMCK) or 3 (tMCK) short $CKM$ enhancers to the promoter. These modified promoters showed lower activity than the CMV promoter in undifferentiated C2C12 cells and comparable activity in differentiated C2C12 cells. In vivo analysis initially confirmed high activity of dMCK and dMCK in TA muscle 1 month after AAV injection of 2-month-old mice, but the promoter's specificity was not confirmed. Further in vivo analyses were made in transgenic mice expressing $LacZ$ under control of the dMCK promoter. These mice showed a high expression level in skeletal muscles, with strong preference for fast-twitch muscles.

**Figure 6. Systemic Delivery of the AAV with the MH Promoter Provides a High Level of Expression in Muscles and a Low Level of Expression in Other Analyzed Tissues**

(A–D) Newborn wild-type mice were injected systemically via the temporal vein with AAV2/9 vectors encoding EGFP under the control of the CMV, MH, or DES promoters. Tissues were analyzed 8 weeks thereafter via qPCR (A and B) and western blotting (C and D). The MH promoter provided high expression and protein levels in muscle tissues, comparable with those for the CMV promoter, and a lower level in other tested tissues, comparable with that for the DES promoter. (A) qPCR analysis of muscle tissues showed comparable expression levels driven by the CMV and MH promoters in the heart and slightly higher levels for CMV than MH in skeletal muscle. (B) qPCR analysis of selected control non-muscle tissues showed the highest expression levels for the CMV promoter. The MH and DES promoters showed comparable expression levels in these tissues. In (A) and (B), each point represents the mean expression level (4 technical replicates) in that particular tissue for each mouse ($n = 5$). The horizontal line represents the mean value ($^*p < 0.01$, $^{**}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$). Values are presented using a log$_{10}$ scale. (C) Western blot analysis of the heart, quadriceps and gastrocnemius muscles, and liver were performed. For the DES promoter, three mice with the highest expression level quantified with qPCR were analyzed, but EGFP was detectable only in the heart. In the heart, the highest protein level was observed for the MH promoter. In skeletal muscle, the EGFP protein levels were slightly higher for the CMV than for the CMV promoter. In the liver, the highest protein level was observed for the CMV promoter, with a barely detectable level for the MH promoter. For the lungs, brain, small intestine, and kidneys, EGFP detection signals were low for all promoters (data not shown). (D) Densitometry analysis of the western blot staining presented in (C). Bars represent the mean EGFP band intensity normalized to loading control bands. Whiskers represent SDs.
In another study, a mouse model of limb-girdle muscular dystrophy 2D was injected with AAV1 encoding the α-sarcoglycan gene under control of the TMCK or CKM promoters. Both artificial promoters led to similar results. Moreover, after intramuscular injection of lentiviral vectors directing GFP expression using DES, CKM, or CMV promoters to newborn mice, the lowest expression level was given by the CKM gene promoter in the injected muscle and the highest in the liver and spleen. Therefore, these CKM-based promoters were also not sufficient for gene therapy.

Based on the troponin T-encoding gene, the ΔUSEx3 promoter was developed. It showed high specificity but low activity in muscle tissue after intramuscular delivery with a helper-dependent adenoviral vector compared with a CMV enhancer/β-actin promoter. Moreover, its activity was 5-fold lower than that of the SPcΔ5-12 promoter, which is a weaker version of artificial the C5-12 promoter.

Altogether, this suggests that our novel MH promoter, which is severalfold more active in both skeletal and heart muscle than the DES promoter, is the most promising alternative to the previously investigated DES-, CKM-, or troponin T-based promoters for gene therapy approaches, especially when expression in the heart is also necessary.

Promoters based on different heart-specific genes also did not show more efficient gene expression in striated muscles. After systemic injection of a lentiviral vector, comparable activity of MLC-2v-, cardiac troponin T-, and αMHC (Myosin heavy chain, alpha isoform)-encoding gene promoters was observed. The most specific one was the αMHC-encoding gene promoter, whereas the slightly more efficient cardiac troponin T gene promoter gave a reporter protein expression level in the liver, lung, and spleen comparable with that of the EF1α promoter. Another study demonstrated about 10-fold higher activity of the DES gene promoter than the αMHC-encoding gene promoter in the heart after systemic delivery of AAV2/9 to newborn mice, but this was still lower than for the CMV promoter. Compared with all of these studies, our results show that the MH promoter could also be a cassette of choice when a high expression level is needed in the heart.

In gene therapy, the long-term expression level has a major effect on the success of the treatment. Therefore, we analyzed the activity kinetics of the MH promoter and its variants in a cell culture model system. Compared with the CMV promoter, the expression given by the MH or DES gene promoters started later, but it increased with time, whereas CMV promoter-driven expression decreased after reaching a peak at 24 h. This is probably because of faster transcript turnover, promoter silencing, or a different promoter’s effect on chromatin structure (Figure 4B). Another key aspect of successful gene therapy is its safety. The MH promoter with AAV2/9 did not show any signs of toxicity, even for higher doses (2.5 × 10^{11} vg for newborn mice, ~2.5 × 10^{13} vg/kg; Figure S3).

The MH promoter may be used in in vitro experimental systems, when a stable and high level is needed in muscle cells, and in further gene therapy such as protein overexpression in cases of genetic deficiency, such as in DMD or Emery-Dreifuss muscular dystrophy, for gene silencing using microRNA, or for gene correction by, e.g., homologous recombination or the CRISPR/Cas system. It can improve the efficiency of therapy in comparison with the combination of promoters and viral vectors mentioned above and the standard promoters used previously.

**MATERIALS AND METHODS**

**Cells**

The HEK293, murine myoblast C2C12, and rat cardiomyocyte H9C2 cell lines were cultured in DMEM with high glucose (4.5 g/L glucose; Lonza). HeLa cervix carcinoma cells and NHDF cells were cultured in minimum essential medium with alpha modification (MEMa; Lonza). Hepatocellular carcinoma HepG2 cells were cultured in Eagle’s minimum essential medium (EMEM; Lonza). HDMECs were cultured in endothelial cell growth medium (EGM-2, Lonza). All media were supplemented with 10% FBS (Sigma), GlutaMAX supplement, antibiotics, and antifungics (Gibco).

The HEK293, HepG2, and HeLa cell lines were purchased from the Cell Line Collection of the Polish Academy of Sciences, which was available through the Institute of Immunology and Experimental Therapy in Wroclaw, Poland. The NHDF cell line was purchased from Lonza. The C2C12 cell line was obtained from the Cell Pathology Department of the University of Wroclaw. The H9C2 cell line was obtained from the Department of Medical Biochemistry of Wroclaw Medical University. The HDMEC cell line was purchased from PromoCell.

To obtain differentiated myotubes, C2C12 cells were seeded to reach 70%–80% confluence, and then a differentiation medium containing 2% horse serum (Sigma) instead of 10% FBS was applied and changed every 2–3 days. Myotubes were analyzed 6–7 days later (Figure S5).

**Mice**

All mouse procedures were performed according to protocols conforming to French laws and regulations concerning the use of animals for research and were approved by an external ethical committee (Ethical agreement 00971.02, French Ministry for High School and Research). Intramuscular injections of 2.5 × 10^{11} vg AAV2/9 in 30 μL PBS were performed into the TA muscle of 2- to 4-day-old wild-type mice (C57BL/6, n = 3). These mice were euthanized 6 weeks post-injection. Systemic injections of 10^{11} vg AAV2/9 into the temporal vein of 2- to 3-day-old wild-type mice (n = 5) were performed. Control mice were injected with PBS only. These mice were euthanized 8 weeks post-injection. Tissue sampling and processing were done post-mortem as described before.

**Plasmids for In Vitro Transfection**

The plasmid pDRIVESLucia-mDesmin encoding secretory luciferase under the control of a mouse desmin-encoding gene promoter (DES-Luc) was purchased from InvivoGen. Further plasmids were prepared by exchanging the mDesmin promoter with a promoter of choice using molecular biology methods described below. To obtain the
MH-Luc plasmid, the sequence encoding the MH promoter was synthesized (Gene Cust) and cloned into the pDRIVE backbone vector digested with the NotI and NheI restriction enzymes (Thermo Fisher Scientific). The CMV promoter was cloned from the pEGFP-C1 plasmid (Clontech) using primers to introduce MluI (5’-AGCGA CGGTATTTATGATCAATTAC-3’) and BamHI (5’-AAT GAGATCCGACCCGGTTACGCTAG-3’) restriction sites (CMV- Luc). The EF1a promoter was cloned from the pWPI plasmid (Addgene) using primers to introduce MluI (5’-TTAACCGGTT GATGGACACGCCATG-3’) and BamHI (5’-TATGGATCTCC AGCA CACCTGAATG-3’) restriction sites (EF1a-Luc).

The variants of the MH promoter were prepared by digesting the MH-Luc plasmid with selected restriction enzymes (Thermo Fisher Scientific) followed by ligation (T4 ligase; Thermo Fisher Scientific). The restriction enzymes were Pmll, Pmel, KpnI, and Spei together with XbaI and BclI. All constructs were verified by sequencing with the primers 5’-TTAAGGGATTTTGGTCATGG-3’ and 5’-CACTGAGATGTGATACTTGTG-3’. Plasmids were amplified with E. coli strain DH5a with the selection antibiotic zeocin (100 μg/mL; InvivoGen) and purified with an endotoxin removal step (midiprep K0481, Thermo Fisher Scientific).

**Plasmids for AAV Production**

AAV2/9 constructs were produced based on pSMD2 plasmids encoding EGFP under the control of the promoters CMV, MH, or the desmin-encoding gene. EGFP was cloned from the pEGFP-C1 plasmid using XhoI and KpnI restriction sites. The MH promoter and desmin-encoding gene promoter were cloned from the pDRIVE plasmid and inserted in place of the CMV promoter in pSMD2-CMV:EGFP cut either with HincII and NheI or HincII and NcoI. All constructs were verified via sequencing with the primers 5’-CACATTGCA TACGTTGATCC-3’ and 5’-CGAGCTTAGTGATACTTGTG-3’. Plasmids were amplified with E. coli strain DH5a with the selection antibiotic ampicillin (100 μg/mL; InvivoGen) and purified with a Maxi-prep kit (740410.10, Macherey-Nagel). AAV2/9 production was done in the MyoVector platform of the Center of Research in Myology (SU-INSERM U974, Paris, France). The viruses were produced by the transfected HEK293FT cells (with packaging plasmids encoding rep2 and cap9), collected from the medium, purified via ultracentrifugation, and quantified using qPCR.

**Transient Transfection and Luminescence Measurement**

Cells were plated on a 96-well plate 24 h before transfection with the aim of obtaining 70% confluence prior to transfection. Cells were transfected using Turbofect reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions as described before.56 For each construct, one transfection mix was prepared for all transfected cell types. The medium was replaced with DNA-Turbofect complexes diluted in ULTRA-MEM (Lonza) supplemented with 2% FBS for 6 h, with 200 ng plasmid and 0.4 μL Turbofect in 100 μL medium per well. Then the medium was replaced with standard medium with 10% FBS. For myotube analysis, C2C12 cells were transfected and then differentiated.

Luciferase activity was measured in the medium prior to its collection, usually 48 h after transfection. 50 μL of substrate (QuantiLuc, InvivoGen) was automatically added to 10 μL of medium and incubated for 4 s. The light emission was measured for 100 ms with a Varioskan Flash instrument (Thermo Fisher Scientific). For each construct, there were 4 technical replicates in at least 2 independent experiments. For kinetic measurements, the medium from cells transfected with pDRIVE plasmids was collected and replaced every 24 h.

After collection of 10 μL medium for luminescence measurement, the cells transfected with pDRIVE plasmids were fixed with 10% trichloroacetic acid for 1 h at 4°C, and the relative amount of protein was measured using the sulphorhodamine B test. The transfection efficiency and total protein amount were measured for normalization of luminescence quantification between the experimental series and the cell lines.

In additional wells, each cell line was transfected with the pEGFP-C1 plasmid only to calculate the transfection efficacy in each series after 48 h. The percentage of EGFP-positive cells was calculated using flow cytometry (BD FACSCalibur). The threshold was set using non-transfected control cells. The following average transfection efficiencies were obtained: 50% for C2C12, 55% for H9C2, 65% for HEK293, 25% for HepG2, 15% for NHDF, 80% for HeLa, and 10% for HDMEC cells.

**qPCR**

Murine tissues were weighed and fragmented with the MP Biomedicals Fast Prep system using Lysing Matrix D as described previously.57 Total RNA was isolated with an RNeasy Fibrous Tissue Mini Kit (QIAGEN) with a DNase digestion step and reverse-transcribed using Maxima Reverse Transcriptase (Thermo Fisher Scientific). The mRNA levels of Efgp and Rlplp0 were measured using specific primers (Efgp, 5’-GAGCTAAAAGGCCAAGGT-3’ and 5’-AAGTCTTG CTGCTTTGTATG-3’; Rlplp0, 5’-AACCCTCCTCCTCACCAGCTT-3’ and 5’-CCCACTTGTCCAACTCC-3’). Real-time qPCR was performed with 50 ng of cDNA using a LightCycler 480 SYBR Green I Master (Roche) with 0.3 μM of each primer in triplicate. The relative expression of Efgp to Rlplp0 was calculated using LightCycler 480 software.

Total DNA (with episcopal DNA) was isolated using a Gentra Puregene kit (QIAGEN), and the viral copy number per cell nucleus was quantified using TaqMan probes and primers for the vector ITR (inverted terminal repeat) sequence and endogenous titin-encoding gene. Absolute quantification was performed with a StepOne real-time PCR system (Thermo Fisher Scientific) relative to the standard curve of a plasmid encoding both ampiclons.

**Electrophoresis and Western Blot Analysis**

Snap-frozen tissue samples were weighed and extracted in RIPA buffer with protease inhibitors (100 μL/10 mg; Cell Signaling Technology) with the MP Biomedicals FastPrep system using Lysing Matrix D. Protein extracts were centrifuged at 14,000 × g for 10 min at
were analyzed from 5,000 bp before the TSS to the end of the intron.

The proteins were visualized using ECL substrate (Bio-Rad) and analyzed with ImageLab (Bio-Rad). The primary antibodies were rabbit anti-EGFP (sc-8334, 1:1,000; Santa Cruz Biotechnology), rabbit anti-human z-tubulin 4a (PA5-29444, 1:5,000; Thermo Fisher Scientific) and rabbit anti-mouse GAPDH (MA5-15738, 1:8,000, Sigma). The secondary antibodies were goat anti-rabbit HRP (111-035-144, 1:10,000; Jackson ImmunoResearch).

Cryosections, Histochemistry, Immunohistochemistry, and Microscopy Imaging

10-µm sections were made using a cryostat (Leica CM3050S). Frozen sections were stained with H&E or Sirius Red using standard methods and visualized under light microscopy for nucleus position, vascularization, and the presence of necrosis or fibrosis.

For fluorescence imaging, sections were fixed with 4% paraformaldehyde (PFA), mounted with Vectashield containing DAPI (Vector Laboratories), and visualized under a Zeiss 510 Meta confocal microscope for analysis of EGFP localization and level.

Bioinformatics Analysis

The selected promoters were analyzed using Cister (Gis-element Cluster Finder), Match 1.0 (TRANSFAC matrix, BIOBASE), Alibaba 2.1 (http://gene-regulation.com/), and ClusterBuster. Genes were analyzed from 5,000 bp before the TSS to the end of the first intron.

Statistical Analyses

Statistical analysis was performed with GraphPad Prism software. For the luciferase analyses, the measurement system was characterized by high repeatability and linearity (Figure S6). For in vitro tests, the activity of each construct was measured with at least 3 independent experiments, 4 technical replicates each. For in vivo tests, 3–5 mice were injected for each vector. Mean values with SD were calculated and are presented on graphs. To calculate statistical significance, Mann-Whitney test, Kruskal-Wallis test with two-sided Dunn’s test for multiple comparisons, or ANOVA test for related measures with the Greenhouse-Geisser correction with Tukey’s test for multiple comparisons were used, depending on particular experimental data distribution.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2019.09.001.

AUTHOR CONTRIBUTIONS

K.P. and R.R. designed the experiments. K.P. performed the bioinformatics analysis and designed the MH promoter under supervision of R.R. K.P. performed the following experiments: preparation of all plasmids, cell transfection and luciferase activity analysis, sample preparation and analysis of mouse tissues using fluorescence microscopy, WB, and qPCR. L.J. performed AAV production. A.T.B. and F.A. performed mouse injections, sacrifice, and tissue collection. M.B. prepared cryosections and IHC staining. K.P., M.M., and R.R. performed data analysis. K.P. performed statistical analysis and data visualization. K.P. wrote the original draft of the manuscript. K.P., A.T.B., M.M., G.B., and R.R. reviewed and edited the manuscript. G.B. and A.T.B. supervised experiments in vivo. K.P. and R.R. secured financial support. R.R. initiated and supervised the project and manuscript preparation.

CONFLICTS OF INTEREST

K.P. and R.R. are authors of a patent (PL415205) concerning the MH promoter.

ACKNOWLEDGMENTS

We would like to thank the Penn Vector Core (Gene Therapy Program, University of Pennsylvania, Philadelphia) for providing the pAAV2/9 plasmid (pSE18-VD29) and Sofia Benkelhafa-Ziyat from the MyoVector platform of the Centre of Research in Myology-UMRS974 (Paris, France) for AAV production, Cécile Peccatte for assistance with AAV quantification and kindly providing the qPCR probes, Ludovic Arandel for assistance with mouse systemic injections, and Yosef Gruenbaum for helpful comments. This study was supported by Wroclaw Research Center EIT+ (POIG.01.01.02-00-003/08) from Regional Development Fund; COST Actions: BM1002 Nanonet: Nanomechanics of Intermediate Filament Network and CA15214 EuroCellNet: An Integrative Action for Multidisciplinary Studies on Cellular Structural Networks; KP was funded by: National Science Center grant ETIUDA: UMO-2014/12/T/0Z3/00504. The preparation and publication of the manuscript were supported by a KNOW grant for the National Leading Scientific Center for Biotechnology from the Polish Ministry of Science and a grant for research for 2019 from the Polish Ministry of Science.

REFERENCES

1. Theudom, A., Rodrigues, M., Bozborough, R., Balalla, S., Higgins, C., Bhattacharjee, R., Jones, K., Krishnamurthi, R., and Fregia, V. (2014). Prevalence of muscular dystrophies: a systematic literature review. Neuroepidemiology 43, 259–268. 2. Chuah, M.K., Collen, D., and VandenDriessche, T. (2001). Gene therapy for hemophilia. J. Gene Med. 3, 3–20.

3. Bohi, D., and Heard, J.M. (2000). Delivering erythropoietin through genetically engineered cells. J. Am. Soc. Nephrol. 11 (Suppl 16), S159–S162.

4. Kreiss, P., Bettan, M., Crouzet, J., and Scherman, D. (1999). Erythropoietin secretion and physiological effect in mouse after intramuscular plasmid DNA electrotransfer. J. Gene Med. 1, 245–250.

5. MacColl, G.S., Novoa, F.J., Marshall, N.J., Waters, M., Goldspink, G., and Bouloux, P.M. (2000). Optimisation of growth hormone production by muscle cells using plasmid DNA. J. Endocrinol. 165, 329–336.
6. Richard, E., Douillard-Guilloux, G., Batista, L., and Caillaud, C. (2008). Correction of
glycogenosis type 2 by muscle-specific lentivector. In Viro Cell. Dev. Biol.
Anim. 44, 397–406.

7. Mendell, J.R., Rodino-Klapac, L.R., Rosales, X.Q., Coley, B.D., Galloway, G., Lewis, S.,
Malik, V., Shilling, C., Byrne, B.J., Conlon, T., et al. (2010). Sustained alpha-sarcogly-
can gene expression after gene transfer in limb-girdle muscular dystrophy, type 2D.
Ann. Neurol. 68, 629–638.

8. Herson, S., Hentati, F., Rigolet, A., Behin, A., Romero, N.B., Leturcq, F., Laforêt, P.,
Maisenbode, T., Amouri, R., Haddad, H., et al. (2012). A phase I trial of adeno-assoc-
iated virus serotype 1 γ-sarcoglycan gene therapy for limb girdle muscular dystro-
phy type 2C. Brain 135, 483–492.

9. Duan, D. (2018). Systemic AAV Micro-dystrophin Gene Therapy for Duchenne
Muscular Dystrophy. Mol. Ther. 26, 2337–2356.

10. Toscano, M.G., Romero, Z., Muñoz, F., Cobo, M., Benabbedallah, K., and Martin, F.
(2011). Physiological and tissue-specific vectors for treatment of inherited diseases.
Gene Ther. 18, 117–127.

11. Weeratna, R.D., Wu, T., Eeke, S.M., Zhang, L., and Davis, H.L. (2001). Designing gene
therapy vectors: avoiding immune responses by using tissue-specific promoters. Gene
Ther. 8, 1872–1878.

12. Neri, M., Torelli, S., Brown, S., Ugo, I., Sabatelli, P., Merlini, L., Spitali, P., Rimessi, P.,
Gualandi, F., Sewry, C., et al. (2007). Dystrophin levels as low as 30% are sufficient to
avoid muscular dystrophy in the human. Neuromuscul. Disord. 17, 913–918.

13. Waldrop, M.A., Gumienny, F., El Husayni, S., Frank, D.E., Weiss, R.B., and Flanigan,
K.M. (2018). Low-level dystrophin expression attenuating the dystrophinopathy
phenotype. Neuromuscul. Disord. 28, 116–121.

14. Wasala, N.B., Yue, Y., Vance, J., and Duan, D. (2017). Uniform low-level dystrophin
expression in the heart partially preserves cardiac function in an aged mouse model of
Duchenne cardiomyopathy. J. Mol. Cell. Cardiol. 102, 45–52.

15. Nott, A., Meislin, S.H., and Moore, M.J. (2003). A quantitative analysis of intron ef-
fects on mammalian gene expression. RNA 9, 607–617.

16. Furger, A., O’Sullivan, J.M., Binnie, A., Lee, B.A., and Proudfoot, N.J. (2002).
Promoter proximal splice sites enhance transcription. Genes Dev. 16, 2792–2799.
expression results in sustained correction of hemophilia B at low vector dose. Mol. Ther. 16, 280–289.

47. Chen, P., Tian, J., Kovesdi, I., and Bruder, J.T. (2008). Promoters influence the kinetics of transgene expression following adenovector gene delivery. J. Gene Med. 10, 123–131.

48. Salva, M.Z., Himeda, C.L., Tai, P.W., Nishiuchi, E., Gregorevic, P., Allen, J.M., Finn, E.E., Nguyen, Q.G., Blankinship, M.J., Meuse, L., et al. (2007). Design of tissue-specific regulatory cassettes for high-level rAAV-mediated expression in skeletal and cardiac muscle. Mol. Ther. 15, 320–329.

49. Rodnot-Klapac, L.R., Lee, J.-S., Mulligan, R.C., Clark, K.R., and Mendell, J.R. (2008). Lack of toxicity of alpha-sarcoglycan overexpression supports clinical gene transfer trial in LGMD2D. Neurology 71, 240–247.

50. Talbot, G.E., Waddington, S.N., Bales, O., Tchen, R.C., and Antoniou, M.N. (2010). Desmin-regulated lentiviral vectors for skeletal muscle gene transfer. Mol. Ther. 18, 601–608.

51. Robert, M.-A., Lin, Y., Bendjelloud, M., Zeng, Y., Dessolin, S., Broussau, S., Larochelle, N., Nailbantoglou, J., Massie, B., and Gilbert, R. (2012). Strength and muscle specificity of a compact promoter derived from the slow troponin I gene in the context of episomal (gutless adenovirus) and integrating (lentiviral) vectors. J. Gene Med. 14, 746–760.

52. Zaremba-Czogalla, M., Dubińska-Magiera, M., and Rzepecki, R. (2011). Laminopathies: the molecular background of the disease and the prospects for its treatment. Cell. Mol. Biol. Lett. 16, 114–148.

53. Dubińska-Magiera, M., Zaremba-Czogalla, M., and Rzepecki, R. (2013). Muscle development, regeneration and laminopathies: how lamins or lamina-associated proteins can contribute to muscle development, regeneration and disease. Cell. Mol. Life Sci. 70, 2713–2741.

54. Dubińska-Magiera, M., Kozioł, K., Machowska, M., Piekarowicz, K., Filipczak, D., and Rzepecki, R. (2019). Emerin Is Required for Proper Nucleus Reassembly after Mitosis: Implications for New Pathogenetic Mechanisms for Laminopathies Detected in EDMD1 Patients. Cells 8, 240.

55. Azibani, F., Brull, A., Arandel, L., Beuvin, M., Nelson, I., Jollet, A., Ziat, E., Prudhon, B., Benkhelifa-Ziyyat, S., Bitoun, M., et al. (2018). Gene Therapy via Trans-Splicing for LMNA-Related Congenital Muscular Dystrophy. Mol. Ther. Nucleic Acids 10, 376–386.

56. Piekarowicz, K., Machowska, M., Dratkiewicz, E., Lorek, D., Madej-Pilarczyk, A., and Rzepecki, R. (2017). The effect of the lamin A and its mutants on nuclear structure, cell proliferation, protein stability, and mobility in embryonic cells. Chromosoma 126, 501–517.

57. Bertrand, A.T., Renou, L., Papadopoulos, A., Beuvin, M., Lacène, E., Massart, C., Ottolenghi, C., Decostre, V., Maron, S., Schlossarek, S., et al. (2012). DelK32-lamin A/C has abnormal location and induces incomplete tissue maturation and severe metabolic defects leading to premature death. Hum. Mol. Genet. 21, 1037–1048.

58. Frith, M.C., Hansen, U., and Weng, Z. (2001). Detection of cis-element clusters in higher eukaryotic DNA. Bioinformatics 17, 878–889.

59. Wingender, E., Chen, X., Hehl, R., Karas, H., Liebich, I., Matys, V., Meinhardt, T., Prüss, M., Reuter, I., and Schacherer, F. (2000). TRANSFAC: an integrated system for gene expression regulation. Nucleic Acids Res. 28, 316–319.

60. Frith, M.C., Li, M.C., and Weng, Z. (2003). Cluster-Buster: Finding dense clusters of motifs in DNA sequences. Nucleic Acids Res. 31, 3666–3668.
Supplemental Information

A Muscle Hybrid Promoter
as a Novel Tool for Gene Therapy

Katarzyna Piekarowicz, Anne T. Bertrand, Feriel Azibani, Maud Beuvin, Laura Julien, Magdalena Machowska, Gisèle Bonne, and Ryszard Rzepecki
**Supplementary Figure S1**
The EF1a promoter driven luciferase was abundantly expressed in all analysed cell types. EF1a was about 5 times more active in muscle cells than MH promoter, however in non-muscle cells EF1a was 30-100 times more active.

**Supplementary Figure S2**
Comparison of promoters’ activities in the days following transfection for (A) H9C2 and (B) HEK 293 cells.
Supplementary Figure S3

Histochemical analyses did not show any damage in muscle tissues after intramuscular injection of AAV9 vectors with different expression cassettes. A) Analysis of Sirius Red staining for fibrosis detection in different muscles, B) example of Sirius Red staining, C) HE staining of injected TA muscles.
Supplementary Figure S4
Analysis of the hearts from different mice after intramuscular injection. Nuclei were stained with DAPI (blue). EGFP was equally distributed within all muscles of injected mouse, with no fiber preference.
Supplementary Figure S5
Sample images of differentiated C2C12 cells, marked in the text as myotubes, after 7 days in differentiation medium.

Supplementary Figure S6
A) The validation of the method’s linearity (luminescence related to transfection efficiency) for C2C12 cells.
B) The validation of the methods’ repeatability for C2C12 cells.