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Adeno-associated virus gene transfer in Morquio A disease – effect of promoters and sulfatase-modifying factor 1

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Mucopolysaccharidosis (MPS) IVA is an autosomal recessive disorder caused by deficiency of the lysosomal enzyme *N*-acetylgalatosamine-6-sulfate sulfatase (GALNS), which leads to the accumulation of keratan sulfate and chondroitin 6-sulfate, mainly in bone. To explore the possibility of gene therapy for Morquio A disease, we transduced the *GALNS* gene into HEK293 cells, human MPS IVA fibroblasts and murine MPS IVA chondrocytes by using adeno-associated virus (AAV)-based vectors, which carry human *GALNS* cDNA. The effects of the promoter and the cotransduction with the sulfatase-modifying factor 1 gene (*SUMF1*) on GALNS activity levels was evaluated. Downregulation of the cytomegalovirus (CMV) immediate early enhancer/promoter was not observed for 10 days post-transduction. The eukaryotic promoters induced equal or higher levels of GALNS activity than those induced by the CMV promoter in HEK293 cells. Transduction of human MPS IVA fibroblasts induced GALNS activity levels that were 15–54% of those of normal human fibroblasts, whereas in transduced murine MPS IVA chondrocytes, the enzyme activities increased up to 70% of normal levels. Cotransduction with *SUMF1* vector yielded an additional four-fold increase in enzyme activity, although the level of elevation depended on the transduced cell type. These findings suggest the potential application of AAV vectors for the treatment of Morquio A disease, depending on the combined choice of transduced cell type, selection of promoter, and cotransduction of *SUMF1*.

Introduction

Mucopolysaccharidosis (MPS) IVA (Morquio A disease; OMIM# 253000) is an autosomal recessive disorder caused by deficiency of *N*-acetylgalatosamine-6-sulfate sulfatase (GALNS; EC 3.1.6.4, UniProt P34059), leading to lysosomal accumulation of glycosaminoglycans, keratan sulfate and chondroitin 6-sulfate, mainly in bone and cornea [1]. Clinical manifestations vary from severe to attenuated forms characterized by systemic skeletal dysplasia, laxity of joints, hearing loss, corneal clouding and heart valvular disease with normal intelligence [2]. Currently, no effective therapies exist for MPS IVA, and only supportive measures and surgical interventions are used to alleviate some manifestations of the disease [2].

Abbreviations
AAT, α_1-antitrypsin promoter; AAV, adeno-associated virus; CMV, cytomegalovirus; EF1, elongation factor 1α; GALNS, *N*-acetylgalatosamine-6-sulfate sulfatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRES, internal ribosomal entry site; LSD, lysosomal storage disease; MPS, mucopolysaccharidosis; SUMF1, sulfatase-modifying factor 1.

Although bone marrow transplantation improves many aspects of the somatic manifestations, it has a limited impact on cardiac, eye and skeletal abnormalities, in addition to the risk of fatal complications [3,4]. Preclinical trials for enzyme replacement therapy have shown significant decreases in keratan sulfate in blood and tissues [5], and clinical trials are in progress. However, patients will require weekly intravenous infusions of the recombinant enzyme, with high costs (over $300 000 annually), and immunological complications are expected for most patients [6].

Gene therapy is a promising alternative approach, and there have been a number of clinical and experimental studies. The success of a gene therapy protocol depends on the selection of the candidate disease, target cell, promoter region and ability to avoid an immune reaction [7]. The cytomegalovirus (CMV) immediate early enhancer/promoter has frequently been used for gene therapy, because of its capacity to induce transgene expression in a wide range of tissues, and the long-term therapeutic levels of expressed proteins observed in different diseases and animal models [8–10]. However, several reports have indicated that the CMV promoter is associated with short-term expression because of promoter silencing and the immune response to the transgene product [11–13]. Eukaryotic promoters [e.g. elongation factor 1α (EF1), muscular creatine kinase, and α_1-antitrypsin (AAT)] have emerged as alternatives to improve the therapeutic effect, to reduce side effects and to induce immunotolerance against gene products [14–16].

Gene therapy studies in animal models of lysosomal storage diseases (LSDs) have shown that after a single vector administration, therapeutic enzyme levels can be maintained with clinical benefits for up to 1.5 years in mice and 7 years in dogs [17–19]. Additionally, in sulfatase-deficient LSDs, the coexpression of a sulfatase gene together with the sulfatase-modifying factor 1 (*SUMF1*) gene has permitted a two-fold to three-fold increase in the corresponding sulfatase enzyme activity. *SUMF1* encodes the enzyme converting serine to formylglycine at the common active site among all human sulfatases [20–22]. MPS IVA is also a candidate disease for gene therapy, owing to the lack of central nervous system involvement [2]. To date, no *in vivo* gene therapy trial has been performed for MPS IVA; one report demonstrated, using a retroviral vector *in vitro*, that transduced cells produced enzyme activity five-fold to 50-fold higher than the baseline enzyme activity in non-transduced cells [23].

In this first study on gene transfer for MPS IVA with the use of adeno-associated virus (AAV)-based vectors, we have compared the expression level of *GALNS* under the control of either the CMV immediate early enhancer/promoter or eukaryotic AAT or EF1 promoter in the presence or absence of human *SUMF1* gene coadministration. We demonstrated that the eukaryotic AAT promoter gives equal or higher enzyme activity levels as that induced by the CMV promoter, and cotransduction with *SUMF1* leads to a substantial elevation of the enzyme activity.

Results

AAV2 vectors with the CMV, AAT or EF1 promoter driving the expression of human *GALNS* cDNA were constructed as described in Experimental procedures (Fig. 1). The CMV–SUMF1 vector was used for all *in vitro* cotransduction experiments, because of the non-tissue-specific profile of the CMV promoter, which may allow comparison of the effects of *SUMF1* coexpression on different cell types. All vector preparations had about 10^{13} vg·mL^{-1} of viral titers, and there was no effect of vector genome size on viral titers. The yield of the vector packing process was 60–80% (Fig. 1).

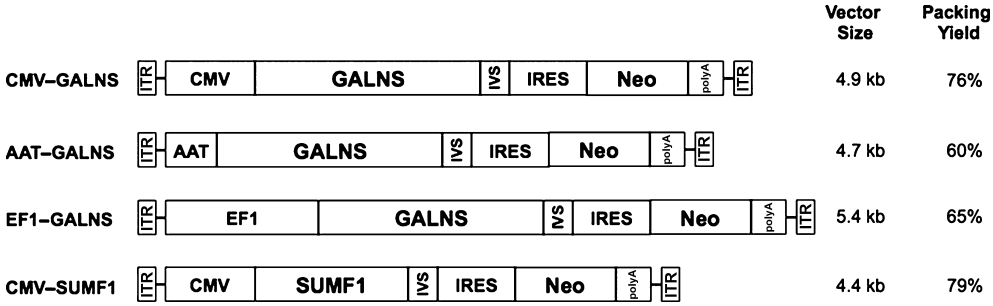

Fig. 1. Structure of CMV–GALNS, AAT–GALNS, EF1–GALNS and CMV–SUMF1 vectors. The AAV-derived vectors contain the inverted terminal repeats (ITRs) from AAV2, the CMV immediate early enhancer/promoter, the human AAT or EF1 promoters, a synthetic intron (IVS), the attenuated IRES from encephalomyocarditis virus, the neomycin phosphotransferase coding sequence (Neo), and the bovine growth hormone poly-A signal (polyA).

Transduction of HEK293 cells

HEK293 cells transduced with CMV–GALNS, AAT–GALNS or EF1–GALNS showed a 13-fold to 30-fold increase in GALNS activity levels in cell lysates, as compared with nontransduced cells (0.63 ± 1.10 U·mg^{-1}, $n = 3$) (Fig. 2A). The enzyme activity was detectable from the second day post-transduction in all transduced cells. In CMV–GALNS-transduced cells, no significant increment ($P > 0.05$) of GALNS activity was observed between days 2 and 10 post-transduction. A peak of the enzyme activity level was observed at day 4 in cells transduced with AAT–GALNS (18.63 ± 1.39 U·mg^{-1}, $n = 3$) and EF1–GALNS (14.57 ± 0.8 U·mg^{-1}, $n = 3$). However, these values decreased by 22% and 46%, respectively, on day 8 (Fig. 2A). At day 10, no significant difference in enzyme activity was observed among the three vectors ($P = 0.062$), and the final enzyme activity levels were 22 times higher than those in nontransduced cells ($P = 0.041$). No enzyme activity was detected in culture medium at any point of the study. All three vectors showed similar efficiencies of gene transfer, regardless of their DNA size (Fig. 2B). RNA analysis showed a similar profile to that observed for the enzyme activity; a peak in expression at day 4 post-transduction, a slight decrease at day 8, and similar levels of expression at day 10 (Fig. 2C). Transduced HEK293 cells increased GALNS mRNA levels by 7–14%, and they were significantly higher ($P < 0.001$) than those observed in nontransduced cells, regardless of the promoter. No statistical difference was observed in *GALNS* expression levels among the different vectors ($P > 0.05$).

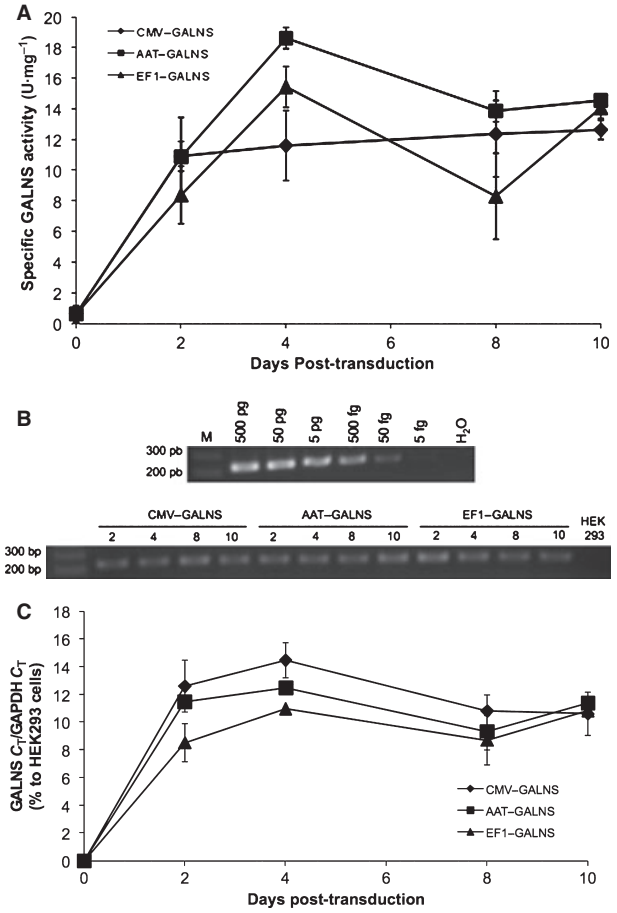

Fig. 2. Transduction of HEK293 cells. (A) HEK293 cells were transduced with 1×10^{10} vg of CMV–GALNS, AAT–GALNS or EF1–GALNS, and the enzyme activity was measured in cell lysates 2, 4, 8 and 10 days post-transduction. (B) Viral DNA was extracted from transfected HEK293 cells 2, 4, 8 and 10 days post-transduction. DNA was amplified with *GALNS* cDNA-specific primers, using 1 μg of total DNA. The standard was obtained with 500 pg to 5 fg, with the plasmid pAAV–CMV–GALNS. Nontransduced HEK293 cells were used as negative controls. (C) Vector mRNA from transduced HEK293 cells was amplified using 1 μg of total RNA. GALNS mRNA was amplified with *GALNS* cDNA-specific primers, and the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used for normalization. cDNAs were quantified by real-time PCR, and results were expressed as the increase of the GALNS C_T/GAPDH C_T ratio as compared with the values observed in nontransduced HEK293 cells (day 0).

Cotransduction of HEK293 cells with CMV–SUMF1

As compared with those cells transduced without CMV–SUMF1, transduction of HEK293 cells with *GALNS* and *SUMF1* in a 1 : 1 ratio gave 2.4-fold, 1.5-fold and 1.5-fold increases in cells cotransduced with CMV–GALNS (28.31 ± 1.52 U·mg^{-1}, $P = 0.006$), AAT–GALNS (28.19 ± 1.74 U·mg^{-1}, $P = 0.012$) and EF1–GALNS (23.69 ± 4.77 U·mg^{-1}, $P = 0.223$), respectively (Fig. 3). A 4.5-fold (51.72 ± 2.80 U·mg^{-1}, $P = 0.001$), 4.8-fold (53.34 ± 2.44 U·mg^{-1}, $P < 0.001$) and 5.3-fold (56.59 ± 8.28 U·mg^{-1}, $P = 0.013$) increases, respectively, were observed when *GALNS* and *SUMF1* were cotransduced in a 1 : 2 ratio (Fig. 3). The GALNS activity levels corresponded approximately to 85 times the levels in nontransduced cells (0.63 ± 1.10 U·mg^{-1}, $n = 3$).

The enzyme activity was detectable in medium when the cells were cotransduced with the CMV–SUMF1 vector (Fig. 3). Coexpression with *SUMF1* in a 1 : 1 ratio provided 0.45 ± 0.08 U·mL^{-1}, 0.18 ± 0.08 U·mL^{-1} and 0.18 ± 0.18 U·mL^{-1} of GALNS activity in media for CMV–GALNS, AAT–GALNS and EF1–GALNS, respectively. The levels increased 1.8-fold

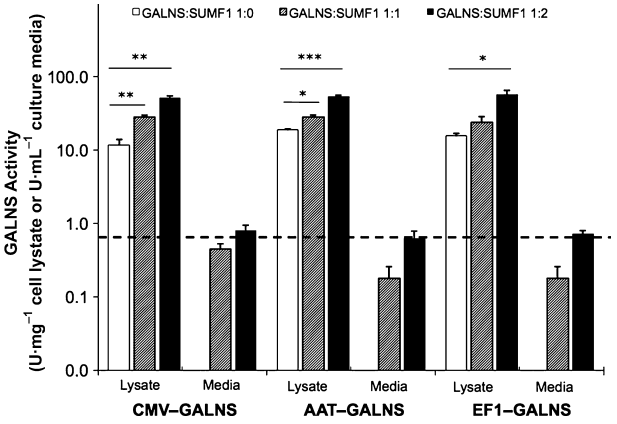

Fig. 3. *SUMF1* coexpression in HEK293 cells. HEK293 cells were cotransduced with 1×10^{10} vg of CMV–GALNS, AAT–GALNS or EF1–GALNS, and CMV–SUMF1 in a 1 : 0, 1 : 1 or 1 : 2 ratio. Activity in cell lysates and culture media was assayed 4 days post-transduction. The dashed line represents the GALNS activity levels in nontransduced HEK293 cells (0.63 ± 1.10 U·mg^{-1}), and no GALNS activity was detected in culture medium from HEK293 cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

(0.81 ± 0.14 U·mL^{-1}), 3.5-fold (0.63 ± 0.16 U·mL^{-1}) and 4.0-fold (0.72 ± 0.08 U·mL^{-1}), respectively, when the cells were cotransduced with a *GALNS/SUMF1* 1 : 2 ratio, as compared with levels in cells transduced with *GALNS/SUMF1* 1 : 1.

Transduction of human MPS IVA fibroblasts and murine MPS IVA chondrocytes

Human MPS IVA fibroblasts

Transduction of human MPS IVA fibroblasts with the CMV–GALNS, AAT–GALNS or EF1–GALNS gave 36.5%, 54.6% and 15.3% of GALNS activity levels in normal fibroblasts (13.47 ± 0.73 U·mg^{-1}, $n = 3$), respectively (Fig. 4A). Furthermore, cotransduction with CMV/SUMF1 in a 1 : 1 ratio led to a 1.5-fold increase of activity in the cells transduced with CMV–GALNS, AAT–GALNS or EF1–GALNS, which gave 60%, 86% or 23% of normal GALNS levels, respectively (Fig. 4A). When *GALNS* and *SUMF1* were cotransduced into the cells in a 1 : 2 ratio, an additional 2.1–2.6-fold increase in enzyme activity was seen. This corresponded to 93.6%, 112% and 39% of the GALNS activity levels of nontransduced normal fibroblasts, respectively. GALNS activity in medium was detected only when *GALNS* and *SUMF1* were cotransduced in a 1 : 2 ratio (Fig. 4A). Although the enzyme levels were lower than those observed in HEK293 cells, they were comparable to those in medium from normal fibroblasts.

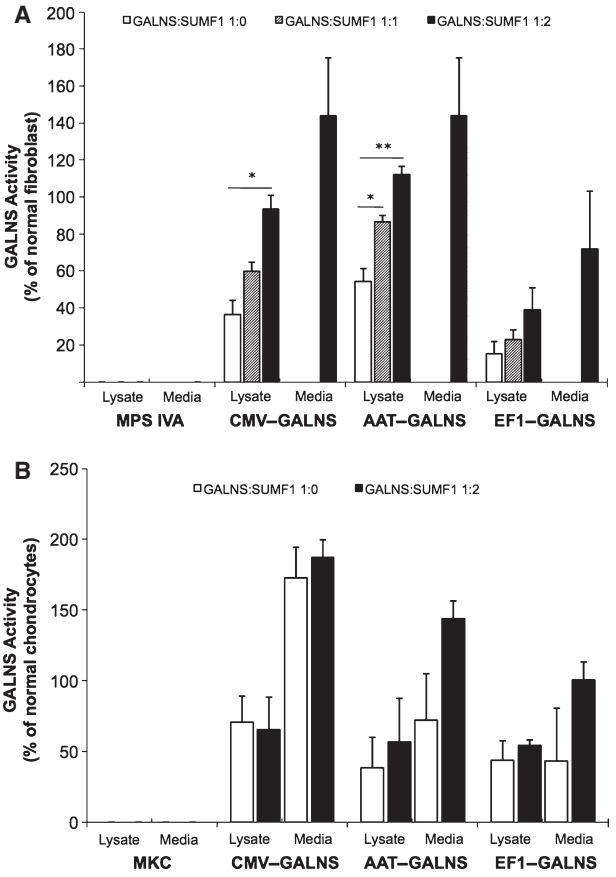

Fig. 4. Human fibroblast and murine chondrocyte transduction. (A) Human MPS IVA fibroblasts and murine MPS IVA chondrocytes were transduced with 1×10^{10} vg of CMV–GALNS, AAT–GALNS or EF1–GALNS with or without CMV–SUMF1 in a 1 : 1 or 1 : 2 ratio. GALNS activity in cell lysates and culture media was measured 4 days post-transduction, and the results are shown as percentages of GALNS activity levels in normal human fibroblasts. (B) Murine MPS IVA chondrocytes were transduced with 1×10^{10} vg of CMV–GALNS, AAT–GALNS or EF1–GALNS with or without CMV–SUMF1 in a 1 : 2 ratio. GALNS activity in cell lysates and culture media was measured 4 days post-transduction, and the results are shown as percentages of GALNS activity levels in normal murine chondrocytes. $^{*}P < 0.05$, $^{**}P < 0.01$.

Murine MPS IVA chondrocytes

In murine MPS IVA chondrocytes, transduction with CMV–GALNS induced up to 70% of the GALNS activity levels of normal chondrocytes (24.12 ± 6.23 U·mg^{-1} versus 34.0 ± 16.47 U·mg^{-1}), whereas AAT–GALNS and EF1–GALNS gave 40% of normal levels (13.16 ± 7.29 U·mg^{-1} and 14.91 ± 4.71 U·mg^{-1}, respectively) (Fig. 4B). Unlike the results observed in HEK293 cells and MPS IVA fibroblasts, cotransduction with *SUMF1* yielded a lesser impact on GALNS

activity, with a 1.3-fold increase in cells cotransduced with AAT–GALNS or EF1–GALNS (Fig. 4B).

GALNS activity in medium from affected murine chondrocytes after treatment with CMV–GALNS reached 230% of the enzyme activity of normal chondrocytes (0.43 ± 0.05 U·mL^{-1} versus 0.19 ± 0.06 U·mL^{-1}), whereas transduction with AAT–GALNS and EF1–GALNS produced 90% (0.18 ± 0.08 U·mL^{-1}) and 60% (0.11 ± 0.09 U·mL^{-1}) of normal GALNS activity, respectively (Fig. 4B). The cells cotransduced with CMV–GALNS and CMV–SUMF1 showed slightly increased GALNS activity in medium, whereas in those cells cotransduced with AAT–GALNS or EF1–GALNS, 2.0-fold and 2.3-fold increases were observed in GALNS activity. These corresponded to 190% and 130% of the enzyme activity in medium from wild-type chondrocytes (Fig. 4B).

Discussion

The aim of this study was to establish the optimal conditions for *in vivo* AAV gene therapy for MPS IVA by evaluating the effects on GALNS enzyme activity of: (a) different promoters; and (b) *SUMF1* coexpression. We have demonstrated that: (a) GALNS activity level was influenced by the promoter and the cell type; (b) eukaryotic AAT and EF1 promoters induced similar or higher GALNS activity levels as those induced by the CMV promoter; (c) unlike previous findings obtained with the CMV promoter [11,24,25], no reductions in mRNA and enzyme activity levels were observed, at least up to 10 days post-transduction, suggesting the absence or delay of gene silencing; and (d) cotransduction with an *SUMF1* vector allowed a further increase in the GALNS enzyme activity.

We selected an AAV2 vector because of its well-established transduction of HEK293 cells [26–28], human skin fibroblasts [27,29,30] and chondrocytes [31], and transduction efficiencies higher than those observed with other AAV serotypes [29,31]. As Compared with other gene therapy vectors, AAV vectors themselves have several advantages: (a) long-term expression; (b) wide-ranging cell and tissue tropism; (c) well-characterized serotypes; (d) lack of pathogenicity; and (e) low immunogenicity [32–34]. In addition, AAV vectors have been used for more than 30 different metabolic diseases, half of which were LSDs, resulting in complete correction of phenotype or substantial improvement of biochemical and phenotypic manifestations without side effects [32]. Previously, Toietta *et al.* [23] reported five-fold to 50-fold increases in normal GALNS activity levels in different cell types when a retroviral vector was used. Although retroviral vectors induced high levels of expression, they could cause insertional mutagenesis [35]. Thus, we selected AAV-based vectors because of their higher efficiency and safer profile [36], although there are a few *in vivo* studies referring to the asymptomatic immune response in clinical trials [37] and the occurrence of hepatocellular carcinoma in MPS VII mice [38].

Effect of promoter and cell type

Promoter selection has been widely studied to date; however, no consensus has been reached [39]. We demonstrated that expression profiles varied, depending on a combination of the cell type and the promoter. In HEK293 cells, no significant difference in GALNS activity was observed among the promoters used, whereas in human fibroblasts and murine chondrocytes, GALNS activity levels were as follows: AAT > CMV > EF1, and CMV > AAT = EF1, respectively. In transduced HEK293 cells, the GALNS enzyme activity showed an approximately 20-fold increase, whereas mRNA levels were increased by between 7% and 14%, resulting in an absence of correlation between GALNS enzyme activities and mRNA levels ($r = 0.377$, $P = 0.226$). The difference between the increases in GALNS enzyme activities and mRNA levels could be explained by the presence of additional sequences within the cassette (Fig. 1). The synthetic intron (IVS) used in our constructs has been associated with improvement in polyadenylation/transport and mRNA processing, which resulted in a six-fold to 50-fold increase in the indicator (CAT) protein [40]. The presence of introns in expression plasmids can also increase by up to 10 times the transport of an mRNA to the cytoplasm [41], or extend its half-life significantly [42]. In addition, the bovine growth hormone poly-A signal has been associated with more efficient post-transcriptional processes than those observed with other poly-A signals, which increase mRNA instability and production of the target protein [43,44]. The results presented in this work agree with previous reports showing that the inclusion of a synthetic intron and the use of the bovine growth hormone poly-A signal allowed high-level production of the indicator protein [44,45]. Finally, the internal ribosomal entry site (IRES) sequence has not been associated with an increase in mRNA stability, but with gene control expression and synthesis of several proteins from a single multicistronic mRNA [46,47].

The CMV promoter has been used frequently in preclinical and clinical protocols of gene therapy [39], because it induces higher expression levels than other promoters [39,48]. High and long-term expression

levels have been achieved in some *in vivo* studies [8–10,13,49,50]. However, in other studies, the CMV promoter has been associated with relatively short-term expression, because of promoter silencing [11,24,51] or downregulation by cytokines [52,53]. These observations were confirmed for adenovirus-derived, retrovirus-derived or plasmid-derived vectors [11,24,25,53]. Previously, we showed that *GALNS* expression was downregulated in HEK293 cells at 4 days post-transfection, using a calcium phosphate method with a plasmid carrying the CMV promoter and human *GALNS* cDNA [54]. In the present study, no reductions in GALNS mRNA and activity levels were observed for 10 days post-transduction of HEK293 cells. This finding suggests the absence or delay of promoter silencing, as some previous data have shown that silencing occurs within the first 6 h or during the first week after gene transfer [11,24,55–59]. Promoters that are not silenced within this period can allow long-term gene expression without subsequent downregulation [55–59]. In addition, preliminary results in the MPS IVA mouse model have shown sustained expression over 3 months after AAV-mediated gene delivery (data not shown). Several reports also indicated long-term expression with the use of AAV vectors with a CMV promoter [8–10,13,49,50], supporting our results.

The reason why CMV promoter silencing does not happen in particular cases, including our study, remains unknown. However, *in vitro* [60] and *in vivo* [61] studies have shown that AAV vectors induce a change in gene expression profile. Genes involving cellular proliferation and differentiation, DNA replication, DNA binding and mRNA transcription are downregulated, whereas immunoregulatory genes are upregulated [60,61]. Further investigations are required to establish gene expression profiles of epigenetic regulatory factors.

Recently, eukaryotic promoters have emerged as an alternative option to achieve long-term expression and immunotolerance induction against the recombinant protein [14,39,51]. The liver-specific AAT promoter has been used in gene therapy for mucopolysaccharidoses [62,63] and hemophilias [12,64]. We have observed that *GALNS* expression in deficient fibroblasts and chondrocytes transduced with AAT–GALNS was compatible with that induced by the CMV–GALNS or EF1–GALNS vector. This is attributed to: (a) the alteration of the expression profile in promoters, especially tissue-specific ones [65], owing to the difference in expression of transcription factors between *in vitro* and *in vivo* cells; and (b) the fact that the AAT promoter used here was a 400 bp fragment of the 3′-end derived from the full-length 1.2 kb fragment (GenBank accession no. D38257.1). Loss of cell specificity of the AAT promoter could be explained by the presence of specific transcription factor sites in the deleted region of 880 bp [54,66,67]. A loss of tissue specificity for the AAT promoter was also reported in a retroviral vector carrying the same AAT promoter fragment used here, driving the expression of the human β-glucuronidase gene (*GUSB*) [63].

The EF1 promoter produced similar GALNS activity levels in HEK293 cells and 1.6-fold to 2.3-fold lower levels in human MPS IVA fibroblasts and murine MPS IVA chondrocytes, respectively, than those obtained with the CMV promoter. These variations were observed in previous studies with the EF1 promoter [68–72].

Coexpression of *SUMF1*

The CMV promoter was selected for all *SUMF1* coexpression experiments, to assess the *SUMF1* coexpression effect objectively without variations associated with the other promoters and the cell types used. In HEK293 cells cotransduced with *GALNS* and *SUMF1* vectors, the enzyme activity approached 4.5-fold of that in cells transduced only with the *GALNS* vector, as previously reported for arylsulfatase A in HEK293 cells [73,74]. In human MPS IVA fibroblasts, *SUMF1* coexpression allowed up to a 2.6-fold increase in GALNS activity in cell lysates. These results are compatible with the elevations of enzyme activity observed for different sulfatases coexpressed with *SUMF1* [20]. Cotransduction with CMV–SUMF1 and any of CMV–GALNS, AAT–GALNS or EF1–GALNS in murine chondrocytes had a lower impact on elevation of enzyme activity than in HEK293 cells and human MPS IVA fibroblasts. These results showed that the effect of *SUMF1* coexpression could vary with the cell type, as previously described [21,74]. Sulfatase activity elevation after cotransduction with an SUMF1 vector has been evaluated and confirmed in media from HeLa, COS and HEK293 cells [20,21,74], but not in medium from primary cell cultures. Here, we have investigated GALNS activity in medium from different cell types cotransduced with the CMV–SUMF1 vector. The results indicated that elevation of GALNS activity in medium depends on the transduced cell type. In HEK293 cells GALNS activity was detectable with both 1 : 1 and 1 : 2 ratios of *GALNS* and *SUMF1*, whereas in MPS IVA fibroblasts, GALNS activity was only detected with a 1 : 2 ratio of *GALNS* and *SUMF1*. Unlike for HEK293 cells and human MPS IVA fibroblasts, GALNS activity was detectable in medium of transduced murine MPS IVA chondrocytes even

without *SUMF1* coexpression within the range of 43–230% of normal activity levels. Cotransduction with CMV–GALNS and CMV–SUMF1 did not markedly increase GALNS activity in medium of murine chondrocytes, whereas AAT–GALNS or EF1–GALNS cotransduction provided twice the normal level of enzyme activity. *In vivo* studies have shown that the coexpression of sulfatases (arylsulfatase A and sulfamidase) and *SUMF1* genes, in a 1 : 1 ratio, produces a significant elevation of enzyme activity [21,22]. However, the optimal ratio between the individual sulfatase and *SUMF1* has not been fully investigated to date. Taken together, all of these data indicate that secretion of GALNS and the effect of *SUMF1* coexpression are affected by cell type, and also demonstrate the importance of defining the optimal ratio of sulfatase and SUMF1 genes.

Bone dysplasia is one of the most important clinical obstacles in Morquio A patients [2]. Therefore, the enzyme and/or vector should be delivered mainly to bone cells. Gene therapy studies for LSDs often use the liver as a 'factory' to produce and secrete the enzyme, which is taken up in nontransduced cells via the mannose 6-phosphate receptor [75,76]. This mechanism of cross-correction has allowed pathology correction in spleen, heart, eye, ear, bone and liver, in MPS I [19,77], MPS II [78] and MPS VII [17,79] animal models. In future *in vivo* studies, we can expect that, after an intravenous infusion of the vector, the liver will be the main transduced tissue [80], and the enzyme will be secreted extracellularly to be taken up by nontransduced cells. Although the biodistribution of AAV2-derived vectors has been well characterized [80], their delivery to bone has not been confirmed. Our preliminary *in vivo* results also suggest that AAV2 vectors are not delivered directly to bone (data not shown). However, we have previously shown that inclusion of a bone-tag sequence in the N-terminus of the mature GALNS enzyme significantly increases the retention time in bone, and allows substantial clearance of the storage material [81]. Therefore, to improve the distribution of the enzyme to bone, an AAV vector encoding a bone-targeting enzyme should be considered.

Conclusions

We have demonstrated that eukaryotic promoters can increase GALNS activity in transduced cells to levels comparable to those obtained with the commonly used CMV promoter. This fact could have a significant impact on the reduction of potential side effects and/or immune reactions against a recombinant protein in *in vivo* experiments. We have also observed that the CMV promoter in an AAV vector may not be silenced, which supports previous studies showing long-term expression with the use of CMV-bearing AAV vectors. Thus, the use of AAV-based vectors could avoid or substantially delay the CMV promoter silencing process by an unknown mechanism. In addition, we showed that *SUMF1* coexpression allowed a substantial increase in GALNS activity in transduced cells and their media, indicating the advantage of coexpression of *SUMF1* and *GALNS*. The effect of *SUMF1* coexpression on the sulfatase activity is influenced by mutual interactions among different types of promoters, target cells, sulfatases and the ratio between the sulfatase and SUMF1. Overall, the current *in vitro* data suggest that combinations of eukaryotic promoters, especially AAT–GALNS and CMV–SUMF1 cotransduction, will be the optimal choices for future *in vivo* studies with MPS IVA mouse models. We will clarify the following issues through future long-term *in vivo* studies: (a) evaluation of silencing of the promoter, and the resultant level of coexpression of *SUMF1* and *GALNS*; and (b) confirmation of targeting of the expressed enzyme into affected chondrocytes and their pathological improvement.

Experimental procedures

Plasmid construction

The pAAV–CMV–GALNS plasmid was previously constructed [27], carrying human *GALNS* cDNA with a CMV promoter flanked by the inverted terminal repeats of AAV2. The pAAV–AAT–GALNS plasmid was constructed by replacement of the CMV promoter in pAAV–CMV–GALNS with a 0.4 kb fragment of the AAT promoter (kindly provided by K. Ponder, Washington University in St Louis). The pAAV–EF1–GALNS plasmid was constructed by replacement of the CMV promoter in pAAV–CMV–GALNS with a 1.2 kb fragment of the EF1 promoter (kindly provided by T. Sferra, Ohio State University) [18]. The pAAV–CMV–SUMF1 plasmid, carrying human *SUMF1* cDNA, was constructed by replacing the *GALNS* cDNA portion in pAAV–CMV–GALNS with the 1.2 kb fragment of human *SUMF1* cDNA.

Production and purification of AAV vectors

AAV vectors were produced by calcium phosphate-mediated cotransfection of pAAV–CMV–GALNS, pAAV–AAT–GALNS or pAAV–CMV–SUMF1 with pXX2 and pXX6-80 (Gene Therapy Center, University of North Carolina at Chapell Hill, NC, USA). HEK293 cells (ATCC CRL-1573) were seeded on 15 cm culture plates, and the culture medium [DMEM (Gibco, Carlsbad, CA,

USA) supplemented with fetal bovine serum 15%, penicillin 100 $U \cdot mL^{-1}$ and streptomycin 100 $U \cdot mL^{-1}$] was removed immediately before starting the transfection. Plasmids were mixed in 18 : 18 : 54 μg ratio (a 1 : 1 : 1 molar ratio) with 0.25 M $CaCl_2$ and 2× HeBS buffer (280 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM Hepes, pH 7.1), and the mixture was immediately dispensed into the culture plates. Forty-eight hours after transfection, cells were harvested, resuspended in AAV lysis buffer (0.15 M NaCl, 50 mM Tris/HCl, pH 8.5), and lysed by three freeze–thaw cycles. The solution was clarified by centrifugation at 4 °C for 20 min. AAV vectors were purified by iodixanol gradient (Sigma-Aldrich, Saint Louis, MO, USA) and affinity chromatography as previously described [82]. Quantification was carried out with a spectrophotometric method, based on the extinction coefficient of the AAV2 capsid proteins and genome [83]. The yield of the packaging process was measured by comparing the experimental $A_{260\ nm}/A_{280\ nm}$ ratio against a hypothetical $A_{260\ nm}/A_{280\ nm}$ ratio for a preparation without empty capsids (100% yield) [83].

In vitro experiments

HEK293 cells, normal human skin fibroblasts or MPS IVA human skin fibroblasts were used. For transduction experiments, 1×10^5 HEK293 cells per well were seeded in 24-well plates and transduced with 1×10^{10} vg (1×10^5 vg per cell) of CMV–GALNS, AAT–GALNS or EF1–GALNS. Nontransduced cells were used as controls. After 24 h, the medium was changed to one containing 0.4 $mg \cdot mL^{-1}$ geneticin (Gibco, Carlsbad, CA, USA). GALNS activity in the medium and cell lysate was measured 2, 4, 8 and 10 days post-transduction. For *SUMF1* coexpression experiments, 1×10^5 HEK293 cells or MPS IVA fibroblasts were seeded in 24-well plates and cotransduced with 1×10^{10} vg (1×10^5 vg per cell) of CMV–GALNS, AAT–GALNS or EF1–GALNS with CMV/SUMF1 in a 1 : 0, 1 : 1 or 1 : 2 ratio. After 4 days, GALNS activity was measured in the medium and cell lysate. The wild-type and $Galns^{-/-}$ mouse chondrocytes were isolated and cultured as previously described [84]. Chondrocytes were grown up to 60–70% confluence to avoid differentiation, and were cotransduced with 1×10^{10} vg (1×10^5 vg per cell) of CMV–GALNS, AAT–GALNS or EF1–GALNS with CMV/SUMF1 in a 1 : 0 or 1 : 2 ratio. GALNS activity was measured for 4 days post-transduction in the medium and cell lysate. All cells were lysed by resuspension in 1% sodium deoxycholate (Sigma-Aldrich, Saint Louis, MO, USA). All transductions were carried out in triplicate.

GALNS enzyme activity

GALNS activity was assayed with 4-methylumbeliferyl-β-D-galactopyranoside-6-sulfate (Toronto Chemicals Research, North York, Canada) as a substrate. The enzyme assay was performed as described previously [85]. One unit was defined as the catalysis of 1 nmol of substrate h^{-1}. GALNS activity was expressed as $U \cdot mL^{-1}$ (medium) or $U \cdot mg^{-1}$ protein (cell lysate), as determined by micro-Lowry assay.

Viral DNA and qRNA

For viral DNA and RNA analysis 2×10^5 HEK293 cells were seeded in six-well plates and cultured as previously described. Cells were transduced with 2×10^{10} vg of CMV–GALNS, AAT–GALNS or EF1–GALNS, and harvested 2, 4, 8 and 10 days post-transduction. All assays were carried out in duplicate. Total DNA and RNA were isolated with the AllPrep DNA/RNA miniprep kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Viral DNA was amplified from 1 μg of total DNA with the primers TOMF23 (5′-acagggccattgatggcctcaacctcct-3′) and TOMF34R (5′-gcttcgtgtggtcttccagattgtgagttg-3′), which amplify a 235 bp fragment of human GALNS cDNA. PCR products were visualized in a 1.5% agarose gel, and band density (intensity per mm^2) was measured using IMAGE J 1.38 x (http://rsb.info.nih.gov/ij/, National Institutes of Health, USA). Band density was compared with a standard curve of pAAV–CMV–GALNS between 500 pg and 5 fg. First-strand cDNA was synthesized using the SuperScript II First-Strand Synthesis System kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, with 1 μg of total RNA. Viral cDNA was quantified by real-time PCR with the Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions, with 20 ng of first-strand product. Threshold cycles (C_T) of *GALNS* amplification curves were normalized to C_T values of human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Results were expressed as the increase of the GALNS C_T/GAPDH C_T ratio as compared with the values observed in nontransduced HEK293 cells.

Statistical analysis

Differences between groups were tested for statistical significance by using Student's *t*-test. An error level of 5% ($P < 0.05$) was considered to be significant. All analyses were performed with SPSS 13.0 for Macintosh (SPSS, Chicago, IL, USA). All results are shown as mean ± standard deviation.

Authors' contributions

C. J. Alméciga-Díaz performed the experiments, helped to conceive and design the experiments and drafted the manuscript. A. M. Montaño conceived and designed the experiments, and helped in analysis of the results and drafting of the manuscript. S. Tomatsu and L. A.

Barrera conceived the study, its design and coordination, and helped to draft the manuscript. All of the authors read and approved the final manuscript.

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References

1 Neufeld E & Muenzer J (2001) The mucopolysaccharidoses. In *The Metabolic and Molecular Bases of Inherited Diseases* (Scriver C, Beaudet A, Sly W & Valle D eds), pp. 3421–3452. McGraw-Hill, New York.
2 Montaño AM, Tomatsu S, Gottesman G, Smith M & Orii T (2007) International Morquio A registry: clinical manifestation and natural course of Morquio A disease. *J Inherit Metab Dis* **30**, 165–174.
3 Ashworth JL, Biswas S, Wraith E & Lloyd IC (2006) Mucopolysaccharidoses and the eye. *Surv Ophthalmol* **51**, 1–17.
4 Vellodi A, Young EP, Cooper A, Wraith JE, Winchester B, Meaney C, Ramaswami U & Will A (1997) Bone marrow transplantation for mucopolysaccharidosis type I: experience of two British centres. *Arch Dis Child* **76**, 92–99.
5 Tomatsu S, Montaño A, Ohashi A, Oikawa H, Oguma T, Dung V, Nishioka T, Orii T & Sly W (2008) Enzyme replacement therapy in a murine model of Morquio A syndrome. *Hum Mol Genet* **17**, 815–824.
6 Beutler E (2006) Lysosomal storage diseases: natural history and ethical and economic aspects. *Mol Genet Metab* **88**, 208–215.
7 Dobbelstein M (2003) Viruses in therapy – royal road or dead end? *Virus Res* **92**, 219–221.
8 Bosch A, Perret E, Desmaris N & Heard JM (2000) Long-term and significant correction of brain lesions in adult mucopolysaccharidosis type VII mice using recombinant AAV vectors. *Mol Ther* **1**, 63–70.
9 Herzog R, Yang E, Couto L, Hagstrom J, Elwell D, Fields P, Burton M, Bellinger D, Read M, Brinkhous K *et al.* (1999) Long-term correction of canine hemophilia B by gene transfer of blood coagulation factor IX mediated by adeno-associated viral vector. *Nat Med* **5**, 56–63.
10 Mah C, Cresawn K, Fraites T, Pacak C, Lewis M, Zolotukhin I & Byrne B (2005) Sustained correction of glycogen storage disease type II using adeno-associated virus serotype 1 vectors. *Gene Ther* **12**, 1405–1409.
11 Collas P (1998) Modulation of plasmid DNA methylation and expression in zebrafish embryons. *Nucleic Acids Res* **26**, 4454–4461.
12 Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JJE, Ozelo MC, Hoots K, Blatt P, Konkle B *et al.* (2006) Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* **12**, 342–347.
13 Nakai H, Herzog R, Hagstrom J, Walter J, Kung S, Yang E, Tai S, Iwaki Y, Kurtzman G, Fisher K *et al.* (1998) Adeno-associated viral vector-mediated gene transfer of human blood coagulation factor IX into mouse liver. *Blood* **91**, 4600–4607.
14 Mingozzi F, Hasbrouck NC, Basner-Tschkarajan E, Edmonson SA, Hui DJ, Sabatino DE, Zhou S, Wright JF, Jiang H, Pierce GF *et al.* (2007) Modulation of tolerance to the transgene product in a non-human primate model of AAV-mediated gene transfer to liver. *Blood* **110**, 2334–2341.
15 Mochizuki S, Mizukami H, Kume A, Muramatsu S, Takeuchi K, Matsushita T, Okada T, Kobayashi E, Hoshika A & Ozawa K (2004) Adeno-associated virus (AAV) vector-mediated liver- and muscle-directed transgene expression using various kinds of promoters and serotypes. *Gene Ther Mol Biol* **8**, 9–18.
16 Nathwani AC, Gray JT, McIntosh J, Ng CYC, Zhou J, Spence Y, Cochrane M, Gray E, Tuddenham EGD & Davidoff AM (2007) Safe and efficient transduction of the liver after peripheral vein infusion of self-complementary AAV vector results in stable therapeutic expression of human FIX in nonhuman primates. *Blood* **109**, 1414–1421.
17 Ponder KP, Melniczek JR, Xu L, Weil MA, O'Malley TM, O'Donnell PA, Knox VW, Aguirre GD, Mazrier H, Ellinwood NM *et al.* (2002) Therapeutic neonatal hepatic gene therapy in mucopolysaccharidosis VII dogs. *Proc Natl Acad Sci USA* **99**, 13102–11307.
18 Sferra T, Backstrom K, Wang C, Rennard R, Miller M & Hu Y (2004) Widespread correction of lysosomal storage following intrahepatic injection of a recombinant adeno-associated virus in the adult MPS VII mouse. *Mol Ther* **10**, 478–491.
19 Traas AM, Wang P, Ma X, Tittiger M, Schaller L, O'Donnell P, Sleeper MM, Vite C, Herati R, Aguirre GD *et al.* (2007) Correction of clinical manifestations of canine mucopolysaccharidosis I with neonatal retroviral vector gene therapy. *Mol Ther* **15**, 1423–1431.
20 Fraldi A, Biffi A, Lombarda A, Visigalli I, Pepe S, Setiembre C, Nusco E, Auricchio A, Naldini L, Ballabio A *et al.* (2007) SUMF1 enhances sulfatase activities in vivo in five sulfatase deficiencies. *Biochem J* **403**, 305–312.
21 Fraldi A, Hemsley H, Crawley A, Lombardi A, Lau A, Sutherland L, Auricchio A, Bollabio A & Hopwood J (2007) Functional correction of CNS lesions in MPS-

IIIA mouse model by intracerebral AAV-mediated delivery of *sulfamidase* and SUMF1 genes. *Hum Mol Genet* **16**, 2693–2702.
22 Kurai T, Hisayasu S, Kitagawa R, Migita M, Suzuki H, Hirai Y & Shimada T (2007) AAV1 mediated co-expression of formylglycine-generating enzyme and arylsulfatase A efficiently corrects sulfatide storage in a mouse model of metachromatic leukodystrophy. *Mol Ther* **15**, 38–43.
23 Toietta G, Severini G, Traversari C, Tomatsu S, Sukegawa K, Fukuda S, Kondo N, Tortora P & Bordignon C (2001) Various cells retrovirally transduced with N-acetylgalactosoamine-6-sulfate sulfatase correct Morquio skin fibroblasts in vitro. *Hum Gene Ther* **12**, 2007–2016.
24 Brooks A, Harkins R, Wang P, Qian H, Liu P & Rubanyi G (2004) Transcriptional silencing is associated with extensive methylation of the CMV promotor following adenoviral gene delivery to muscle. *J Gene Med* **6**, 395–404.
25 Prosch S, Stein J, Staak K, Liebenthal C, Volk HD & Kruger DH (1996) Inactivation of the very strong HCMV immediate early promoter by DNA CpG methylation in vitro. *Biol Chem Hoppe Seyler* **377**, 195–201.
26 Foti S, Samulski R & McCown T (2009) Delivering multiple gene products in the brain from a single adeno-associated virus vector. *Gene Ther* **16**, 1314–1319.
27 Gutierrez M, Garcia-Vallejo F, Tomatsu S, Ceron F, Alméciga-Díaz C, Domínguez M & Barrera L (2008) Construction of an adenoassociated virus-derived vector for the treatment of Morquio A disease. *Biomedica* **28**, 448–459.
28 Ito T, Yamamoto S, Hayashi T, Kodera M, Mizukami H, Ozawa K & Muramatsu S (2009) A convenient enzyme-linked immunosorbent assay for rapid screening of anti-adeno-associated virus neutralizing antibodies. *Ann Clin Biochem* **46**, 508–510.
29 Han Z, Berendzen K, Zhong L, Surolia I, Chouthai N, Zhao W, Maina N, Srivastava A & Stacpoole P (2008) A combined therapeutic approach for pyruvate dehydrogenase deficiency using self-complementary adeno-associated virus serotype-specific vectors and dichloroacetate. *Mol Genet Metab* **93**, 381–387.
30 Teo E, Cross K, Bomsztyk E, Lyden D & Spector JA (2009) Gene therapy in skin: choosing the optimal viral vector. *Ann Plast Surg* **62**, 576–580.
31 Goodrich L, Choi V, Carbone B, McIlwraith C & Samulski R (2009) Ex vivo serotype-specific transduction of equine joint tissue by self-complementary adeno-associated viral vectors. *Hum Gene Ther* **20**, 1697–1702.
32 Alexander I, Cunningham S, Logan G & Cristodoulou J (2008) Potential of AAV vectors in the treatment of metabolic disease. *Gene Ther* **15**, 831–839.
33 Carter B (2005) Adeno-associated virus vectors in clinical trials. *Hum Gene Ther* **16**, 541–550.
34 Wu Z, Asokan A & Samulski R (2006) Adeno-associated virus serotypes: vector toolkit for human gene therapy. *Mol Ther* **14**, 316–327.
35 Hacein-Bey-Abina S, Garrigue A, Wang GP, Soulier J, Lim A, Morillon E, Clappier E, Caccavelli L, Delabesse E, Beldjord K *et al.* (2008) Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest* **118**, 3132–3142.
36 Edelstein ML, Abedi MR & Wixon J (2007) Gene therapy clinical trials worldwide to 2007 – an update. *J Gene Med* **9**, 833–842.
37 Zaiss AK & Muruve DA (2005) Immune responses to adeno-associated virus vectors. *Curr Gene Ther* **5**, 323–331.
38 Donsante A, Miller DG, Li Y, Vogler C, Brunt EM, Russell DW & Sands MS (2007) AAV vector integration sites in mouse hepatocellular carcinoma. *Science* **317**, 477.
39 Papadakis E, Nicklin S, Baker A & White S (2004) Promoters and control elements: designing expression cassettes for gene therapy. *Curr Gene Ther* **4**, 89–113.
40 Huang M & Gorman C (1990) Intervening sequences increase efficiency of RNA 3′ procesing and accumulation of cytoplasmic RNA. *Nucleic Acids Res* **18**, 937–947.
41 Valencia P, Dias AP & Reed R (2008) Splicing promotes rapid and efficient mRNA export in mammalian cells. *Proc Natl Acad Sci USA* **105**, 3386–3391.
42 Zhao C & Hamilton T (2007) Introns regulate the rate of unstable mRNA decay. *J Biol Chem* **282**, 20230–20237.
43 Azzoni AR, Ribeiro SC, Monteiro GA & Prazeres DM (2007) The impact of polyadenylation signals on plasmid nuclease-resistance and transgene expression. *J Gene Med* **9**, 392–402.
44 Xu ZL, Mizuguchi H, Ishii-Watabe A, Uchida E, Mayumi T & Hayakawa T (2002) Strength evaluation of transcriptional regulatory elements for transgene expression by adenovirus vector. *J Control Release* **81**, 155–163.
45 Yew NS, Wysokenski DM, Wang KX, Ziegler RJ, Marshall J, McNeilly D, Cherry M, Osburn W & Cheng SH (1997) Optimization of plasmid vectors for high-level expression in lung epithelial cells. *Hum Gene Ther* **8**, 575–584.
46 Stoneley M & Willis AE (2004) Cellular internal ribosome entry segments: structures, trans-acting factors and regulation of gene expression. *Oncogene* **23**, 3200–3207.
47 Hennecke M, Kwissa M, Metzger K, Oumard A, Kroger A, Schirmbeck R, Reimann J & Hauser H (2001) Composition and arrangement of genes define the strength of IRES-driven translation in bicistronic mRNAs. *Nucleic Acids Res* **29**, 3327–3334.

48 Vargas J Jr, Gusella GL, Najfeld V, Klotman ME & Cara A (2004) Novel integrase-defective lentiviral episomal vectors for gene transfer. *Hum Gene Ther* **15**, 361–372.
49 Kim I, Józkowicz A & Piedra P (2001) Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector. *Proc Natl Acad Sci USA* **98**, 13282–13287.
50 Watson G, Sayles J, Chen C, Elliger S, Elliger C, Raju N, Kurtzman G & Podsakoff G (1998) Treatment of lysosomal storage disease in MPS VII mice using a recombinant adeno-associated virus. *Gene Ther* **5**, 1642–1649.
51 Toniatti C, Bujard H, Cortese R & Ciliberto C (2004) Gene therapy progress and prospects: transcription regulatory systems. *Gene Ther* **11**, 649–657.
52 Ghazizadeh S, Carroll JM & Taichman LB (1997) Repression of retrovirus-mediated transgene expression by interferons: implications for gene therapy. *J Virol* **71**, 9163–9169.
53 Qin L, Ding Y, Pahud DR, Chang E, Imperiale MJ & Bromberg JS (1997) Promoter attenuation in gene therapy: interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression. *Hum Gene Ther* **8**, 2019–2029.
54 Alméciga-Díaz C, Rueda-Paramo M, Espejo A, Echeverri O, Montano A, Tomatsu S & Barrera L (2009) Effect of elongation factor 1α promoter and SUMF1 over in-vitro expression of N-acetylgalactosamine-6-sulfate sulfatase. *Mol Biol Rep* **36**, 1863–1870.
55 Chan K, Wu S, Nissom P, Oh S & Choo A (2008) Generation of high-level stable transgene expressing human embryonic stem cell lines using Chinese hamster elongation factor-1 alpha promoter system. *Stem Cells Dev* **17**, 825–836.
56 Gerolami R, Uch R, Jordier F, Chapel S, Bagnis C, Bréchot C & Mannoni P (2000) Gene transfer to hepatocellular carcinoma: transduction efficacy and transgene expression kinetics by using retroviral and lentiviral vectors. *Cancer Gene Ther* **7**, 1286–1292.
57 Jenke A, Scinteie M, Stehle I & Lipps H (2004) Expression of a transgene encoded on a non-viral episomal vector is not subject to epigenetic silencing by cytosine methylation. *Mol Biol Rep* **31**, 85–90.
58 Krishnan M, Park J, Cao F, Wang D, Paulmurugan R, Tseng J, Gonzalgo M, Gambhir S & Wu J (2006) Effects of epigenetic modulation on reporter gene expression: implications for stem cell imaging. *FASEB J* **20**, 106–108.
59 Zhao G, Karageorgos L, Hutchinson R, Hoopwood J & Hemsley K (2010) Genetic manipulation of murine embryonic stem cells with enhanced green fluorescence protein and sulfatase-modifying factor I genes. *Cytotherapy* **12**, 400–407.
60 Stilwell JL & Samulski RJ (2004) Role of viral vectors and virion shells in cellular gene expression. *Mol Ther* **9**, 337–346.
61 McCaffrey AP, Fawcett P, Nakai H, McCaffrey RL, Ehrhardt A, Pham TT, Pandey K, Xu H, Feuss S, Storm TA *et al*. (2008) The host response to adenovirus, helper-dependent adenovirus, and adeno-associated virus in mouse liver. *Mol Ther* **16**, 931–941.
62 Chung S, Ma X, Liu Y, Lee D, Tittiger M & Ponder KP (2007) Effect of neonatal administration of a retroviral vector expressing a-L-iduronidase upon lysosomal storage in brain and other organs in mucopolysaccharidosis I mice. *Mol Genet Metab* **90**, 181–192.
63 Wang B, O'Malley TM, Xu L, Vite C, Wang P, O'Donnell PA, Ellinwood NM, Haskins ME & Ponder KP (2006) Expression in blood cells may contribute to biochemical and pathological improvements after neonatal intravenous gene therapy for mucopolysaccharidosis VII in dogs. *Mol Genet Metab* **87**, 8–21.
64 Mount J, Herzog R & Tillson M (2002) Sustained phenotypic correction of hemophilia B dogs with a factor IX null mutation by liver-direct gene therapy. *Blood* **99**, 2670–2675.
65 Rodríguez-Antona C, Donato M, Boobis A, Edwards R, Watts P, Castell J & Gómez-Lechón M (2002) Cytochrome P450 expression in human hepatocytes and hepatoma cell lines: molecular mechanisms that determine lower expression in cultured cells. *Xenobiotica* **32**, 505–520.
66 Alon U (2007) Network motifs: theory and experimental approaches. *Nat Rev Genet* **8**, 450–461.
67 Sandelin A, Carninci P, Lenhard B, Ponjavic J, Hayashizaki Y & Hume D (2007) Mammalian RNA polymerase II core promoters: insights from genome-wide studies. *Nat Rev* **8**, 424–436.
68 Gill D, Smyth S, Goddard C, Pringle I, Higging C, Coledge W & Hyde S (2001) Increased persistence of lung gene expression using plasmids containing the ubiquitin C or elongation factor 1a promoter. *Gene Ther* **8**, 1539–1546.
69 Liu Y, Okada T, Nomoto T, Ke X, Kume A, Ozawa K & Xiao S (2007) Promoter effects of adeno-associated viral vector for transgene expression in the cochlea in vivo. *Exp Mol Med* **39**, 170–175.
70 Song S, Emburry J, Laipis P, Berns K, Crawford J & Flotte T (2001) Stable therapeutic serum levels of human alpha-1 antitrypsin (AAT) after portal vein injection of recombinant adeno-associated virus (AAV) vectors. *Gene Ther* **8**, 1299–1306.
71 Teschendorf C, Warrington K, Siemann D & Muzyczka N (2002) Comparison of the EF-1 alpha and the CMV promoter for engineering stable tumor cell lines using recombinant adeno-associated virus. *Anticancer Res* **22**, 3325–3330.

72 Xu L, Daly T, Gao C, Flotte T, Song S, Byrne B, Sands M & Ponder K (2001) CMV-β-actin promoter directs higher expression from an adeno-associated viral vector in the liver than the cytomegalovirus or elongation factor 1a promoter and results in therapeutic levels of human factor X in mice. *Hum Gene Ther* **12**, 563–573.
73 Cosma M, Pepe P, Annunziata I, Newbold R, Grompe M, Parenti G & Ballabio A (2003) The multiple sulfatase deficiency gene encodes an essential and limiting factor for the activity of sulfatases. *Cell* **113**, 445–456.
74 Takakusaki T, Hisayasu S, Hirai Y & Shimada T (2005) Coexpression of formylglycine-generating enzyme is essential for synthesis and secretion of functional arylsulfatase A in a mouse model of metachromatic leukodystrophy. *Hum Gene Ther* **16**, 929–936.
75 Ponder K & Haskins M (2007) Gene therapy for mucopolysaccharidosis. *Expert Opin Biol Ther* **7**, 1333–1345.
76 Sands M & Davidson B (2006) Gene therapy for lysosomal storage diseases. *Mol Ther* **13**, 839–849.
77 Liu Y, Xu L, Henning A, Kovacs A, Fu A, Chung S, Lee D, Wang B, Herati R, Ogilvem J *et al*. (2005) Liver-directed neonatal gene therapy prevents cardiac, bone, ear, and eye disease in mucopolysaccharidosis I mice. *Mol Ther* **11**, 35–47.
78 Cardone M, Polito VA, Pepe S, Mann L, D'Azzo A, Auricchio A, Ballabio A & Cosma MP (2006) Correction of Hunter syndrome in the MPSII mouse model by AAV2/8-mediated gene delivery. *Hum Mol Genet* **15**, 1225–1236.
79 Mango RL, Xu L, Sands MS, Vogler C, Seiler G, Schwarz T, Haskins ME & Ponder KP (2004) Neonatal retroviral vector-mediated hepatic gene therapy reduces bone, joint, and cartilage disease in mucopolysaccharidosis VII mice and dogs. *Mol Genet Metab* **82**, 4–19.
80 Zincarelli C, Soltys S, Rengo G & Rabinowitz JE (2008) Analysis of AAV serotypes 1–9 mediated gene expression and tropism in mice after systemic injection. *Mol Ther* **16**, 1073–1080.
81 Tomatsu S, Montano AM, Dung V, Ohashi A, Oikawa H, Oguma T, Orii T, Barrera L & Sly W (2010) Enhacement of drug delivery: enzyme-replacement therapy for murine Morquio A syndrome. *Mol Ther* **18**, 1094–1102.
82 Zolotukhin S, Byrne B, Mason E, Zolotukhin I, Potter M, Chesnut K, Summerford C, Samulski R & Muzyczka N (1999) Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Ther* **6**, 973–985.
83 Sommer M Jr, Smith PH, Parthasarathy S, Isaacs J, Vijay S, Kieran J, Powell SK, McClelland A & Wright JF (2003) Quantification of adeno-associated virus particles and empty capsids by optical density measurement. *Mol Ther* **7**, 122–128.
84 Tomatsu S, Orii K, Vogler C, Nakayama J, Levy B, Grubb J, Gutierrez M, Shim S, Yamaguchi S, Nishioka T *et al*. (2003) Mouse model of N-acetylgalactosamine-6-sulfate sulfatase deficiency (Galns–/–) produced by targeted disruption of the gene defective in Morquio A disease. *Hum Mol Genet* **12**, 3349–3358.
85 van Diggelen O, Zhao H, Kleijer W, Janse H, Poorthuis B, Pelt Jv, Kamerling J & Galjaard H (1990) A fluorometric enzyme assay for the diagnosis of Morquio disease type A (MPS IVA). *Clin Chem Acta* **187**, 131–140.