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A Muscle Hybrid Promoter as a Novel Tool for Gene Therapy

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Gene therapy is a promising strategy to cure 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.

INTRODUCTION

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 year[1]) 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 5×10^{13} vg/kg (vector genome number per kilogram), whereas, in currently ongoing trials for Duchenne muscular dystrophy (DMD), higher doses were chosen (reviewed in Duan[9]). 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 treatment[13,14]), 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 fibers[15,16]).

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

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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.[23]

Previously developed synthetic, modified, or hybrid promoters such as C5-12,[24] CK6–CK9,[25,26] and tMCK[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-muscular cell types *in vitro*. 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).[28] 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 −1,262 to −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 *EF1a* 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 *EF1a* gene promoter, which is a strong constitutive promoter. Because it was severalfold 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

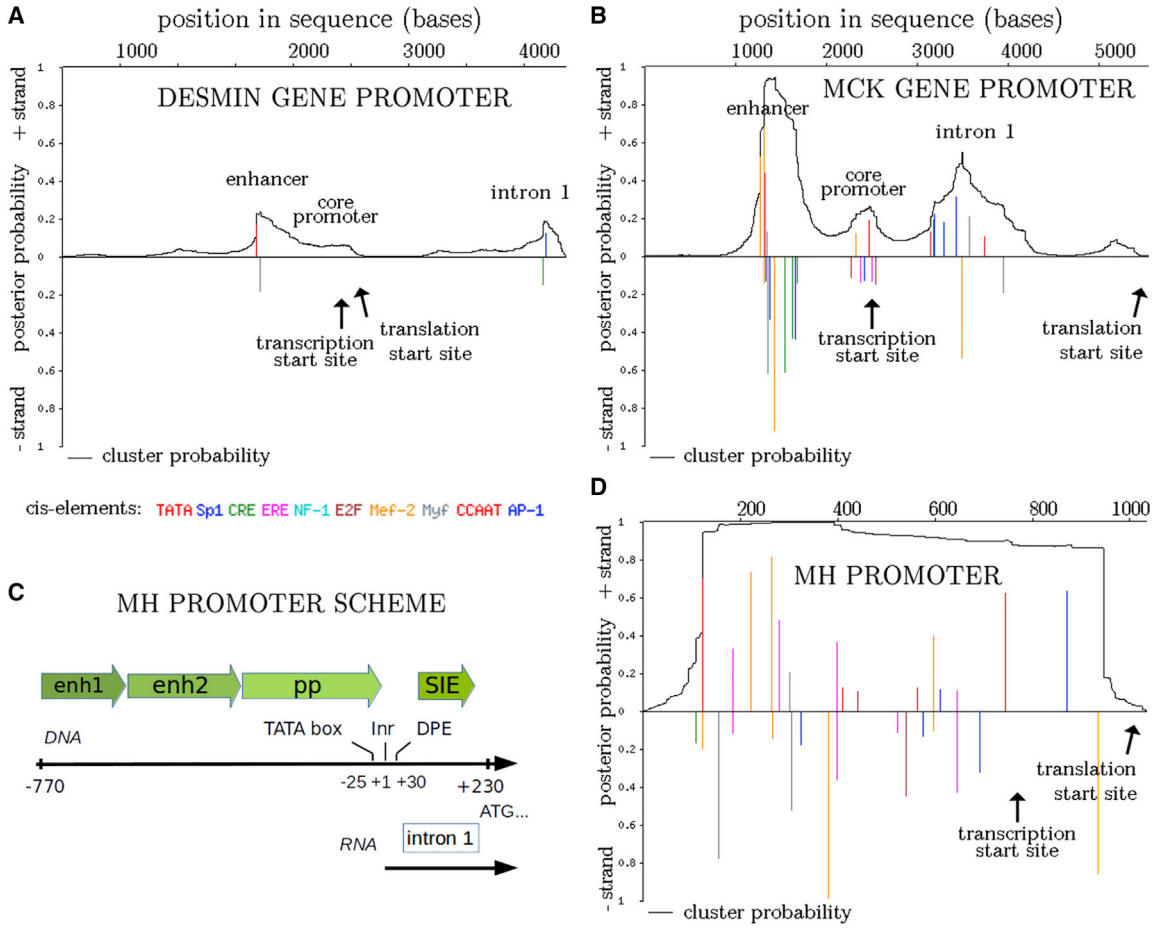

Figure 1. The MH Promoter Is Composed of 4 Main Modules that Together Are Predicted to Efficiently Bind Transcription Factors

(A) Analysis of the *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 *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 *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 *Des* gene enhancer (enh1); (2) the *Ckm* gene enhancer (enh2); (3) the *Ckm* gene core promoter (with modifications within the proximal promoter [pp]); and (4) a designed intron consisting of a SIE derived from the *Ckm* gene. Moreover, the 5′ UTR derived from the *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.

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 *Des* gene-based enhancer (enh1); (2) the *Ckm* gene-based enhancer (enh2); (3) both the *Des* and *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

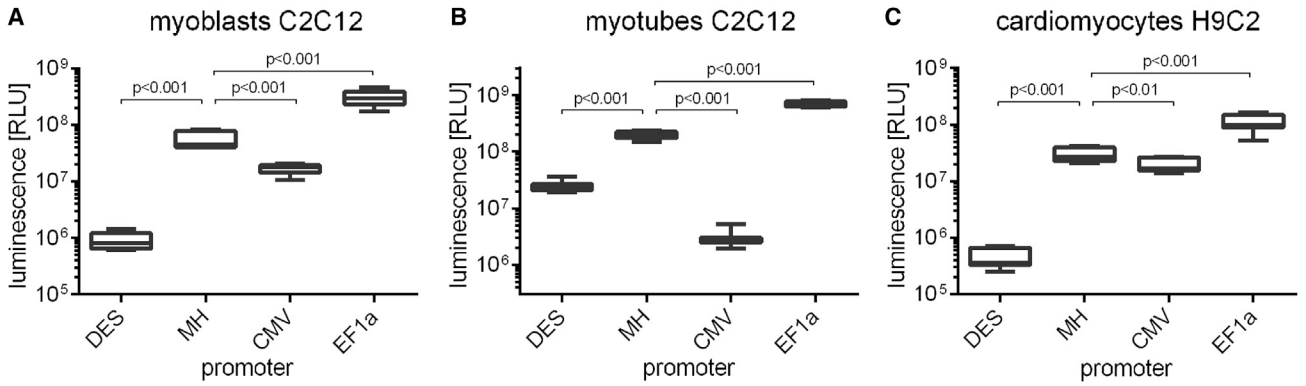

Figure 2. The MH Promoter Is Highly Active in Muscle Cells

(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 $\log_{10}$ scale.

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,[35] it was still expected to increase expression efficiency.[36] 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

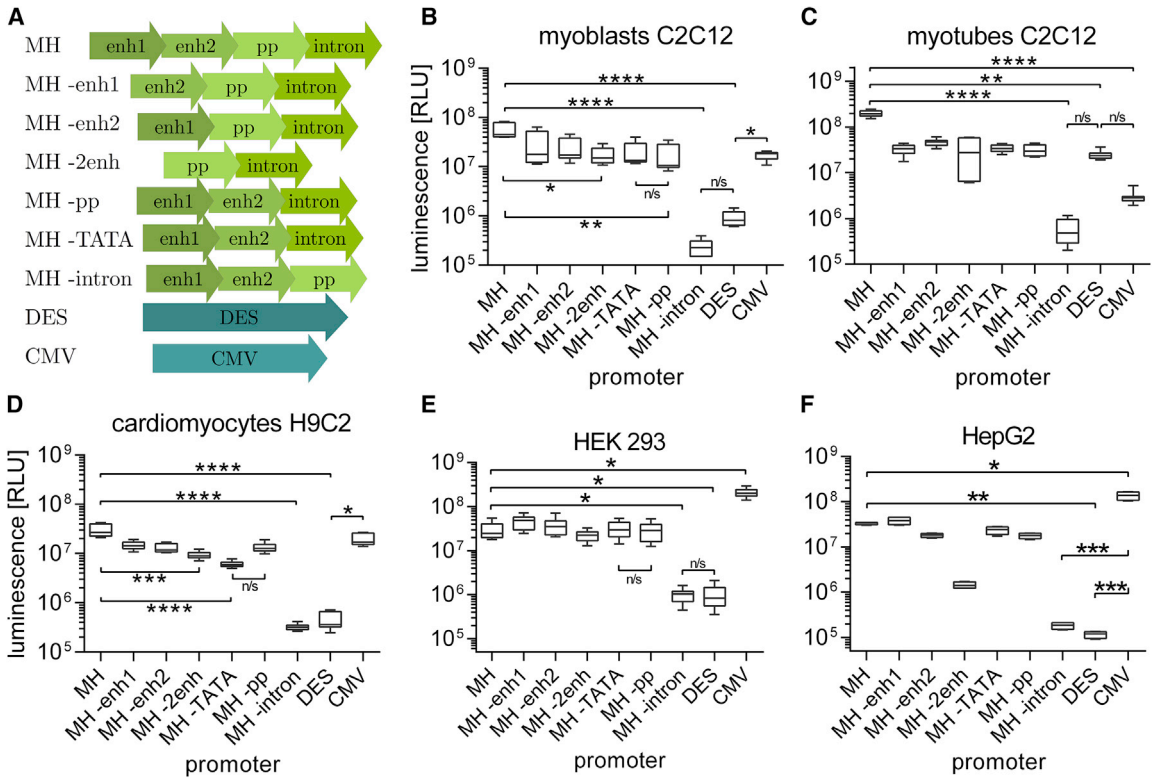

Figure 3. The Key Element of the MH Promoter Is an Intron-Containing SIE

(A) A set of MH promoter variants lacking particular module(s) was prepared. (B–F) Cells were transfected, and luciferase activity was measured using luminescence in the following cell lines: (B) C2C12 myoblasts, (C) C2C12 myotubes, (D) H9C2 cardiomyocytes, (E) HEK293 cells, and (F) HepG2 cells. Deletion of any element decreased MH promoter activity at least by half in all muscle cells but not in HEK293 or HepG2 cells. Deletion of the intron module decreased MH promoter activity most significantly, to a much greater degree than deletion of the core/pp. When the pp was deleted, there was no difference in whether the TATA box remained or was deleted, suggesting that this regulatory cassette has low significance when other cassettes (Inr or DPE) are in proximity. 3 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 (*$p < 0.01$; **$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$; n/s, not significant). Values are presented using a $\log_{10}$ scale.

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 MH 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

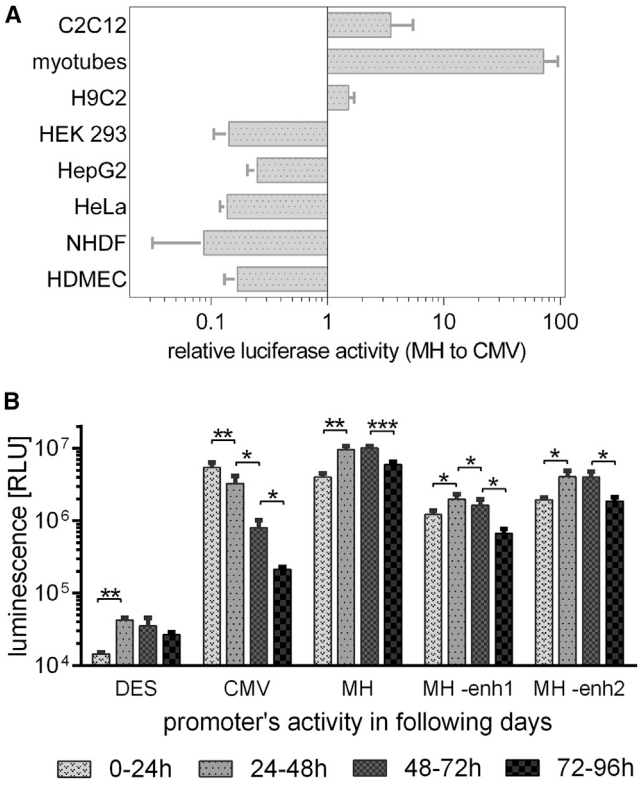

Figure 4. MH Promoter Activity and Specificity

(A) The MH promoter is specific for muscle cells. The MH promoter was more active than the CMV promoter in muscle cells (67-fold difference in myotubes). The activity of the MH promoter was at least 5-fold lower than the activity of the CMV promoter in all control non-muscle cell lines (HEK293, NHDF, HepG2, HeLa, and HDMEC cells). The MH promoter was more active than the *DES* promoter in all the examined cell lines (data not shown). Bars represent mean values with SD. 3 biological replicates were performed, each in 4 technical replicates normalized to transfection efficiency and total protein amount. Values are presented using a $\log_{10}$ scale. (B) The MH promoter has favorable kinetics activity in C2C12 cells. C2C12 cells were transfected with constructs encoding secretory luciferase under control of various promoters, and luciferase activity was measured based on luminescence in the days following transfection. MH promoter-controlled expression starts later than that of the CMV promoter, but its expression lasts longer. The luminescence intensity decrease after 72 h is due to plasmid loss in cells after division because C2C12 cells proliferate very rapidly. 3 biological replicates were performed, with 4 technical replicates each. Bars represent mean values with SDs (*$p < 0.01$, **$p < 0.05$, ***$p < 0.001$). Values are presented using a $\log_{10}$ scale.

the CMV and *DES* promoters (Figures 6C and 6D). In skeletal muscles, qPCR analyses showed 3- to 6-fold higher expression levels for the CMV promoter than for the MH promoter and low levels for the *DES* promoter (Figure 6A). The protein levels were slightly higher for the MH promoter but not detected for the *DES* promoter (Figures 6C and 6D).

As expected, qPCR analysis of non-muscular tissues showed higher *Egfp* expression for the CMV promoter than for both the MH and *DES* gene promoters. The expression level was 2-fold lower in the liver, 2 times lower in the intestine, 6 times lower in the lung, 8 times lower in the brain, and 20 times lower in the kidney for the MH promoter than for the CMV promoter. The *DES* promoter provided mRNA expression levels comparable with the MH promoter in these tissues (Figure 6B).

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.[24]

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.[24,41,42] 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 efficiency[43–45]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

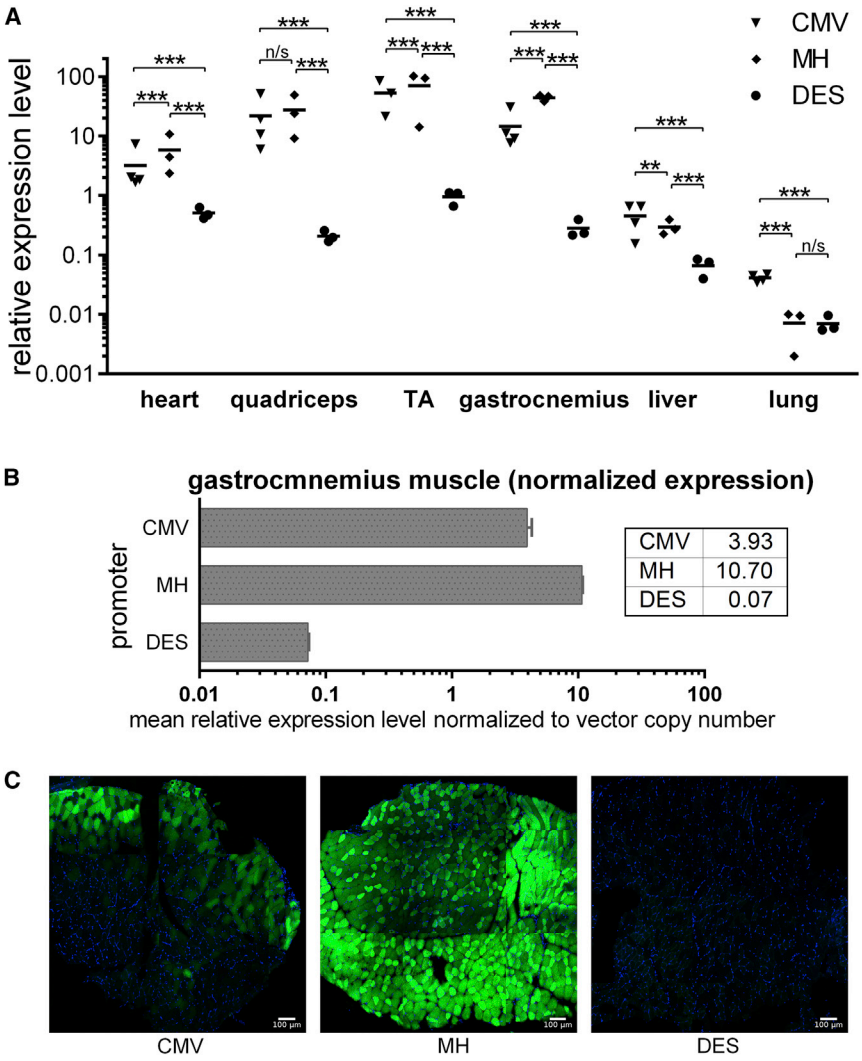

Figure 5. The MH Promoter Is Highly Active in Both Skeletal and Heart Muscle *In Vivo* after Intramuscular Delivery

Newborn wild-type mice were injected into *TA* muscle with AAV2/9 encoding EGFP under control of the MH (n = 3), *DES* (n = 3), or CMV (n = 4) promoters. Tissues were analyzed 6 weeks thereafter. The MH promoter provided the highest expression level in muscle tissue and lower expression compared with the CMV promoter in the liver and lungs. The *DES* promoter always led to the lowest expression in all tissues. (A) qPCR analysis showed that the MH promoter provides the highest expression levels in comparison with CMV or *DES* promoters in muscle tissues. The opposite effect was observed in the lungs and liver, where CMV promoter-driven expression was higher than for the MH promoter. *DES* promoter-controlled expression was the lowest in all tissues and comparable with MH in the lung. Each point represents the mean expression level in particular tissue for each mouse in 4 technical replicates. The horizontal line represents the mean value for all mice (*$p < 0.01$, **$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$). Values are presented using a $\log_{10}$ scale. (B) For gastrocnemius muscle, the *Egfp* expression level was normalized to both the *Rplp0* expression level and to the vector copy number, showing that the MH promoter provides an expression level 2.7 times higher than the CMV promoter and 150 times higher than the *DES* promoter in cells transduced with the AAV. (C) Analysis of representative injected *TA* muscles from mice treated with different vectors. Nuclei were stained with DAPI (blue). When EGFP (green) was detected, it was homogeneously distributed throughout the muscle section.

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,[47] 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.[25] Its variants, CK7, CK8, and CK9,[26] 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.[48] Although CK8, MHCK7, or minimal *CKM* promoters are currently used in DMD gene therapy clinical trials, the necessary AAV vector

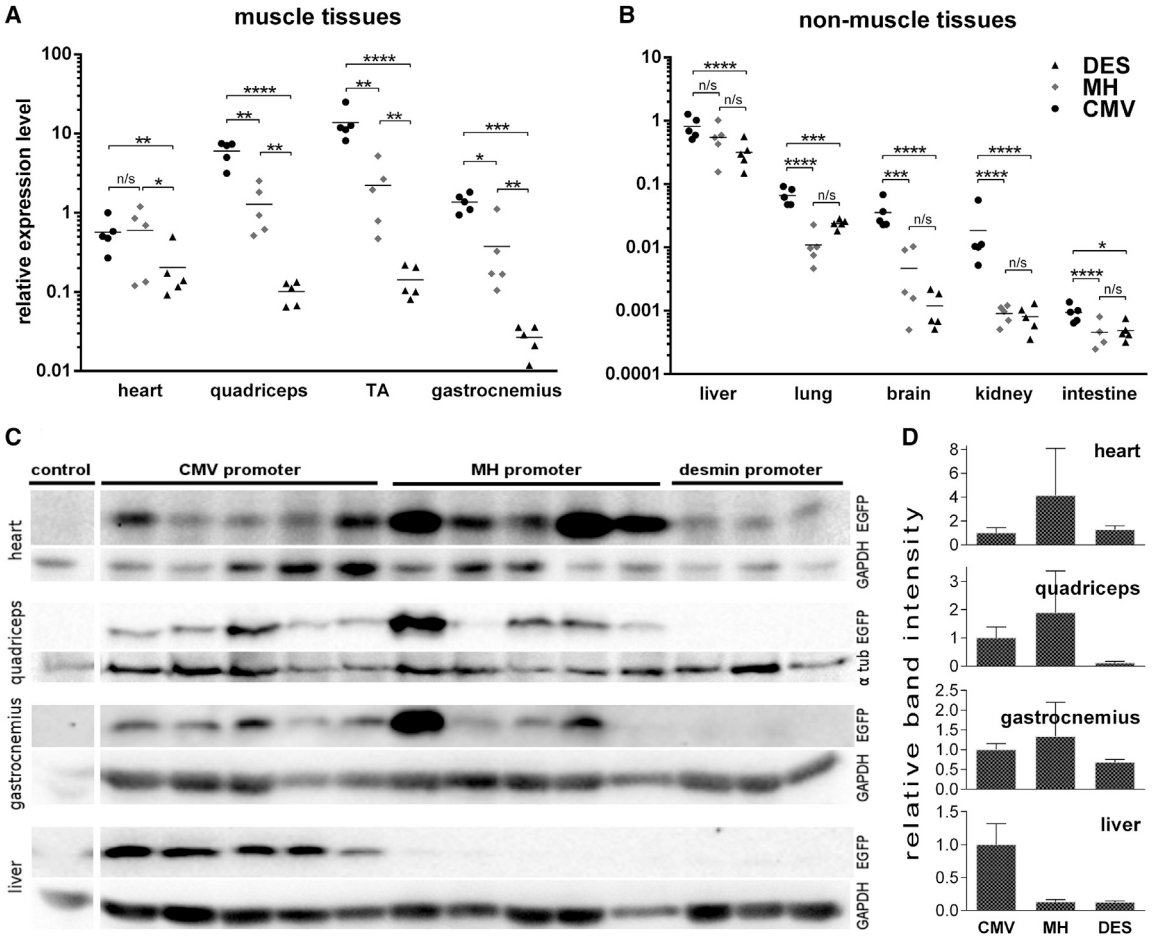

Figure 6. Systemic Delivery of the AAV with the MH Promoter Provides a High Level of Expression in Muscles and a Low Level of Expression in Other Analyzed Tissues

(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 MH 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.

doses are high (at least 5×10^{13} vg/kg) and side effects are observed, even resulting in temporary hold of one trial.[9] 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.[22] 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 tMCK and dMCK in *TA* muscle 1 month after AAV injection of 2-month-old mice, but the promoter's specificity was not confirmed.[27] 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 and low activity in the heart and diaphragm.[27] 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.[49] 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.[50] 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.[51]

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 *EF1a* promoter.[17] 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.[18] 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, $\sim 2.5 \times 10^{13}$ 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,[52–54] 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 (MEMα; 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 antimycotics (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/6J, 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.[55]

Plasmids for *In Vitro* Transfection

The plasmid pDRIVE5Lucia-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 *Not*I and *Nhe*I restriction enzymes (Thermo Fisher Scientific). The CMV promoter was cloned from the pEGFP-C1 plasmid (Clontech) using primers to introduce *Mlu*I (5′-AGCGA CGCGTTATTAATAGTAATCAATTAC-3′) and *BamH*I (5′-AAT GAGGATCCGACCGGTAGCGCTAG-3′) restriction sites (CMV-Luc). The EF1a promoter was cloned from the pWPI plasmid (Addgene) using primers to introduce *Mlu*I (5′-TTAACGCGT CAGTGGGCAGAGC-3′) and *BamH*I (5′-TATGGATCCTCACGA CACCTGAAATG-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 *PmlI*, *PmeI*, KpnI, and *SpeI* together with *XbaI* and *BclI*. All constructs were verified via sequencing with the primers 5′-TTAAGGGATTTTGGTCATGG-3′ and 5′-CAACA GCAATACAGATGAG-3′. Plasmids were amplified with *E. coli* strain DH5α with the selection antibiotic zeocin (100 μg/mL; InvivoGen) and purified with an endotoxin removal step (midi-prep 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 *Xho*I 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 *Hinc*II and *Nhe*I or *Hinc*II and *Nco*I. All constructs were verified via sequencing with the primers 5′-CCATTGCAT ACGTTGTATCC-3′ and 5′-CGAGCTTAGTGATACTTGTG-3′. Plasmids were amplified with *E. coli* strain DH5α 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 *Egfp* and *Rplp0* were measured using specific primers (*Egfp*, 5′-GACGTAAACGGCCACAAGTT-3′ and 5′-AAGTCGTG CTGCTTCATGT-3′; *Rplp0*, 5′-ACCTCCTTCTTCCAGGCTTT-3′ and 5′-CCCACCTTGTCTCCAGTCTTT-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 *Egfp* to *Rplp0* was calculated using LightCycler 480 software.

Total DNA (with episomal DNA) was isolated with 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 amplicons.

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

4°C. The supernatants were quantified with a BCA Protein Assay Kit (Thermo Fisher Scientific), lysed in Laemmli buffer at 96°C for 10 min, separated via SDS-PAGE on 6%–12% gels, transferred to nitrocellulose membranes, and blocked in 5% non-fat milk in PBS with 0.75% Tween 20 for 1 h at room temperature. Primary antibodies were applied overnight at 4°C in blocking buffer, and secondary antibodies conjugated with horseradish peroxidase (HRP) were applied for 1 h at room temperature in blocking buffer.

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 α-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, Immunochemistry, 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 (Cis-element Cluster Finder),[58] Match 1.0 (TRANSFAC matrix, BIOBASE),[59] Alibaba 2.1 (http://gene-regulation.com/), and ClusterBuster.[60] 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.

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