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Strong ubiquitous micro-promoters for recombinant adeno-associated viral vectors

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Significant progress has been made in developing recombinant adeno-associated virus (rAAV) for clinical gene therapy. While rAAV is a versatile gene delivery platform, its packaging limit of 4.7 kb limits the diseases it can target. Here, we report two unusually small promoters that enable the expression of larger transgenes than standard promoters. These micro-promoters are only 84 (MP-84) and 135 bp (MP-135) in size but have activity in most cells and tissues comparable to the CAG promoter, the strongest ubiquitous promoter to date. MP-84- and MP-135-based rAAV constructs displayed robust activity in cultured cells from the three different germ-layer lineages. In addition, reporter gene expression was documented in human primary hepatocytes and pancreatic islets and in multiple mouse tissues *in vivo*, including brain and skeletal muscle. MP-84 and MP-135 will enable the therapeutic expression of transgenes currently too large for rAAV vectors.

INTRODUCTION

Adeno-associated virus (AAV) is currently the safest and most effective gene delivery vector owing to its low immunogenicity, ability to transduce a broad range of host cell types, and capability of long-term expression of transgenes.[1] AAVs are a small single-stranded DNA virus and various AAV capsid serotypes have a broad spectrum of tissue and cell tropism. The AAV genome of ~4,700 nt contains two 145 nt inverted terminal repeat (ITR) sequences and two open reading frames, *rep* and *cap*, that encode several isoforms of replication proteins, capsid proteins, and the assembly activating protein (AAP) for capsid assembly.[2] Despite these gene products, AAVs are unable to complete their life cycle by themselves but require helper viruses such as adenoviruses or herpes viruses for their genome replication. Precisely these properties, the inability to self-replicate and the simplicity of their genomic structure, have been valuable properties of AAVs for their development as a successful therapy vector. In recombinant AAVs (rAAVs) the *rep* and *cap* genes are replaced by a gene expression cassette containing a promoter, a transgene, and the poly(A) transcription termination signal. The packaging of rAAV genomes is achieved by a pseudo-typing strategy in which the cargo DNA between two ITRs of AAV2 origin is packaged with a capsid from either another natural serotype or a recombinant capsid. The pseudo-typed rAAV approach has been highly efficient to enhance transduction efficiency in target cells.[3] Both capsid and the gene expression units are two crucial components for efficient gene therapy vectors.

Thus far, capsid engineering has been extensively explored to target specific types of cells and tissues employing selected mutagenesis or a directed evolution screening method.[3] By comparison, efforts to find promoters that are suitable for AAV have been less extensive, partly because well-established universal promoters were able to drive strong gene expression in various mammalian host cells. In fact, a recent survey showed that over 50% of rAAVs in 106 clinical settings used three universal promoters, the cytomegalovirus (CMV) promoter, which is the abbreviated term herein for the CMV enhancer and promoter, the chicken beta-actin (CBA) promoter, and the CAG promoter, which is a synthetic fusion of the CMV enhancer and the CBA promoter.[4,5] Moreover, the majority of clinical trials for tissue- and organ-specific disorders used one of these established universal promoters to achieve high transgene expression.[4,5] While these promoters have strong ubiquitous activity in various cell types, their size ranges from ~500 (CMV) to 1,000 bp (CAG) and larger, and such large sizes can be disadvantageous for rAAV gene therapy due to the limited payload that can be packaged by the virion. Studies have reported the maximum packaging capacity of rAAVs in the range of 5.2–5.6 kb, which is slightly larger than the natural AAV genome size of 4.9 kb.[6,7] Nonetheless, the packaging capacity of AAVs is much smaller than other viral vectors such as lentivirus or adenovirus, which are often used in clinical trials. Using the CAG promoter, which is the most preferred promoter in clinical trials, the available space for a transgene in an rAAV genome is less than 3.5 kb. Many genetic diseases and cancers involve genes with large transcripts exceeding 4 kb, which is too large to deliver in a single AAV and requires two or three AAVs in so-called split gene AAV vectors.[6,8–11] Whether single or split AAVs, promoters much smaller than the currently available universal promoters increase the chance to deliver large cargo DNAs as well as improve the packaging efficiency and vector titers, which strongly decrease with cargo DNAs larger than the wild-type AAV genome.[12]

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Here, we report two unusually small promoters that we have named micro-promoters, MP-84 and MP-135, which are only 84 and 135 bp, respectively. The activity of these micro-promoters is robust, and their activity is as strong as or only slightly weaker than the much larger CAG promoter in numerous cell types and tissues both *in vitro* and *in vivo*. The DNA sequences of the micro-promoters originate from the human insulin and glucagon promoter regions, respectively. Unexpectedly, the activity of the micro-promoters is superior to their much larger full-length promoters, and their robust activity is specific to AAV, requiring close proximity to an AAV2 ITR in both plasmid and virus configurations. Using the fluorescent reporter gene mRFP, we detected strong activity of the micro-promoters in human islet endocrine cells, hepatocytes, brain, and muscle tissues, among others. Micro-promoters may enable the packaging of larger genes than is possible with using conventional large universal promoters in AAVs.

RESULTS

Micro-promoters derived from human endocrine promoters exhibit strong activity in AAV vectors

The DNA sequences of MP-84 and MP-135 are derived from the promoters of the human insulin and glucagon genes located in chromosomes 11 and 2, respectively (Figure 1A). Both the insulin and glucagon genes are among the most highly regulated genes in the human body and are the most abundantly expressed genes in beta and alpha cells in pancreatic islets, respectively. MP-84 consists of 84 bp and contains a canonical TATA box, the transcription start site (+1), and a potential CAAT box. The short sequence immediately upstream of the TATA box contains predicted binding sites for the transcription factors PDX1 and PUR-1.[13,14] The human glucagon promoter is less well studied than the human insulin promoter and has been characterized mostly by sequence comparison studies with its rodent orthologs.[15,16] MP-135 is 135 bp long and contains a promoter (110 bp) and a sequence (25 bp) downstream of the transcription start site. The 110 bp sequence contains a TATA box and an upstream sequence with a binding site for the transcription factor FOX.[15,16]

We recently reported the robust activity of MP-84, which was much stronger than the full-length insulin promoter (363 bp) in islet endocrine cells upon AAV expression.[17] Such unexpectedly strong promoter activity was also observed with AAV-expressed MP-135 in islet endocrine cells. Like MP-84, MP-135 is much stronger than the full-length glucagon promoter (650 bp) in AAV (Figure S1). Another common property of these micro-promoters is universal activity that is not specific to any of the islet endocrine cell types. The strong activity of the micro-promoters in all types of endocrine cells prompted us to question whether their activity is cell type specific or comparable to the established universal promoters such as CMV and CAG. The CAG promoter used in this study is a shorter version (~1 kb) of the full-length CAG promoter (1.7 kb) and includes the CMV enhancer (249 bp) and the CBA promoter (687 bp) but not the rabbit globin intron. The promoters were cloned individually into AAV vectors with an identical genetic background, which was comprised of two ITRs, a chimeric intron, the fluorescence reporter gene mRFP, a WPRE (woodchuck hepatitis virus posttranscriptional regulatory element), and a poly(A) signal (Figure 1B). The CMV and CAG promoters are renowned for strong universal activity in many cell types and, for that reason, are frequently used in clinical trials with AAV gene therapy vectors.[5] All AAVs in this study were packaged with the recombinant capsid KP1, which transduces human pancreatic islets with high efficiency.[18]

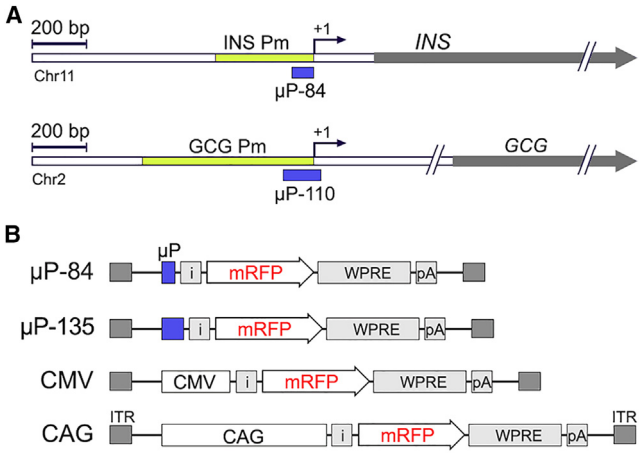


Figure 1. Schematic representation of the locations of MP-84 and MP-135 in the human genome and rAAV constructs used in this study

(A) The DNA sequences of the micro-promoters (blue) originated from the human genomic insulin and glucagon promoter regions (green), respectively. Both micro-promoters contain the core promoter with a TATA box, and MP-135 contained an additional 25 bp downstream of the transcription start (+1). (B) AAV ITR sequences (gray) are from serotype 2. Promoters were inserted upstream of the reporter gene mRFP, and the WPRE was used as stuffer DNA. A mini-intron (i) was present between the promoters and the reporter gene.

AAVs containing different promoters were first tested in both cultures of mouse alpha cells αTC1 and mouse insulinoma MIN6 cells. After the transduction, αTC1 cells were harvested at day 2, due to the fragility of the cells under longer culture conditions, and MIN6 cells at day 3. The promoter activity was assessed by the expression levels of mRFP, which was measured as the median fluorescence intensity (MFI), as the midpoint of fluorescence intensity of the population determined by flow cytometry analysis. A high MFI value indicates high mRFP expression from a promoter. Overall, all three promoters, MP-84, MP-135, and CAG, showed strong activities in both mouse cell lines. Surprisingly, the micro-promoters outperformed the CAG promoter in αTC1 cells with MFI values ~2- to 3-fold higher than for the CAG reference (Figures 2A and 2B). In comparison, the activity of the micro-promoters was similar to the CAG promoter in MIN6 cells. We further quantified the mRFP expression per intracellular AAV vector genome (vg) in MIN6 cells by quantitative PCR (qPCR) methods (Figure S2). Statistical analysis showed no significant differences in the activity between the micro- and the CAG promoter in both AAVs with capsid KP1 and DJ (Figure S2).

To test if the strong mRFP expression from the micro-promoters, and the CAG promoter, results from the transcription capability of the AAV2-ITR, we quantified mRFP expression from an AAV

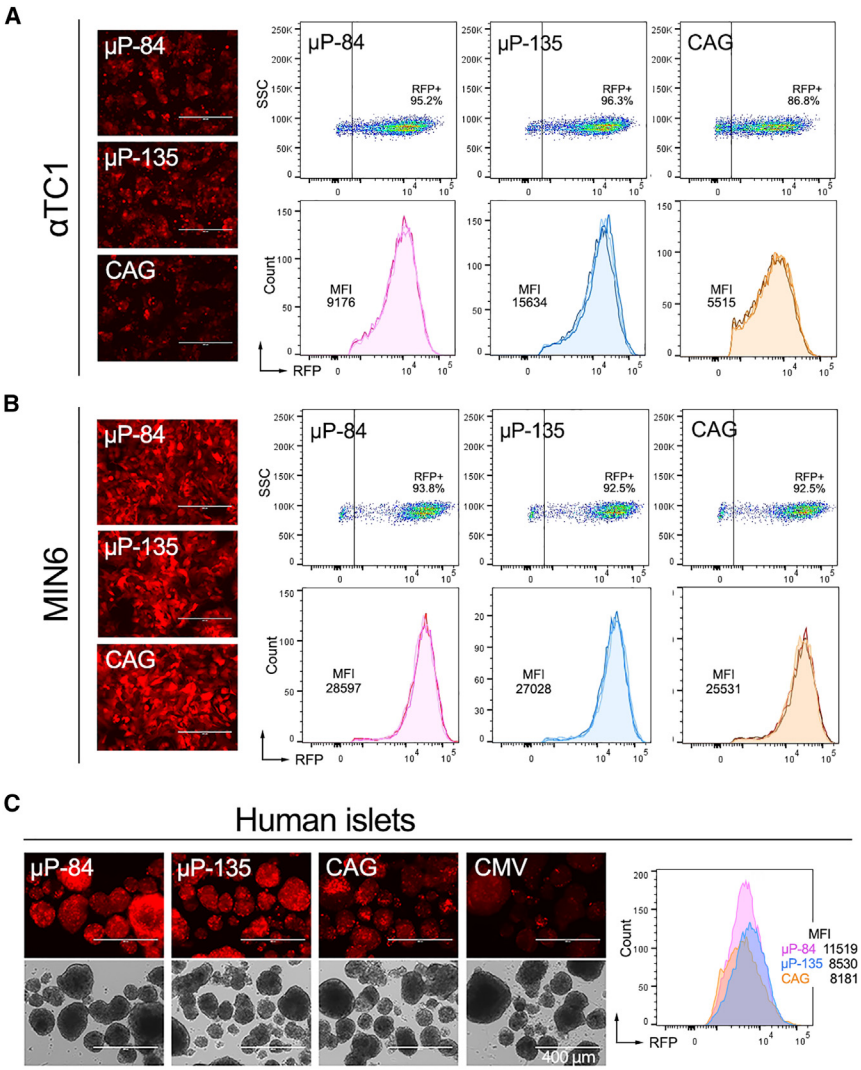


Figure 2. The micro-promoters show strong activity in mouse endocrine cell lines and human islet cells

rAAVs containing either micro-promoters or the reference promoters CMV and CAG were compared. (A and B) αTC1 mouse alpha (A) and MIN6 mouse insulinoma cells (B) were transduced with rAAVs and cultured for 2 (αTC1) or 3 (MIN6) days. Flow cytometry was performed to measure expression levels of mRFP, shown as the median fluorescence intensity (MFI). MFI values represent the mean value of the triplicates in each sample. (C) Intact human islets were transduced with AAVs and cultured for 4 days prior to the flow cytometry (shown in the box with MFI measurements). MP-84, MP-135, and the CAG promoter showed strong activity in all islet cells, while the CMV promoter had much weaker activity.

without any promoter. The AAV without a promoter showed a moderate level of mRFP expression in MIN6 cells, yet it was significantly lower compared with the AAVs containing MP-84 (Figure S3).

We next tested the micro-promoters in intact human islets. The islets were transduced with AAVs and cultured for 4 days. Previously, we have shown that the MP-84 and CAG promoters are active in all human islet cell types.[17] Likewise, MP-135 was active in all islet cells (Figure S4). Therefore, the MFI was measured from the mixed population of all islet cells that were dissociated for the flow cytometry. In human islet cells, the micro-promoters expressed high levels of mRFP, similar to the CAG promoter. Surprisingly the CMV promoter was much weaker than the other three promoters in human islets, as shown by fluorescence microscopy, and therefore was not subjected to the MFI measurement (Figure 2C).

The micro-promoters have strong activity in human primary hepatocytes but weaker activity in cells of other germ line origins

The unexpected, strong activity of the micro-promoters in endocrine cells led to the question of whether these promoters are also active in other endodermal lineage cells such as hepatocytes. Human primary hepatocytes were plated 24 h prior to transduction with AAVs (KP1 capsid) with the micro- and the CAG promoters. MFI was measured after 4 days in culture. Unlike in endocrine cells, the activity of the micro-promoters was not quite as strong as the CAG promoter, which was ~2 to 3-fold higher than the other two micro-promoters (Figure 3A). The time course of mRFP expression showed that the transcriptional activity from the CAG promoter was surprisingly rapid in these cells, as mRFP expression was visible within 24 h after transduction. In contrast, the micro-promoters showed mRFP expression 2 days after the AAV transduction (Figure S5). Despite the slower start, mRFP expression from the micro-promoters gradually increased, and high expression levels of mRFP were observed at day 4 posttransduction (Figure 3A).

Overall, the micro-promoters showed a robust transcription activity in cells of endodermal origin, islet endocrine cells, and hepatocytes. We asked whether the micro-promoters are also active in cells that originate from different germ layers since different genetic and cellular compositions display distinct regulatory machineries for gene expression. A primary culture of PD220 human fibroblasts was tested as an example of mesoderm-derived cells and both human neuroblastoma SH-SY5Y cells and rat glioma C6 cells as examples of the ectodermal origin. Overall, both micro-promoters showed clearly detectable mRFP expression that was weaker than the CAG promoter in these cell lines (Figure 3B). As in hepatocytes, mRFP expression from the CAG promoter was visible within 24 h after the AAV

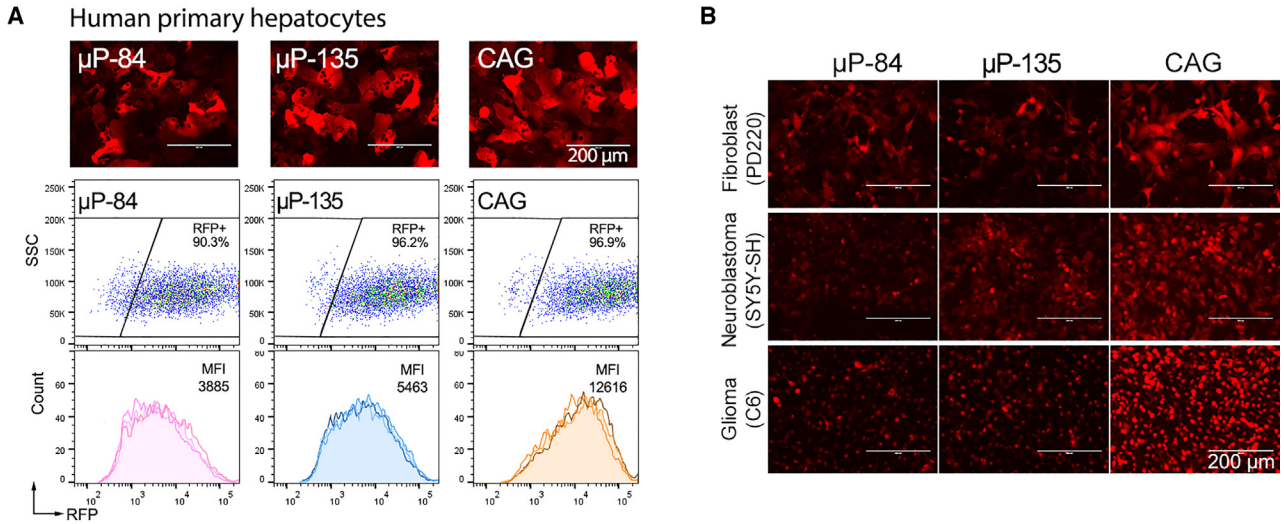


Figure 3. Activity of the micro-promoters in human primary hepatocytes and in cell lines

(A) Micro-promoters expressed high levels of mRFP in human hepatocytes but were not as strong as the CAG promoter. The rAAV transduced hepatocytes were cultured for 4 days prior to the flow cytometry. (B) The micro-promoters had weaker activity in cells of mesodermal (PD220 fibroblasts) or ectodermal origin (SY5Y-SH and C6). Microscopic images were taken 1 week following rAAV transduction.

transduction and remained strong in these cells, while the weak mRFP expression from the micro-promoters was slow and remained weak throughout the culture period. Although both micro-promoters showed reduced activity in these cells compared with the endocrine cells, MP-135 appeared to express a slightly higher level of mRFP than MP-84 (Figure 3B).

Activity of the micro-promoters is independent of the WPRE and intron but dependent on the AAV ITR

The AAV constructs used in this study include an intron and WPRE (Figures 1B and 4A). While WPRE functions to enhance the transgene expression, the primary role in our AAV vectors was stuffer DNA to increase the size of the DNA cargo for packaging. This stuffer DNA was needed particularly for the unusually small micro-promoters. Since the micro-promoters showed strong activity in endocrine cells, we questioned if the promoter activity was due to the enhanced transcriptional and posttranscriptional activity of the intron and WPRE in rAAV vectors. To investigate the promoter activity in the absence of these two regulators, rAAVs were constructed in which the intron was deleted or the WPRE was replaced with a new stuffer DNA, a DNA segment from intron 4 of the human ubiquitin gene (Figure 4B). The activity of the micro-promoters from these AAVs was assessed along with their parental AAVs in MIN6 cells. rAAVs with MP-84 showed no change in MFI of mRFP in the absence of the intron (Figure 4A). In contrast, the rAAV containing MP-135 without an intron showed a mild but significant decrease in MFI to 78% of the parental AAV (Figure 4A). Despite the decrease in mRFP expression in the absence of an intron, the promoter activity from MP-135 was still high (Figure 4A). Unlike in MIN6 cells, the activity of the micro-promoters did not change in the absence of an intron and remained high in αTC1 cells (Figure S6). Together, presence of the intron did not alter the expression levels from either micro-promoter. The positive regulation of WPRE in gene expression was evident when we measured MFI with the AAVs with or without WPRE. Deletion of WPRE showed a decrease in mRFP levels to 61% (MP-84) and 60% (MP-135) of their parental AAV with WPRE (Figure 4B). Despite this decrease in fluorescence intensity, the overall activity of the micro-promoters was still strong, and they expressed high levels of mRFP.

The micro-promoters require ITR sequences from AAV for their activity

We further examined whether the micro-promoters were active from a plasmid or a lentiviral vector. To test the efficacy in a plasmid, the micro-promoters were inserted into the plasmid pCDNA3.1, by replacing the existing CMV promoter, and mRFP was inserted as a reporter gene. Upon transfection of HEK293 cells with the pCDNA3.1 plasmid containing the CMV promoter, cells showed a robust expression of mRFP within 24 h. However, mRFP expression was not visible with the micro-promoters even 3 days after transfection, suggesting that the micro-promoters do not promote expression from the plasmid (Figure 4C). To assess the activity from lentivirus, the micro-promoters were inserted with an mRFP reporter into the FUGW lentiviral vector in place of the human ubiquitin-C (UBC) promoter. The mRFP reporter expression was monitored by lentiviral transduction of HEK293 cells. Five days after transduction, only a negligible level of mRFP was detected from the lentiviral vectors with the micro-promoters. In contrast, the lentivirus with the UBC promoter showed strong mRFP expression (Figure 4D). Together, these experiments showed that the micro-promoters are not functional in plasmids or lentivirus. Surprisingly, however, the AAV plasmid vectors with the micro-promoters expressed high levels of

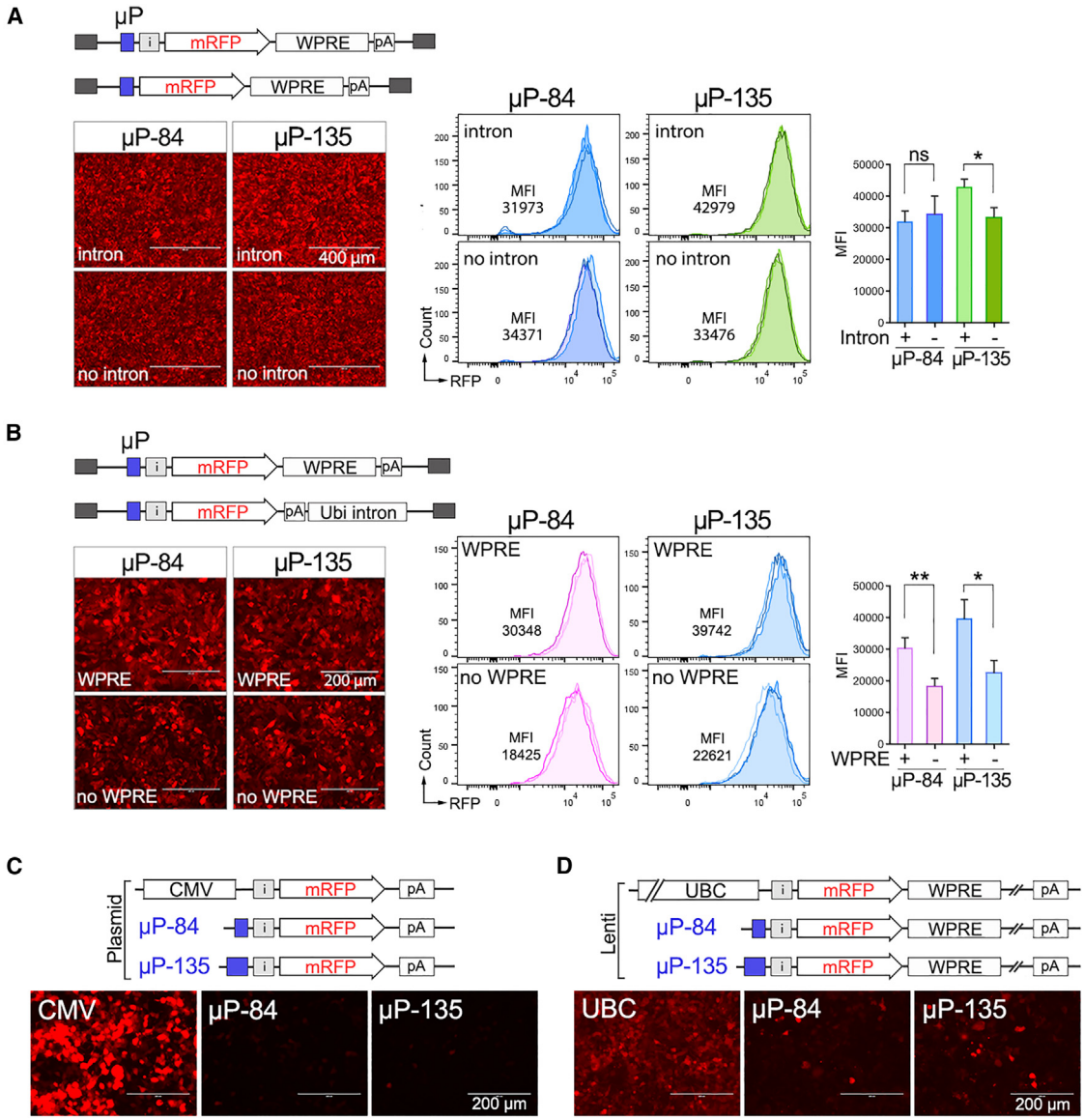


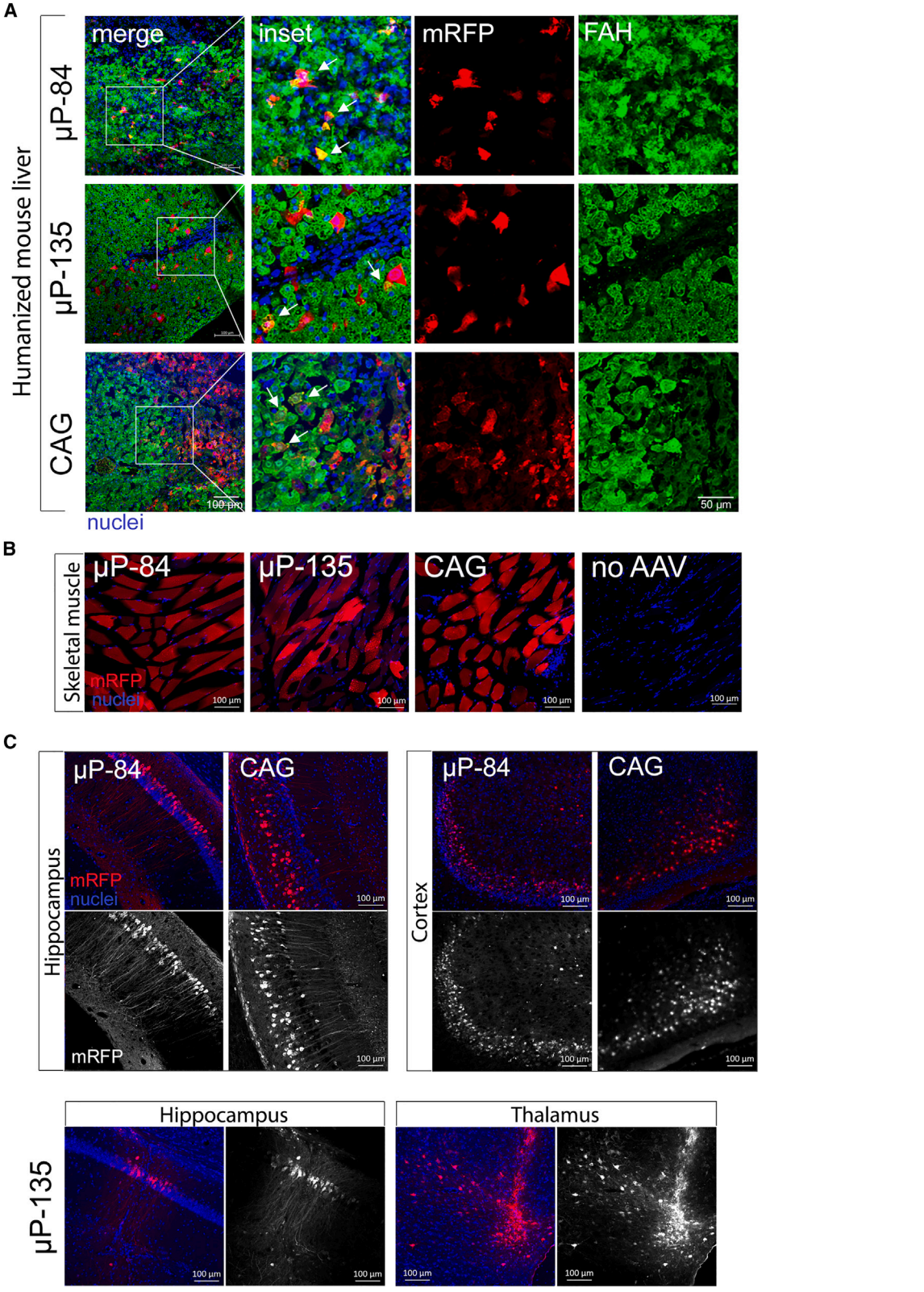
Figure 4. Effect of an intron, the WPRE, and AAV ITR on micro-promoter activity

(A) The activity of MP-84 was high regardless of the presence of an intron, while MP-135 showed a mild decrease. (B) The presence of WPRE in AAV enhanced mRFP expression from AAVs containing the micro-promoters in MIN6 cells. Replacement of WPRE in the AAV vector with an intron segment from the ubiquitin gene (Ubi intron) showed a decrease in gene expression, but activity was still high as shown in MFI measured by flow cytometry. Analyses were performed with triplicate samples, and the mean MFI values are shown in the box. Statistical analyses by t test (*$p < 0.05$, **$p < 0.001$, n.s. not significant $p > 0.05$). (C) The micro-promoters were not active in a plasmid lacking AAV2 ITRs. Microscopic images show HEK293 cells transfected with plasmids expressing mRFP from the indicated promoters. (D) The micro-promoters had negligible to low activity in a lentiviral vector. HEK293 cells were transduced with lentiviruses expressing mRFP under the indicated promoters. Microscopic images were taken 1 week following transduction. Both the promoters CMV (C) and UBC (D) were used to as the control promoter.

mRFP upon transfection of HEK293 and MIN6 cells. (Figure S7). These results were intriguing since they suggest that AAV sequences are required for the transcriptional activity of the micro-promoters. The only DNA structures that remained in our AAV constructs were two ITRs of AAV2 origin. Thus, it is plausible that a DNA sequence or a structural component of the ITR is responsible for generation of transcriptional activity from these micro-promoters.

Transgene expression driven by the micro-promoters is strong and stable for long term in tissues *in vivo*

Promoter properties such as strong, universal, and long-term activity are crucial assets for gene delivery vectors to successfully express transgenes in clinical applications. In cultured cells, the micro-promoters displayed a modest to strong promoter activity that was similar to the CAG promoter activity in some cell types. To find out if the micro-promoters



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were also active in *in vivo* settings, we tested AAVs containing these promoters in mice. In these experiments, the AAV containing the CAG promoter was also tested to compare the promoter activity.

To determine the activity from the micro-promoters in human hepatocytes, we employed liver-humanized mice. Mice were retro-orbitally injected with 10^{11} vg AAV, which was packaged with the capsid KP1, and the liver tissues were analyzed 2 weeks after injection by confocal microscopy of the liver tissues. An antibody recognizing human fumarylacetoacetate hydrolase (FAH) was used to distinguish the transplanted human hepatocytes from the mouse cells. Overall, all AAVs showed similarly high expression levels of mRFP in both human and mouse hepatocytes (Figure 5A). These data are consistent with the results obtained with cultured hepatocytes and indicate that robust and durable expression can be achieved in this important cell type. As the AAVs with capsid KP1 showed that transduction occurred predominantly in mouse hepatocytes in these experiments, we also tested AAVs generated with the human tropic capsid LK03 in liver-humanized mice to examine mRFP expression in human hepatocytes only (Figure S8). MP-84 packaged with LK03, as the CAG control promoter, also exhibited high mRFP expression in human hepatocytes (Figure S8). We next tested the micro-promoters in tissues and cells that are differentiated from other germ layers. The skeletal muscle tissues are developed from the mesoderm. In *in vitro* experiments with fibroblasts, cells of the mesodermal lineage, the micro-promoters showed transcription activities that were noticeably weaker than the CAG promoter (Figure 3B). To investigate the promoter activities *in vivo*, AAVs, at a dose of 10^{10} vg per mouse, were administered by intramuscular injection into the hind limbs of 3-month-old mice, and mRFP expression was examined 4 weeks after the injection. Confocal microscopy of the muscle tissues showed robust expression of mRFP from all three promoters (Figure 5B). These data suggest that the micro-promoters are able to drive very strong gene expression in the skeletal muscle tissues in mice.

To determine the ability of the micro-promoters in ectoderm-derived tissues, we injected 10^9 vg directly into the lateral ventricle of neonatal mouse brain. The mouse brain is not fully developed at postnatal day 1, and the space in the lateral ventricles allows injection of AAVs.[19,20] AAVs are then taken up by adjacent tissue layers that develop to form the different parts of the brain. Here, we examined mRFP expression in the cortex and hippocampus 8 weeks after the injection. Both the MP-84 and the CAG promoters showed overall high levels of mRFP expression in neurons throughout the cortex and hippocampus regions (Figure 5C). In MP-135-injected mice, however, we repeatedly failed to detect any neurons expressing mRFP. Instead, we detected cells expressing high levels of mRFP that were restricted to venous-like structures in these mice (Figure S9). We then questioned if MP-135 activity is suppressed in the brains of young mice because we injected AAVs at postnatal day 1 and the mRFP expression was examined at 2 months of age. To explore the possibility of MP-135 activity in neural tissue of older mice, we injected AAV with MP-135 into the cortex of a 10-week-old mouse brain. Confocal microscopy 3 weeks after the injection showed mRFP expression mainly in neurons in the hippocampus and thalamus and a small number of neurons in the cortex along the injection sites (Figure 5C). Together, these experiments showed that both MP-84 and MP-135 are capable of transgene expression in mouse and human cells *in vivo*. Although the properties of these two micro-promoters differ from each other, such as the timing of activity in neurons, these promoters showed strong activity in many cells and tissues, not unlike the CAG promoter.

DISCUSSION

Here, we show that two micro-promoters, MP-84 and MP-135, are highly expressed in many cells and tissues in the context of AAV2 genomes. One of the striking characteristics of the micro-promoters is their size. These promoters are unconventionally small, yet capable of driving high expression of transgenes from AAV. To our knowledge, there are no other promoters this small that are ubiquitously active and strong in many, but not all, cell types. Among the established promoters, the mouse phosphoglycerate kinase (mPGK) promoter (551 bp), the human synapsin (hSYN) promoter (499 bp), and the mouse methyl-CpG-binding protein-2 (MeCP2) promoter (229 bp) are considered small promoters.[21,22] The hSYN and the MeCP2 promoters do not have universal activity but preferentially express in neurons. The micro-promoters MP-84 (84 bp) and MP-135 (135 bp) described here represent a significant advance in the toolbox of AAV-delivered gene therapy since their small sizes allow packaging of larger transgenes than can be currently expressed from other ubiquitous promoters. While the micro-promoters had very strong activity in many cell types, our current study sampled only a limited number of tissues. We did not test their activity in the retina, pulmonary epithelium, motor neurons, myocardium, or endothelial cells, which are all important targets for gene therapy. Nonetheless, the data provided here suggest that these promoters should be considered for large transgenes. They certainly seem promising for gene expression in the endocrine pancreas, brain, liver, and skeletal muscle.

Figure 5. *In vivo* activity of the micro-promoters

(A) Promoter activities in liver-humanized mice. Overall transduction efficiency of AAVs generated with KP1 capsid was ~10%, but some human hepatocytes (arrows) expressed bright mRFP in mice injected with either the micro- or the CAG promoters. The human hepatocytes were immune-labeled with the antibody specific to human FAH (green). The liver tissues were stained with Hoechst33342 for nuclei (blue). Nuclei stained but FAH^- cells are mouse liver cells. (B) Mouse skeletal muscle show high levels of mRFP from all AAVs in mouse skeletal muscle 4 weeks after injection. Although not quite as strong as the CAG promoter, the micro-promoters showed high activity in muscle tissues. (C) The micro-promoters were also active in neurons of different regions in mouse brain. Similar to the CAG promoter, MP-84 showed high mRFP expression in the cortex and hippocampus. Intraventricular injection of AAVs was performed at postnatal day 1, and the confocal images were taken from 2-month-old mouse brain (top panel). The MP-135-injected brain did not show mRFP expression with neonatal brain injection. However, the administration of rAAVs in brain of mature (10-week-old) mice showed strong mRFP expression in neurons in the hippocampus and thalamus at 3 weeks following the injection (bottom panel).

An additional novel finding of this study is the AAV dependency of the micro-promoters because this property implicates a new function of the AAV ITR. ITRs across all AAV serotypes have a conserved T-shaped structure, and their main functions are to provide a *cis*-acting element for the replication origin and to serve as the packaging signal. ITRs are also known for their intrinsic promoter activity. Accordingly, AAV2 ITRs are able to drive expression of large DNAs (~4.5 kb) such as the CFTR gene and CRISPR-Cas9 gene editing machinery from promoter-less AAV vectors, which were tested because of their limited capacity of ~4.7 kb DNA cargo.[2,12,23] In addition to the promoter activity, multiple transcription start sites (TSSs) have been mapped throughout the ITR sequences, although a canonical TATA box has not been found.[2] In our study, the AAV vectors contained AAV2 ITRs, and both micro-promoters contain a core promoter with a canonical TATA box. The DNA sequences upstream of the TATA box are 54 (MP-84) and 84 bp (MP-135) long, which are considerably smaller than conventional enhancers with multiple transcription factor binding sites. Initially, we did not expect any promoter activity from the micro-promoters since they contain little of their respective enhancer regions. In fact, the micro-promoters do not have any enhancer activity, which is consistent with their lack of activity in plasmid or lentivirus vectors. Therefore, it is plausible that the AAV-dependent activity of the micro-promoters is caused by the ITR functioning as an enhancer for these promoters. Supporting the role of the ITR as an enhancer, we observed a starkly reduced activity of the micro-promoters when inserted into an AAV vector that contains a modified left ITR containing a deletion of 12 bp near the RBE (Rep b binding element) sequence (Figure S10).[2]

The fact that AAV is able to drive robust expression from our micro-sized promoters suggests a potential methodology to develop other similarly strong micro-promoters. This study showed that the micro-promoters have the strongest activity in endocrine cells, in their endogenous genetic backgrounds. Their activity was somewhat reduced yet was still strong in hepatocytes, cells that share an endodermal origin with the endocrine cells. In cells from other than endodermal origins, the micro-promoters had more variable activity. In general, tissue-specific enhancers regulate promoter activity for the highest tissue-specific expression. At present, we do not know whether the micro-promoters have any residual DNA elements that contribute to robust activity in the endocrine cells. However, it would be interesting if the core promoter region of other strong tissue-specific promoters—albumin, for example—exerts strong activity in their endogenous tissue, similar to our micro-promoters in endocrine cells.

MATERIALS AND METHODS

Human islet and cell cultures

Human pancreatic islets were obtained from the Integrated Islet Distribution Program (IIDP) funded by the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK). Islets were cultured in low-glucose-containing CMRL1066 medium with supplements as described in suspension culture dish.[17] HEK293 (ATCC), C6 rat glioma cells (ATCC), and PD220 primary human fibroblast cells (OHSU Fanconi Anemia Research Materials) were cultured in DMEM with high glucose supplemented with 10% fetal bovine serum (FBS). MIN6 mouse insulinoma (ATCC) cells were grown in DMEM with high glucose and 15% FBS and αTC1 mouse clone 6 alpha cells (ATCC) in DMEM with low glucose (1 g/L), 15% HEPES, and nonessential amino acid. Primary human hepatocytes were plated in hepatocyte plating medium (Lonza) at a density of 80,000 cells/cm^2, and media were changed to culture media consisting of HBM basal medium and HCM supplement (Lonza) 18 h after the plating. SH-SY5Y human neuroblastoma (ATCC) cells were grown in culture media recommended by ATCC.

Construction, production, transduction, and injection of rAAVs

All rAAV vectors used in this study were single-stranded AAVs with ITRs from the AAV serotype 2. The micro-promoters MP-84 and MP-135 were cloned using synthetic DNA oligonucleotides containing sequences of top and bottom strands of the promoters and hybridized to form a duplex DNA. The CAG promoter used in this study is a shorter version (~1 kb) of the full-length CAG promoter (1.7 kb) and includes the CMV enhancer (249 bp) and the CBA promoter (687 bp) but not the rabbit globin intron. Production and packaging of AAV were performed using HEK293 cells using pAD5 adeno helper plasmid, pAAV-KP1 Rep-Cap plasmid,[18] and AAV transfer plasmid as described.[17] Cultured cells and islets were transduced with rAAVs at a multiplicity of infection (MOI) of 10^5 vg per cell. Islet transduction was performed following the method as described.[17] For systemic delivery of AAVs, two liver-humanized mice were injected with each AAV sample (10^{11} vg viral particles) via the retro-orbital venous sinus. The promoter activities of MP-84, MP-135, and the CAG promoter in skeletal muscle were each tested in two of three month-old mice. Intramuscular injection was performed with 10^{10} vg rAAVs into the hind limbs of the mice. Intraventricular injection into neonatal mouse brain was performed in two (MP-84 and the CAG promoter) and three (MP-135) litters. The size of the litter varied between 5 and 9 pups. Each litter was injected with the same AAV with 10^9 vg in 1 μL volume, following the published method.[20] mRFP expression was monitored by harvesting one or two mouse brains at 1 and 2 weeks of age and two of an 8-week-old brain for each AAV sample. The confocal images were taken from an 8-week-old mouse brain. Injection of a targeted region in the cortex of adult mouse (two 10-week-old mice) brains was also done with 10^9 vg. Transduction of HEK293 cells with lentivirus was performed in the presence of a polycation polybrene at a final concentration of 8 μg/mL.[24] Procedures involving in vivo test in mice were approved and performed following the guidelines and regulations by OHSU IACUC.

Flow cytometry, preparation of tissues, and microscopy

For flow cytometry, cells were grown in 12-well plates for 2 to 4 days, dissociated with trypsin, and fixed with 4% paraformaldehyde (PFA). For each rAAV vector, the MFI of mRFP was measured from cells grown in three separate wells of a culture plate (technical triplicates). Intact islets were transduced with rAAVs first by incubation at 4°C for 1–2 h prior to incubation at 37°C for 4 days as described.[17] Data were analyzed with FlowJo software, and statistical analysis (t test) was done with GraphPad Prism software. Expression of mRFP in live cells was visualized using an EVOS cell imaging system (Life Technology). A whole-body perfusion fixation method was used on

anesthetized mice before harvesting tissues. Tissues were further fixed overnight at 4°C in 4% PFA and transferred to 30% sucrose before OCT cryoembedding tissues (Sakura). Frozen tissues were sectioned for nuclear staining (Hoechst33342) for all tissues and immunostaining of human FAH in hepatocytes. Confocal microscopy (Zeiss LSM700) was performed to visualize mRFP expression.

DATA AVAILABILITY

The data supporting the findings in this study are available from the corresponding author and the other co-authors.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2023.05.013.

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AUTHOR CONTRIBUTIONS

S.C. and M.G. designed the research and experiments and wrote the paper. S.C., L.W., M.N., B.L., D.E., and D.L.M were involved in experiments.

DECLARATION OF INTERESTS

This potential conflict of interest has been reviewed and managed by OHSU. S.C. and M.G. have filed a provisional application for patent for the promoters MP-84 and MP-135.

REFERENCES

1. Bulcha, J.T., Wang, Y., Ma, H., Tai, P.W.L., and Gao, G. (2021). Viral vector platforms within the gene therapy landscape. Signal Transduct. Targeted Ther. *6*, 53. https://doi.org/10.1038/s41392-021-00487-6.
2. Earley, L.F., Conatser, L.M., Lue, V.M., Dobbins, A.L., Li, C., Hirsch, M.L., and Samulski, R.J. (2020). Adeno-associated virus serotype-specific inverted terminal repeat sequence role in vector transgene expression. Hum. Gene Ther. *31*, 151–162. https://doi.org/10.1089/hum.2019.274.
3. Domenger, C., and Grimm, D. (2019). Next-generation AAV vectors-do not judge a virus (only) by its cover. Hum. Mol. Genet. *28*, R3–R14. https://doi.org/10.1093/hmg/ddz148.
4. Kuzmin, D.A., Shutova, M.V., Johnston, N.R., Smith, O.P., Fedorin, V.V., Kukushkin, Y.S., van der Loo, J.C.M., and Johnstone, E.C. (2021). The clinical landscape for AAV gene therapies. Nat. Rev. Drug Discov. *20*, 173–174. https://doi.org/10.1038/d41573-021-00017-7.
5. Au, H.K.E., Isalan, M., and Mielcarek, M. (2021). Gene therapy advances: a meta-analysis of AAV usage in clinical settings. Front. Med. *8*, 809118. https://doi.org/10.3389/fmed.2021.809118.
6. Wu, Z., Yang, H., and Colosi, P. (2010). Effect of genome size on AAV vector packaging. Mol. Ther. *18*, 80–86. https://doi.org/10.1038/mt.2009.255.
7. Hermonat, P.L., Quirk, J.G., Bishop, B.M., and Han, L. (1997). The packaging capacity of adeno-associated virus (AAV) and the potential for wild-type-plus AAV gene therapy vectors. FEBS Lett. *407*, 78–84. https://doi.org/10.1016/s0014-5793(97)00311-6.
8. Sahakyan, A.B., and Balasubramanian, S. (2016). Long genes and genes with multiple splice variants are enriched in pathways linked to cancer and other multigenic diseases. BMC Genom. *17*, 225. https://doi.org/10.1186/s12864-016-2582-9.
9. McClements, M.E., and MacLaren, R.E. (2017). Adeno-associated virus (AAV) dual vector strategies for gene therapy encoding large transgenes. Yale J. Biol. Med. *90*, 611–623.
10. Lopes, I., Altab, G., Raina, P., and de Magalhães, J.P. (2021). Gene size matters: an analysis of gene length in the human genome. Front. Genet. *12*, 559998. https://doi.org/10.3389/fgene.2021.559998.
11. Marrone, L., Marchi, P.M., and Azzouz, M. (2022). Circumventing the packaging limit of AAV-mediated gene replacement therapy for neurological disorders. Expet Opin. Biol. Ther. *22*, 1163–1176. https://doi.org/10.1080/14712598.2022.2012148.
12. Flotte, T.R., Afione, S.A., Solow, R., Drumm, M.L., Markakis, D., Guggino, W.B., Zeitlin, P.L., and Carter, B.J. (1993). Expression of the cystic fibrosis transmembrane conductance regulator from a novel adeno-associated virus promoter. J. Biol. Chem. *268*, 3781–3790.
13. Lew, A., Rutter, W.J., and Kennedy, G.C. (2000). Unusual DNA structure of the diabetes susceptibility locus IDDM2 and its effect on transcription by the insulin promoter factor Pur-1/MAZ. Proc. Natl. Acad. Sci. USA *97*, 12508–12512. https://doi.org/10.1073/pnas.97.23.12508.
14. Hay, C.W., and Docherty, K. (2006). Comparative analysis of insulin gene promoters: implications for diabetes research. Diabetes *55*, 3201–3213. https://doi.org/10.2337/db06-0788.
15. Philippe, J., Drucker, D.J., Knepel, W., Jepeal, L., Misulovin, Z., and Habener, J.F. (1988). Alpha-cell-specific expression of the glucagon gene is conferred to the glucagon promoter element by the interactions of DNA-binding proteins. Mol. Cell Biol. *8*, 4877–4888. https://doi.org/10.1128/mcb.8.11.4877-4888.1988.
16. Sharma, S.K., Leinemann, U., Ratke, R., Oetjen, E., Blume, R., Dickel, C., and Knepel, W. (2005). Characterization of a novel Foxa (hepatocyte nuclear factor-3) site in the glucagon promoter that is conserved between rodents and humans. Biochem. J. *389*, 831–841. https://doi.org/10.1042/BJ20050334.
17. Chai, S., Kim, Y., Galivo, F., Dorrell, C., Wakefield, L., Posey, J., Ackermann, A.M., Kaestner, K.H., Hebrok, M., and Grompe, M. (2022). Development of a beta cell-specific expression control element for recombinant adeno-associated virus. Hum. Gene Ther. *33*, 789–800. https://doi.org/10.1089/hum.2021.219.
18. Pekrun, K., De Alencastro, G., Luo, Q.J., Liu, J., Kim, Y., Nygaard, S., Galivo, F., Zhang, F., Song, R., Tiffany, M.R., et al. (2019). Using a barcoded AAV capsid library to select for clinically relevant gene therapy vectors. JCI Insight *4*, e131610. https://doi.org/10.1172/jci.insight.131610.
19. Passini, M.A., and Wolfe, J.H. (2001). Widespread gene delivery and structure-specific patterns of expression in the brain after intraventricular injections of neonatal mice with an adeno-associated virus vector. J. Virol. *75*, 12382–12392. https://doi.org/10.1128/JVI.75.24.12382-12392.2001.
20. Kim, J.Y., Ash, R.T., Ceballos-Diaz, C., Levites, Y., Golde, T.E., Smirnakis, S.M., and Jankowsky, J.L. (2013). Viral transduction of the neonatal brain delivers controllable genetic mosaicism for visualising and manipulating neuronal circuits in vivo. Eur. J. Neurosci. *37*, 1203–1220. https://doi.org/10.1111/ejn.12126.
21. Gray, S.J., Foti, S.B., Schwartz, J.W., Bachaboina, L., Taylor-Blake, B., Coleman, J., Ehlers, M.D., Zylka, M.J., McCown, T.J., and Samulski, R.J. (2011). Optimizing promoters for recombinant adeno-associated virus-mediated gene expression in the peripheral and central nervous system using self-complementary vectors. Hum. Gene Ther. *22*, 1143–1153. https://doi.org/10.1089/hum.2010.245.
22. Nieuwenhuis, B., Haenzi, B., Hilton, S., Carnicer-Lombarte, A., Hobo, B., Verhaagen, J., and Fawcett, J.W. (2021). Optimization of adeno-associated viral vector-mediated transduction of the corticospinal tract: comparison of four promoters. Gene Ther. *28*, 56–74. https://doi.org/10.1038/s41434-020-0169-1.
23. Haberman, R.P., McCown, T.J., and Samulski, R.J. (2000). Novel transcriptional regulatory signals in the adeno-associated virus terminal repeat A/D junction element. J. Virol. *74*, 8732–8739. https://doi.org/10.1128/jvi.74.18.8732-8739.2000.
24. Denning, W., Das, S., Guo, S., Xu, J., Kappes, J.C., and Hel, Z. (2013). Optimization of the transductional efficiency of lentiviral vectors: effect of sera and polycations. Mol. Biotechnol. *53*, 308–314. https://doi.org/10.1007/s12033-012-9528-5.