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The Cellular Processing Capacity Limits the Amounts of Chimeric U7 snRNA Available for Antisense Delivery

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Many genetic diseases are induced by mutations disturbing the maturation of pre-mRNAs, often affecting splicing. Antisense oligoribonucleotides (AONs) have been used to modulate splicing thereby circumventing the deleterious effects of mutations. Stable delivery of antisense sequences is achieved by linking them to small nuclear RNA (snRNAs) delivered by viral vectors, as illustrated by studies where therapeutic exon skipping was obtained in animal models of Duchenne muscular dystrophy (*DMD*). Yet, clinical translation of these approaches is limited by the amounts of vector to be administered. In this respect, maximizing the amount of snRNA antisense shuttle delivered by the vector is essential. Here, we have used a muscle- and heart-specific enhancer (MHCK) to drive the expression of U7 snRNA shuttles carrying antisense sequences against the human or murine DMD pre-mRNAs. Although antisense delivery and subsequent exon skipping were improved both in tissue culture and in vivo, we observed the formation of additional U7 snRNA by-products following gene transfer. These included aberrantly 3′ processed as well as unprocessed species that may arise because of the saturation of the cellular processing capacity. Future efforts to increase the amounts of functional U7 shuttles delivered into a cell will have to take this limitation into account.

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INTRODUCTION

A variety of mRNAs with different exon composition, stability or subcellular localization, as well as small RNAs with regulatory roles can be produced from a single transcription unit, thus increasing the complexity of genome information.[1] A large proportion of disease-associated mutations in humans affect the processing of pre-mRNAs[2] often by disturbing the delicate balance of alternative splicing, which is observed in over 90% of transcription units.[3] The modulation of pre-mRNA splicing has been proposed as an approach to compensate the deleterious effect of mutations.[4] It can be achieved with a high-specificity, using antisense oligoribonucleotides (AON) that mask key splicing signals through Watson-Crick pairing with the pre-mRNA. The successful AON-mediated restoration of correct splicing has been reported in a number of cellular and animal models of genetic diseases.[5] In particular cases, AONs can be exploited to induce exon skipping in such a way that it removes deleterious mutations or re-establish a reading frame disrupted by a deletion. This approach is actively pursued in clinical trials involving patients with Duchenne muscular dystrophy (DMD).[6–9]

A limitation of AON-based therapies for treating chronic genetic diseases such as DMD is the need for repeated administration of high doses of oligonucleotides (up to several mg/kg). A sustained intracellular production of antisense sequences can be obtained using modified small nuclear (sn) RNAs such as U1, U2, or U7 delivered by viral vectors.[10–12] We and others have demonstrated the efficacy of chimeric snRNAs for sustained therapeutic exon skipping in animal models of DMD, following local or systemic gene delivery with AAV vectors targeting skeletal muscles, heart, and brain.[13–16] Yet, clinical applications to DMD patients by systemic administration of AAV vectors carrying a modified snRNA will require very high doses that are potentially toxic and represent a challenge for the current capacity of vector manufacturing. Reducing the injected dose is paramount to the success of current translational studies that aim at implementing vector mediated exon skipping into the clinic. It may be achieved in part by using the appropriate AAV serotype for muscle and heart delivery,[14] but also by optimizing the amount of chimeric snRNAs transcribed and assembled into active snRNPs.

The Sm-class snRNA genes are transcribed by RNA polymerase II in association with a specialized complex called Integrator. The polymerase initiate transcription on short bipartite promoters composed of proximal and distal sequence elements (PSE and DSE, respectively), while the Integrator complex recognizes signals in the internal stem-loop structures of the coding sequence, as well as in a conserved 3′ box and mediates co-transcriptional 3′ processing.[17] The snRNAs are then exported to the cytoplasm where they undergo further modifications and assemble with Sm proteins before re-entry into the nucleus. Proper assembly requires a consensus Sm binding site.[18] U7 is a non-spliceosomal snRNA that functions as a processing factor of the non-polyadenylated replication dependent histone mRNA. It is the shortest and simplest of

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snRNAs and it includes a 5′ antisense sequence recognizing the histone pre-mRNA, a non-consensus Sm-binding site and a stem-loop structure containing determinants for the co-transcriptional 3′ endonucleolytic cleavage. The low abundance of the U7 small nuclear ribonucleoproteins (snRNP) is reversed when a consensus spliceosomal Sm sequence is introduced (SmOPT) and substitutions of the original 5′ sequences result in abundant and stable shuttles for ectopic antisense delivery.[10,19]

Here, we have added a strong muscle- and heart-specific transcriptional enhancer upstream of the DSE in chimeric *U7* genes carrying antisense sequences for exon skipping on the human or murine *DMD* pre-mRNAs. We have observed that although the amount of transcripts produced by the U7 promoter is readily enhanced, many of them are not processed and may not be assembled in snRNPs with exon-skipping activity on the *DMD* gene pre-mRNA. In addition, the SmOPT sequence required for stabilizing the U7 transcript induces the generation of 3′-processed species missing 20 nucleotides (nt), whose stability or activity may be affected. We conclude that the potential for enhancing exon-skipping activity of chimeric U7 cassettes is limited by the snRNA processing capacity of the host cell.

RESULTS

Design and validation of an enhanced U7 cassette

Brun *et al*. have shown that transcription from the U7 promoter can be increased by placing an enhancer upstream of the Distal Sequence Element.[20] Based on this observation, we have constructed a U7 cassette in which a strong muscle-specific transcriptional enhancer was inserted (**Figure 1a**). Salva *et al*. have designed such an enhancer by fusing elements from the α-myosin heavy chain (α-MHC) and the muscle creatin kinase (MCK) enhancers, and shown that, in combination with the CK promoter it results in a very strong and mostly tissue-specific enhancement of gene expression when used in AAV vectors.[21] We have assembled a similar enhancer [muscle- and heart-specific enhancer (MHCK), see Materials and Methods] and its activity was first validated in murine C2C12 myoblasts using an AAV vector carrying green fluorescent protein (GFP) under the control of the human PGK promoter (**Supplementary Figure S1**). The MHCK enhancer was then introduced upstream of a U7snRNA cassette containing an antisense sequence able to induce skipping of exon 51 on the human *DMD* pre-mRNA by masking an exonic splicing enhancer (U7ex51)[22] (**Figure 1a**). Lentiviral vectors carrying U7ex51 with or without the enhancer were constructed and human CHQ myoblasts were transduced with increasing multiplicities of infection (MOI, see Materials and Methods). Three days post-transduction, cells were differentiated into myotubes for 3 days and exon-skipping efficiency was analyzed by reverse transcription (RT)-PCR. The efficiency of skipping was evaluated by estimating the relative intensities of the skipped versus nonskipped diagnostic bands appearing after electrophoresis of RT-PCR products on agarose gels (**Figure 1b**). The results indicate a dose-dependent exon skipping for both vectors, with a higher activity for the one containing the MHCK enhancer, detectable at lower doses.

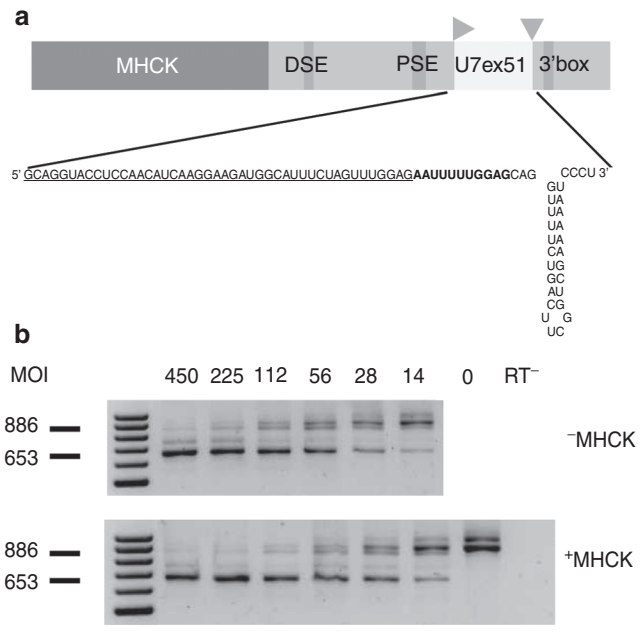

Figure 1 Muscle- and heart-specific enhancer (MHCK) enhances U7ex51-mediated exon skipping on the Duchenne muscular dystrophy (*DMD*) pre-mRNA in cultured human myotubes. (**a**) U7ex51 expression is driven by the U7 promoter containing distal and proximal sequence elements (DSE and PSE, respectively, shaded boxes). The MHCK enhancer[21] has been placed upstream. The rightward arrowhead indicates the site of transcription initiation and the downward arrowhead is the site of 3′ processing, activated by recognition of the 3′box (shaded). The sequence of the U7ex51 transcript is shown, including an antisense sequence (underlined) that corresponds to an exonic splicing enhancer (ESE) in exon 51 of the human *DMD* pre-mRNA (h51AON1: 5′-UCAAGGAAGAUGGCAUUUCU-3′).[22] The optimized Sm binding sequence (SmOPT) is shown in bold. (**b**) Reverse transcription and nested PCR detection of *DMD* exon 51 skipping after transduction of human CHQ cells with lentiviral vectors containing the U7ex51 (upper panel) or the MHCK-U7ex51 (lower panel) cassettes, at increasing multiplicities of infection (MOI) Controls include nontransduced cells (0) and no reverse transcriptase (RT–). The native human *DMD* mRNA containing exon 51 is detected as a 886-bp fragment and the skipped mRNA yields a 653-bp product.

In order to test the efficiency of the MHCK enhancer in the skeletal muscle of mdx mice, we introduced it into an AAV vector containing the U7DTex23 cassette that carries antisense sequences able to induce skipping of exon 23 in the murine *Dmd* pre-mRNA[13] (**Figure 2a**). AAV 2/5 vectors were produced with or without enhancer and used to transduce murine C2C12 myoblasts (see Materials and Methods). The U7DTex23 transcripts were analyzed by northern blot on total RNA isolated from transduced cells and normalized to the quantity of U2 snRNA (**Figure 2b**). Unexpectedly, two small RNA species reacted with a probe in the *Dmd* antisense region (**Figure 2a** and Materials and Methods). The predicted 86 nt U7DTex23 transcript was present along with equivalent quantities of a shorter species of ~66 nt. Altogether, the amounts of chimeric U7 RNA were enhanced in the presence of the MHCK sequences. This effect was clearly visible at the lower vector dose, presumably because nonsaturating levels of transcripts are made under these conditions. Of note, high-molecular weight RNA recognized by

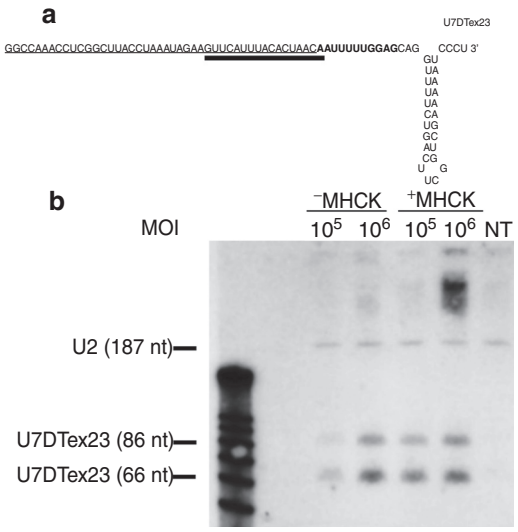

Figure 2 Enhancement of U7DTex23 small nuclear RNAs (snRNAs) expression in murine C2C12 cells. (**a**) The sequence of the U7DTex23 transcript is shown, including two antisense sequences (underlined) that encompass the donor splice site of exon 23 (SD23, shown in italics) and the branch point region of intron 22 (BP22) on the murine Duchenne muscular dystrophy (*Dmd*) pre-mRNA.[13] The optimized Sm-binding sequence (SmOPT) is shown in bold. The probe used for the northern blot analysis in (**b**) is underlined in bold. (**b**) Northern blot analysis of U7DTex23 transcripts expressed in C2C12 cells transduced with AAV2/5-U7DTex23 (–MHCK) or AAV2/5-MHCK-U7DTex23 (+MHCK) at different multiplicities of infection (MOI) (10^5 or 10^6 viral genome per cell). NT, nontransduced cells. The blot was hybridized with two 32P end-labeled probes recognizing the U2 snRNA and the U7DTex23 chimera (underlined in bold in (a)), respectively. U2 snRNA shows up as a 187-nt band. Two bands of 86 nt and ~66-nt react with the U7DTex23 probe.

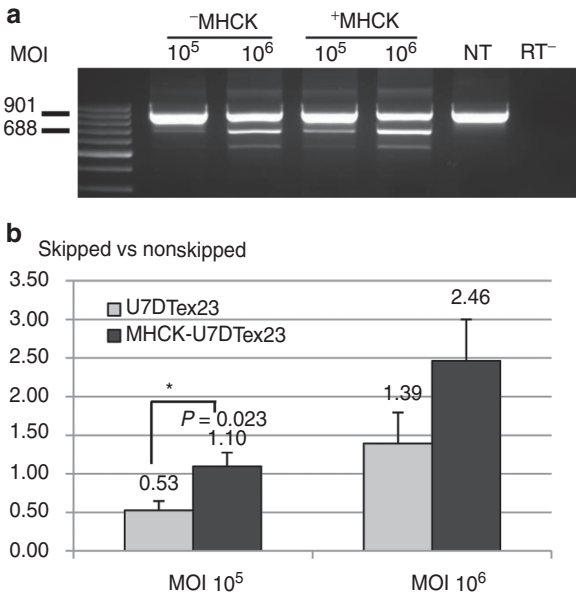

Figure 3 U7-mediated exon 23 skipping on the Duchenne muscular dystrophy (*Dmd*) pre-mRNA. (**a**) Reverse transcription and nested PCR detection of *Dmd* exon 23 skipping after transduction of C2C12 cells with AAV2/5-U7DTex23 (–MHCK) or AAV2/5-MHCK-U7DTex23 (+MHCK) with different multiplicities of infection (MOI) (10^5 or 10^6 viral genome per cell). Controls include nontransduced cells (NT) and no reverse transcriptase (RT–). The native *Dmd* mRNA containing exon 23 is detected as a 901-bp fragment and the skipped mRNA yields a 688-bp product. Results are representative of at least three independent transductions. (**b**) Analysis of mouse exon 23 skipping by quantitative PCR. The graph represents the ratio between the exon 22–24 junction (skipped mRNA species) and the exon 22–23 junction (full-length mRNA species). Quantification was performed on mRNA extract from C2C12 cells transduced with AAV2/5-U7DTex23 or AAV2/5-MHCK-U7DTex23 at the indicated MOI. A significant difference is observed at MOI 10^5 as shown by the *P* value ($P = 0.023$), according to Student's t-test. Error bars are shown as mean ± SEM ($n = 6$). MHCK, muscle- and heart-specific enhancer.

the probe accumulated at the high dose and in the presence of the enhancer. Further analysis indicated that it contained sequences 3′ of the U7-coding sequence and therefore represented unprocessed primary U7 RNA, possibly transcribed from tandem or circle copies of the AAV vector genome[23] (**Supplementary Figure S2**). It suggests that the integrator-mediated formation of the U7 snRNA 3′end may be limiting when the transcript is too abundant.

RNAs from transduced myoblasts were also analyzed by RT-PCR and quantitative RT-PCR (**Figure 3**). Consistently, the results indicate that the MHCK sequences increase exon 23 skipping, particularly at the lower dose of AAV.

The SmOPT sequences induce an additional processing of the U7snRNA cassette

To investigate the origin of the short RNA species detected with the antisense probe, variants of the U7snRNA cassette were analyzed. Human 293T cells were transfected with plasmids expressing an unmodified U7 which retains its natural 5′ sequences targeting Histone H1 mRNA or U7SmOPT, the parent construct for all U7 antisense shuttles.[19] An additional shorten transcript was generated only in the presence of the SmOPT mutations, suggesting that they were responsible for the extra processing (**Supplementary Figure S3**). Since probes at the 5′ extremity of the U7snRNA were used, the experiment also indicated that the processing removes the 3′ extremity. U7DTex23 cassettes with or without the SmOPT mutations were compared in AAV2/5 transductions of C2C12 myoblasts (**Figure 4**). The inclusion of the SmOPT mutations resulted in higher levels of chimeric U7[19] and the 3′ deleted U7 transcripts were enhanced accordingly. The higher molecular weight species were also proportionally increased in the presence of the SmOPT mutations. The cassette containing the original Sm sequences yielded lower levels of transcripts, which were not further processed in 3′, confirming that the SmOPT mutations determine this modification.

The amounts of the different U7-derived species (86 nt, 66 nt, and over 200 nt) were evaluated by densitometry of the northern blot showed in **Figure 4**. The 86 nt species represented 30% and 40% of all transcripts in lanes 1 and 2, respectively. The difference was due to the lower amounts of high-molecular weight unprocessed species at the lower MOI. In contrast, when the original Sm sequence was present, the 86-nt band accounted for >85% of total signal.

RT-PCR analysis of the *Dmd* pre-mRNA skipping was consistent with the U7snRNA expression analysis. The SmOPT construct induced a strong exon 23 skipping whereas the Sm construct was almost inactive (**Figure 4**, lower panel).

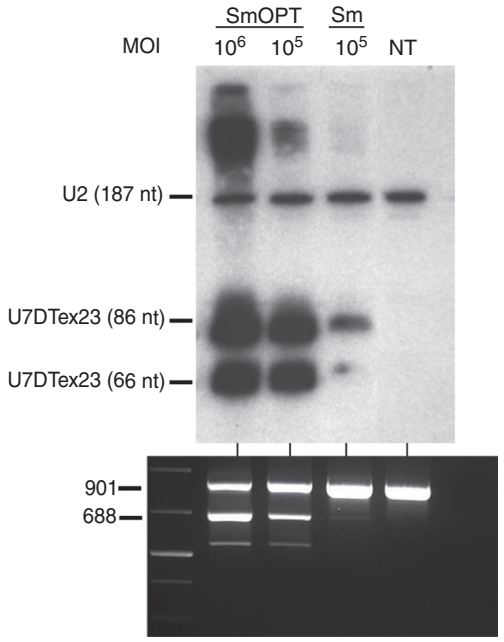

Figure 4 The SmOPT sequence induces an additional 3′ processing of the U7 small nuclear RNA (snRNA). Northern blot analysis of U7DTex23 transcripts expressed in C2C12 cells transduced with AAV2/5-U7DTex23 containing the SmOPT sequence (AAUUUUUGGAG, **Figure 1a**), or the native U7 Sm sequence (AAUUUGUCUAG). NT, nontransduced cells. The blot was hybridized with two 32P end-labeled probes recognizing the U2 snRNA and the U7DTex23 chimera, respectively. U2 snRNA shows up as a 187-nt band. Two bands of 86 nt and ~66-nt react with the U7DTex23 probe. The lower panel presents the analysis of exon 23 skipping performed in the same RNA samples. The native *Dmd* mRNA containing exon 23 is detected as a 901-bp fragment and the skipped mRNA yields a 688-bp product.

MHCK enhances dystrophin rescue in the skeletal muscle of mdx mice

In order to determine the efficiency of the MHCK containing construct *in vivo*, AAV2/8 vectors with or without the MHCK enhancer were produced. Mdx mice received doses of 10^{11}, 2×10^{10}, or 5×10^{9} viral genomes into the tibialis anterior. One month after injection, mice were sacrificed and protein and RNA were isolated from the injected muscles. U7DTex23 and U2snRNA were analyzed by northern blot, and exon 23 skipping efficiency was evaluated by RT-PCR and quantitative RT-PCR (**Figure 5a,b**). The northern blot analysis indicated that the processed U7 species was present and that the MHCK resulted in a modest increase of U7 levels in the muscle. This increase resulted in slightly higher efficiencies of skipping, although the differences analyzed by quantitative PCR were not significant. A stronger evidence of the MHCK enhancement was obtained by analyzing Dystrophin rescue in the injected tissues by western blot. At each dose analyzed, animals receiving the MHCK vector had three- to fivefold increased levels of rescued Dystrophin (**Figure 6**).

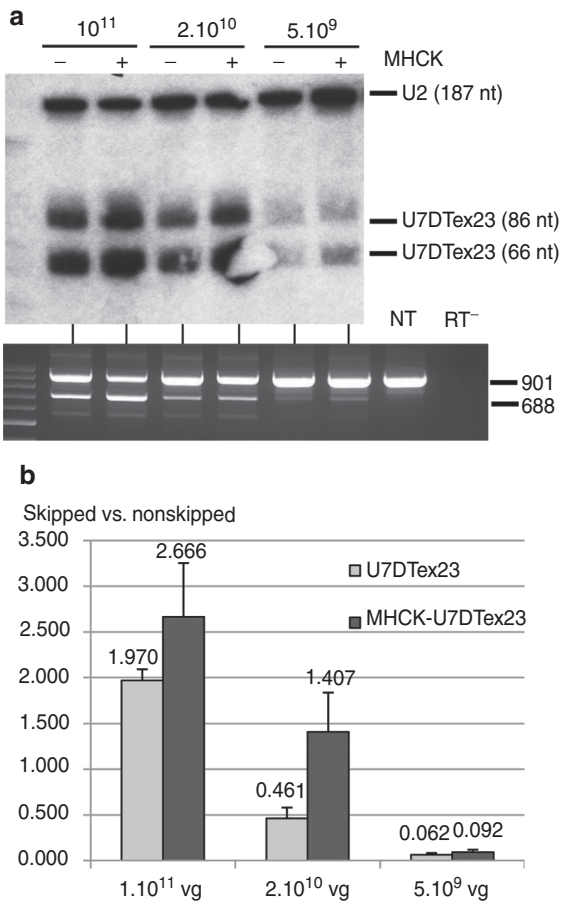

Figure 5 Muscle- and heart-specific enhancer (MHCK) enhances U7DTex23-mediated exon skipping on the Duchenne muscular dystrophy (*Dmd*) pre-mRNA in the muscle of mdx mice. (**a**) Northern blot analysis of U7DTex23 transcripts expressed in mdx mice muscles treated with AAV2/8-U7DTex23 (⁻MHCK) or AAV2/8-MHCK-U7DTex23 (⁺MHCK). Total injected vector doses in vector genomes are indicated on top. The blot was hybridized with two 32P end-labeled probes recognizing the U2 small nuclear RNA (snRNA) and the U7DTex23 chimera, respectively. U2 snRNA shows up as a 187-nt band. Two bands of 86 nt and ~66-nt react with the U7DTex23 probe. The lower panel presents the analysis of exon 23 skipping performed in the same RNA samples. The native *Dmd* mRNA containing exon 23 is detected as a 901-bp fragment and the skipped mRNA yields a 688-bp product. NT, nontransduced muscle; RT–, no reverse transcriptase. Results are representative of at least three independent transductions. (**b**) Quantitative PCR analysis of exon 23 skipping in RNA samples prepared from injected muscles. The graph represents the ratio between the exon 22–24 junction (skipped mRNA species) and the exon 22–23 junction (full-length mRNA species). No statistically significant difference could be observed between the groups (Student's t-test, $P > 0.05$). Error bars are shown as mean ± SEM ($n = 3$ per cohort).

DISCUSSION

The successful clinical translation of AAV-based approaches for exon skipping in diseases such as DMD will depend on our ability to improve the therapeutic index of the vectors. The effective dose should be sufficient to reach extended skeletal muscle territories and to introduce enough transcriptionally active copies of the vector genome in each cell nucleus, without compromising safety. Minimizing the dose will therefore be a combination of using an AAV vector with the appropriate tropism[14] and optimizing the snRNA transcription unit.

The promoters of *snRNA* genes are compact—around 250 base pairs (bp) in length—and very active. For example, the U1 promoter was shown to be equivalent to the immediate

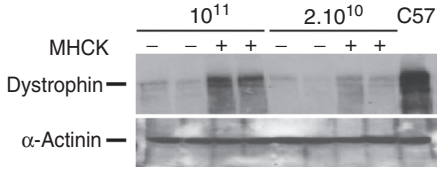

Figure 6 Muscle- and heart-specific enhancer (MHCK)-mediated enhancement of Duchenne muscular dystrophy (*Dmd*) exon skipping in mdx mice leads to an increased rescue of Dystrophin protein in treated muscles. Western blot analysis of dystrophin levels in muscles of mdx mice treated with AAV2/8-U7DTex23 ($^-$MHCK) or AAV2/8-MHCK-U7DTex23 ($^+$MHCK), at doses of 2×10^{10} or 1×10^{11} vg/muscle. Samples from two different animals are analyzed for each condition. The wild type C57 mouse Dystrophin is shown as a positive control and the endogenous α-actinin is detected for normalization.

early CMV enhancer/promoter for expressing a *lacZ* reporter gene.[24] Although the related U7 promoter is of comparable strength,[12,19,25] Brun *et al.* have shown that it can be boosted by a muscle-specific MCK enhancer.[20] Here, we have introduced a strong dual muscle- and heart-specific transcriptional enhancer (MHCK) upstream of a U7 shuttle for antisense sequences in order to evaluate the improvement of exon-skipping efficiency associated with a higher number of chimeric snRNA molecules in cultured myogenic cells and in the mouse skeletal muscle.

The effect of the enhancer on the amounts of snRNA produced in cultured cells or in vivo is modest relative to the one reported in association with the CK promoter and 5′ untranslated region.[21] This is likely due to the high constitutive activity of the U7 promoter. We have observed that transcription enhancement by the MHCK sequences also occurs in 293 cells, indicating that the ubiquitous nature of the U7 promoter overrides the tissue specificity of the enhancer (**Supplementary Figure S4**). This is consistent with reports showing that both MCK and MHC enhancers lose their tissue specificity when combined with constitutive promoters.[26]

Recombinant AAV genomes are found as either linear or circular monomers and concatemers. High doses of AAV vectors result in the formation of concatemers of the vector genome.[23,27] With increasing doses of vector applied to myogenic cells we observe the formation of high-molecular weight RNA species containing internal U7 sequences as well as sequences in the 3′ box. These are consistent with unprocessed read through transcripts generated from AAV concatemers. A similar dose-dependent accumulation of unprocessed U7 transcripts has been reported following injection of U7-based constructs in Xenopus oocytes and it was attributed to a saturation of the U7 3′ end processing machinery.[19,25] The fact that high-molecular weight U7 transcripts were not observed in the injected muscle is probably due to the lower MOI reached in vivo, but could also reflect a higher U7 processing capacity in muscle fibers compared to cultured myotubes.

Our data also indicate the presence in transfected or AAV transduced cells of a U7 species lacking 20 nt at the 3′ end. Such 3′-deleted forms of U7 chimeras have been observed previously[12] and we show here that they are a consequence of the SmOPT mutations. U7 is normally assembled into non-spliceosomal snRNP where a specific core of Sm proteins interacts with the Sm sequence.[28] Changing this sequence to SmOPT redirects U7 transcripts into snRNPs containing the canonical Sm proteins, which accumulate to a much higher levels than unmodified U7 transcripts.[19] As shown in **Figure 4**, the efficiency of U7 shuttles for antisense delivery critically depends on the presence of an SmOPT sequence.[10,29] The shortened U7 transcripts could be generated by a misprocessing of the 3′ end associated with mutations in the Sm sequences, as observed for U1.[30] So far however, only mutations in the 3′ box or in the U7 stem loop are known to affect processing.[31] More likely, the packaging of U7SmOPT into a spliceosomal snRNP may expose a cryptic nucleolytic site in the stem loop and trigger a partial cleavage or degradation during particle maturation.[18] It is unlikely that the misprocessed U7SmOPT species remain properly assembled into functional snRNPs and therefore functional for antisense delivery. Moreover, the shortened U7 RNAs are probably destabilized since the 3′ stem loop of snRNAs acts as a protection against exonucleases. Therefore, the steady-state ratio of full-length versus misprocessed U7 RNA observed by northern blot may in fact underestimate the proportion of transcripts that undergo removal of the terminal 20 nt.

Our study indicates that potentially inactive by-products of U7SmOPT shuttles may be produced following gene transfer. Further understanding of the formation of these molecules may allow improving the potency of U7-based chimera for antisense delivery.

MATERIALS AND METHODS

Primer oligonucleotides

F1	5′-TTTACTAGTCCCTTCAGATTAAAAATAACTGA-3′
R1	5′-CCCGTAGTGGGACAGCAGGGCCCCAAGGTT-3′
F2	5′-CCCTGCTGTCCCACTACGGGTCTAGGCTGC-3′
R2	5′-CCCTCTAGAGATCCACCAGGGACAGGGTTAT-3′
FMCKdel63	5′-CCCCAACACCTGCTGCCTGCTAAAAATAACCCTG TCCCTGGTGG-3′
RMCKdel63	5′-CCACCAGGGACAGGGTTATTTTTAGCAGGCAGCA GGTGTTGGGG-3′
FDTex23Sm	5′gcttacctaaatagaagttcatttacactaacaatttgtctagcaggttttc tgacttcg-3′
RDTex23Sm	5′cgaagtcagaaaacctgctagacaaattgttagtgtaaatgaacttctat ttaggtaagc-3′
HIV_LTR_F	5′-AGCTTGCCTTGAGTGCTTCAA-3′
HIV_LTR_R	5′-AGGGTCTGAGGGATCTCTAGTTACC-3′
DTex23F	5′-GGCCAAACCTCGGCTTA-3′
DTex23R	5′-GTTAGTGTAAATGAACTTC-3′
PolyA-F	5′-ATTTTATGTTTCAGGTTCAGGGGGAGGTG-3′
PolyA-R	5′-GCGCAGAGAGGGAGTGGACTAGT-3′
Ex46Fo	5′-AGGAAGCAGATAACATTGCT-3′
Ex53Ro	5′-TTTCATTCAACTGTTGCCTC-3′
Ex47Fi	5′-TTACTGGTGGAAGAGTTGCC-3′
Ex52Ri	5′-TGATTGTTCTAGCCTCTTGA-3′
Ex20Fo	5′-CAGAATTCTGCCAATTGCTGAG-3′
Ex26Ro	5′-TTCTTCAGCTTGTGTCATCC-3′
Ex20Fi	5′-CCCAGTCTACCACCCTATCAGAGC-3′
Ex26Ri	5′-CCTGCCTTTAAGGCTTCCTT-3′

Vector constructions. The MHCK enhancer was obtained by fusing the 188 bp long-murine α-MHC enhancer, and the 207 bp murine MCK enhancer, as described by Salva *et al*.[21] The sequences were amplified from C2C12 genomic DNA using the PCR Master Mix (Promega, Madison, WI) following the manufacturer's instructions, and primers F1 and R1 (α-MHC enhancer) or F2 and R2 (MCK enhancer). The two amplicons were fused by PCR using primers F1 and R2 and introduced upstream of the U7snRNA cassette in the AAV-U7DTex23 vector, previously described as AAV-U7-SD23/BP22.[13] The α-MHC-MCK fusion was further modified by deleting a 63 bp region as described,[21] using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and the primers FMCKdel63 and RMCKdel63.

A deletion of three cytosines between the left and right E-boxes of the MCK enhancer was observed in the final AAV-MHCK-U7DTex23 construct, compared to the published NCBI sequence (GenBank: AF188002.1). This deletion was also observed in C2C12 genomic DNA and was considered a polymorphism.

U7ex51 was derived from U7DTex23 with the mouse dystrophin antisense sequences replaced by a sequence targeting a splicing enhancer (underlined) in human dystrophin exon 51 (5′-GCAGGTACCTCCAACATCAAGGAAGATGGC ATTTCTAGTTTGGAG-3′).[22] U7ex51 was introduced in the pRRLSIN-cPPT-PGK-GFP-WPRE lentiviral vector[32] to create pRRLSIN-U7ex51. The MHCK enhancer was introduced upstream of the U7snRNA cassette to generate pRRLSIN-MHCK-U7ex51.

The SmOPT site of the U7DTex23 sequence was changed into the canonical Sm sequence in the AAV-U7DTex23 vector by site-specific mutagenesis using the primers FDTex23Sm and RDTex23Sm.

The scAAV-PGK-GFP plasmid was constructed by introducing the PGK-GFP fragment from pRRLSIN-cPPT-PGK-GFP-WPRE into the self-complementary vector scAAV-LP1-hFIX,[33] from which the LP1-hFIX sequence was removed. The MHCK enhancer was inserted upstream of the PGK promoter to obtain scAAV-MHCK-PGK-GFP.

Lentiviral vectors preparation. Lentiviral virions were produced by calcium phosphate transfection of HEK293T cells with the envelope plasmid pMD.G (VSV-G[34]), the packaging plasmids pHDM-gpm2 (codon-optimized HIV-1 Gag-Pol) and pRC/CMV-REV1B (REV) (both gifts from Dr Jeng-Shin Lee, Harvard Medical School), and the vector plasmid pRRLSIN-U7ex51. Viral supernatants were harvested 20, 28, and 36 hours post-transfection, centrifuged 5 minutes at 1,500 rpm, filtered through a 0.45-µm filter, and ultracentrifuged 2 hours at 19,500 rpm at 12 °C. Pellets were resuspended in phosphate-buffered saline (PBS) 1% bovine serum albumin and stored at –80 °C. The infectious titers were determined by quantitative PCR using the SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies, Carlsbad, CA) and primers specific for the lentiviral genome HIV_LTR_F and HIV_LTR_R. The albumin endogenous gene was used for normalization.

AAV vectors preparations. AAV vectors were produced by calcium phosphate transfection of HEK293T cells with the transgene plasmid, the capsid plasmid AAV2/8 (LTAAVhelp2-8[35]) or AAV2/5 (Napoli 2/5[36]), and the plasmid for adenovirus helper function (pHGTI-Adeno1[37]). After 72 hours cells and media were harvested and centrifuged at 1,800 rpm for 10 minutes at 4 °C and the pellets resuspended in 40 ml of TD buffer (140.4 mmol/l NaCl, 4.9 mmol/l KCl, 0.7 mmol/l K_2HPO_4, 3.4 mmol/l $MgCl_2$, 24.7 mmol/l Tris—pH 7.5). After five freeze/thaw cycles of the suspension (30 minutes at –80 °C followed by 30 minutes at 37 °C) 2,000 U of benzonase were added, the suspension was incubated for 30 minutes at 37 °C and centrifuged for 20 minutes at 3,000*g*. The supernatant was filtered through a 0.45-µm filter and the AAV preparations were purified by affinity chromatography using the AKTA explorer chromatography system (GE Healthcare, Waukesha, WI) and a prepacked AVB sepharose affinity column (HiTrap; GE Healthcare). The collected fractions of AAV vectors sample were pooled and dialysed in PBS at 4 °C for 16 hours. The viral genome titers were assessed by real-time PCR using the SYBR Green PCR Master Mix (Applied Biosystems). For titration of the AAV-U7DTex23 and AAV-MHCK-U7DTex23 vectors, primers specific of the DTex23 antisense sequence were used: DTex23F and DTex23R. For the scAAV-PGK-GFP and scAAV-MHCK-PGK-GFP vectors, primers in the polyA sequence were used: PolyA-F and PolyA-R.

Cell culture and transduction. All the cells were cultivated at 37 °C and 5% CO_2. 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen). CHQ cells were grown in F-10 medium (Invitrogen) supplemented with 20% fetal calf serum (Invitrogen). Differentiation of CHQ cells was induced in DMEM without pyruvate (Invitrogen) supplemented with 100 µg/ml of human apolipotransferrin (Sigma, St Louis, MI) and 10 µg/ml of bovin insulin (Sigma). C2C12 cells were grown in DMEM supplemented with 20% fetal calf serum (Invitrogen). Differentiation of C2C12 was induced in DMEM supplemented with 2% horse serum (Sigma).

For lentiviral vector transduction, 30,000 CHQ cells were seeded in each well of a 12-wells plate. After 24 hours, the lentiviral vector preparation was administered in a total volume of 250 µl of proliferation medium at the chosen MOI. After 6-hour incubation 1 ml of proliferation medium was added per well. Seventy two hours post-transduction the medium was replaced with differentiation medium for 72 additional hours. Cells were then harvested for analysis.

For AAV vector transduction, 100,000 C2C12 cells were seeded in each well of a 12-wells plate. The following day proliferation medium was changed to differentiation medium and the cells were left to differentiate into myotubes for a week. The cells were then transduced with the AAV preparation at the chosen MOI in a total volume of 300 µl of medium without serum. After 6 hours of incubation 1 ml of differentiation medium was added per well. Cells were incubated for 7 days in differentiation medium before harvesting for analysis.

Western blot. For dystrophin detection total protein was extracted from muscle samples with Newcastle buffer (3.8% SDS, 75 mmol/l Tris–HCl pH 6.7, 4 mol/l urea, 10% βmercaptoethanol, 10% glycerol, 0.001% bromophenol blue) and quantified using the bicinchoninic acid protein assay kit, according to the manufacturer's instructions (Perbio Science, Brebieres, France). Samples were denatured at

95 °C for 5 minutes before 100 µg of protein was loaded in a 5% polyacrylamide gel with a 4% stacking gel. Gels were electrophoresed for 4–5 hours at 100 V and blotted to a PVDF membrane overnight at 50 V. Blots were blocked for 1 hour with 10% nonfat milk in PBS–Tween (PBST) buffer. Dystrophin and α-actinin proteins were detected by probing the membrane with 1:100 dilution of NCL-DYS1 primary antibody (monoclonal antibody to dystrophin R8 repeat; NovoCastra) and 1:200 dilution of α-actinin primary antibody (Santa Cruz Biotechnology), respectively. An incubation with a mouse horseradish peroxidase-conjugated secondary antibody (1:2,000) or goat horseradish peroxidase-conjugated secondary antibody (1:160,000) allowed visualization using ECL Analysis System (GE Healthcare). Membranes were converted to numerical pictures by scanning.

RNA isolation and nested RT-PCR analysis. RNA were isolated from cells or muscle sections using TriZol Reagent according to the manufacturer's instructions (Invitrogen). RT-PCR was carried out on 300 ng of total RNA using the Access RT-PCR System (Promega). For human sequences analysis external primers Ex46Fo and Ex53Ro were used. The cDNA synthesis was carried out at 45 °C for 45 minutes, directly followed by the primary PCR of 30 cycles of 94 °C (30 s), 55 °C (1 minute) and 72 °C (2 minutes). Two microlitres of the RT-PCR product was used for the nested PCR with the internal primers Ex47Fi and Ex52Ri for 22 cycles under identical cycling conditions. The same nested PCR conditions were used for mouse sequences analysis, with external primers Ex20Fo and Ex26Ro, and internal primers Ex20Fi and Ex26Ri. Products were analyzed on 2% agarose gel.

Quantitative PCR. For each sample, 1 µg of total RNA was used for RT using the SuperScript II RT system (Invitrogen) and random hexamers as the priming nucleotides, according to the manufacturer's instructions. For quantitative PCR analysis, 10 ng of the resulting cDNA was used per well. Comparative CT quantitative analysis of the exon 22-23 junction (Taqman Assay Mm01216934_m1; Applied Biosystems) and the exon 22-24 junction (Fw: CTGAATATGAAATAATGGAGGAGAGACTCG, Rev: CTTCAGCCATCCATTTCTGTAAGGT, probe: FAM-ATGTGATTCTGTAATTTCC-NFQ) was performed on each RNA sample. Reactions were performed in triplicate with the appropriate non-template controls using the cycle conditions: 95 °C for 10 minutes, and 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute.

Data analysis. For each sample, the CTs for the 22–24 assay (skipped dystrophin) are normalized to the CT for the 22–23 assay (nonskipped dystrophin) to generate a delta-CT value. This involves the subtraction of the 22–23 CT from the 22–24 Ct. The delta CT can be used to calculate the relative abundance in skipped dystrophin RNA species relative to full-length species.

An unpaired Student's *t*-test was used to assess the statistical significance of differences between the samples treated with AAV-U7DTex23 and the samples treated with AAV2-MHCK-U7DTex23. A P value <0.05 was considered statistically significant. All results are expressed as mean values ± SEM.

Northern blot. Northern blots were realized on 7 µg of total RNA. Samples of total RNA were denaturated at 70 °C for 15 minutes and loaded on a 15% Novex TBE-Urea precast gel (Invitrogen) along with 1 µl of γ-32P-labeled RNA Decade Markers (Ambion, Austin, TX). Gels were electrophoresed for 1 hour at 180 V in 2.5× TBE buffer, and transferred to a Hybond-N+ membrane (Amersham, UK) for 2 hours at 390 mA in 2× TBE buffer. The membrane was UV-crosslinked with 100 mJ of energy and prehybridized in 10 ml of Rapid Hyb buffer (Amersham) containing salmon sperm DNA (50 µg/ml) at 42 °C. For radiolabeling of the complementary DNA probe 50 pmol of oligonucleotide, U7DTex23 (5′-32P-GTTAGTGTAAATGAACTTC-3′) or endogenous U2snRNA (5′-32P-CTGATAAGAACAGATAC-3′), was end-labeled using γ-32P-ATP (Amersham, 6 000 Ci/mmol) and T4 PolyNucleotide Kinase (New England BioLabs, Ipswich, MA) for 1 hour at 37 °C. The probe was purified from unincorporated label using G-25 MicroSpin Columns (Amersham), and heated for 1 minute at 95 °C before addition to the hybridization solution and incubation overnight at 42 °C. The following day 3 successive washes of increasing stringency were realized for 15 minutes at 42 °C before placing the blots in a cassette with an autoradiogram. The films were exposed for 72 hours.

MDX mice injection. Five-month-old mdx mice were anesthetized with isoflurane and injected in the tibialis anterior with 5×10^9, 2×10^{10}, or 10^{11} viral genome copies of AAV2/8-U7DTex23 or AAV2/8-MHCK-U7DTex23 ($n = 3$ for each condition). For each mouse, contra-lateral muscle was injected with PBS as a negative control. Mice were sacrificed 4 weeks after injection and tibialis anterior muscles were isolated and snap frozen in liquid nitrogen-cooled isopentane. All animal experiments were carried out in Biomedical Science Building, University of Oxford, Oxford, UK and performed according to the guidelines and protocols approved by the Home Office.

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Supplementary Material

Figure S1. The MHCK enhancer increases GFP expression in myogenic cells.
Figure S2. Dose-dependent accumulation of read through U7 RNAs transcripts generated from AAV vector genome concatemers.
Figure S3. Northern blot analysis of (**a**) U7DTex23 and (**b**) U7 transcripts expressed in 293T cells transfected with AAV constructs containing either the SmOPT sequence (AAUUUUGGAG, **Figure 1a**), or the native U7 Sm sequence (AAUUUGUCUAG).
Figure S4. The MHCK enhancer loses its tissue specificity when coupled with a ubiquitous promoter.

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