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Research Article

U7 snRNAs induce correction of mutated dystrophin pre-mRNA by exon skipping

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Abstract. Most cases of Duchenne muscular dystrophy are caused by dystrophin gene mutations that disrupt the mRNA reading frame. Artificial exclusion (skipping) of a single exon would often restore the reading frame, giving rise to a shorter, but still functional dystrophin protein. Here, we analyzed the ability of antisense U7 small nuclear (sn)RNA derivatives to alter dystrophin pre-mRNA splicing. As a proof of principle, we first targeted the splice sites flanking exon 23 of dystrophin pre-mRNA in the wild-type muscle cell line C_2C_{12} and showed precise exon 23 skipping. The same strategy was then successfully adapted to dystrophic immortalized *mdx* muscle cells where exon-23-skipped dystrophin mRNA rescued dystrophin protein synthesis. Moreover, we observed a stimulation of antisense U7 snRNA expression by the murine muscle creatine kinase enhancer. These results demonstrate that alteration of dystrophin pre-mRNA splicing could correct dystrophin gene mutations by expression of specific U7 snRNA constructs.

Key words. Duchenne muscular dystrophy; dystrophin; exon skipping; gene therapy; pre-mRNA; U7 snRNA.

Duchenne muscular dystrophy (DMD) is one of the most common muscular dystrophies, caused by mutations in the X chromosomal dystrophin gene. The major muscle dystrophin form is encoded by 79 exons that give rise to a 427-kDa protein. Shorter isoforms are found in other tissues including heart and brain. Together with the complex of dystrophin-associated proteins, dystrophin connects the intracellular cytoskeleton to the extracellular matrix [for reviews, see refs 1–3]. Mutations in the dystrophin gene that lead to DMD are mainly genomic deletions or duplications, less often point mutations. They commonly result in a premature stop codon leading to the absence of functional dystrophin at the sarcolemma and finally to muscle fiber necrosis. In the allelic Becker muscular dystrophy (BMD), dystrophin gene mutations generally do not disrupt the open reading frame (ORF). Thus, the functionally indispensable carboxy-terminal domains are still expressed [1].

However, dystrophin protein is detectable in some 'revertant' muscle fibers in biopsies from DMD patients. These fibers probably arise from an additional somatic mutation or/and alternative splicing process that eliminates the mutation by an exon-skipping mechanism [4, 5]. Revertant fibers have also been detected in the *mdx* mouse, a well-established animal model of DMD [6, 7]. Here, a nonsense mutation in exon 23 of the dystrophin gene causes a premature stop of protein expression [8]. In skeletal muscle of these mice, several alternatively spliced dystrophin mRNA species have been found which lack the

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mutated exon 23 [6, 7]. Most of these mRNAs retained the ORF.

Despite sound knowledge of the molecular background of DMD, as yet there is no cure. Experimental approaches to treatment include the upregulation of the dystrophin homologue utrophin [9], muscle precursor [10] and stem cell [11] transplantation, and gene replacement strategies [12–15]. However, efforts to increase utrophin expression have been futile [16, 17] and muscle precursor cell transplantation has not yet proven efficient in humans [13, 18]. So far, gene replacement therapy has had severe limitations mainly due to the difficulty in delivering the transgenes efficiently and providing sustained expression of the transfected genes in a tissue-specific, properly regulated manner [2, 19–21].

These considerations have fostered new ideas for gene therapy to cure DMD. Rather than replacing the mutated dystrophin gene with a functional homologue, one might correct the mutated gene transcript by favoring an alternative splicing process that eliminates the premature stop codon generated by the mutation. In principle, more than 65% of DMD deletions are amenable to exon-skipping-based therapy [22]. Thus, for many DMD mutations, skipping of the mutated exon would lead to expression of an at least semi-functional dystrophin protein, thereby ameliorating the phenotype or even curing the disease. Important in this context is that several groups have reported in-frame deletions within the dystrophin gene of human subjects that remained without or with only minor clinical phenotype, demonstrating that skipping one or several exons within the huge central domain of the protein does not necessarily lead to functional impairment [1, 23].

Antisense oligonucleotides targeted to specific splice sites of the dystrophin gene have been reported to induce exon skipping in primary cultures of *mdx* muscle cells [24, 25] and in injected muscles of *mdx* mice [26]. However, non-specific side effects of such oligonucleotides have been reported and little is known about possible long-range toxic or immunological complications [27, 28]. Moreover, their clinical use also requires repeated or even continuous administration to obtain a lasting effect [27, 28].

Thus, in vivo production of antisense RNA from small transgenes is an attractive alternative to the administration of antisense oligonucleotides, but it is still in a relatively early, experimental stage. So far, short RNA transcripts have been used in cell culture studies as carriers of ribozymes to cleave regulatory elements of viral RNA, preferentially of the human immunodeficiency virus (HIV) [29]. In another cell culture approach, a small nuclear RNA (snRNA), U1 snRNA, was modified to bind to regulatory elements of HIV to block viral replication [30]. Here, we propose to use modified U7 snRNAs to induce exon skipping in mutated dystrophin precursor mRNA in order to restore the reading frame.

U7 snRNA is incorporated into small nuclear ribonucleoprotein (snRNP) particles and, as such, is involved in the maturation of histone pre-mRNAs by recognizing its target via base-pairing sequence complementary to the histone 3′ untranslated region [31]. Some of us have modified the wild-type U7 snRNA to change its target specificity and make it an antisense tool. By converting the non-canonical Sm sequence into the consensus SmOPT sequence found in major snRNAs, the modified U7 molecule is prevented from recruiting the U7-specific proteins. It thereby loses the ability to perform the endonucleolytic cleavage during the process of histone pre-mRNA maturation. Moreover, three times more U7 SmOPT snRNA than wild-type U7 snRNA accumulates in the nucleus, and U7 SmOPT snRNA assembled more efficiently into the U7 snRNP [32, 33]. The base-pairing sequence of the U7 snRNA can be modified to bind to any RNA target of interest [34, 35]. Modified U7 snRNAs have already been shown to induce the skipping of an aberrant exon created by certain thalassemic mutations in the human β-globin gene [34, 35]. There, antisense sequences targeted to the 5′ and 3′ splice sites, expressed from a modified U7 SmOPT snRNA gene, led to skipping of the aberrant exon and restored the correct splicing pattern. We then found that skipping of the aberrantly spliced exon was most efficient when the antisense U7 snRNA derivative was capable of binding to two different targets, thereby forcing the pre-mRNA into a looped secondary structure [35].

Here, we applied this double-target strategy to induce skipping of the mutated dystrophin exon 23 in immortalized muscle cells of the *mdx* mouse. The correction of pre-mRNA led to the expression and sarcolemmal accumulation of dystrophin protein by these cells. The results of this study have been published in part previously in abstract form [36, 37].

Materials and methods

Plasmid constructs

New antisense sequences complementary to targets surrounding mouse dystrophin exon 23 were introduced at the 5′ end of the SmOPT variant of the mouse U7 snRNA gene, present in a pSP64 vector background [38], using PCR-based mutagenesis as described elsewhere [35]. For stable transfections, anti-dystrophin U7 gene cassettes were subcloned into a derivative of pCMV-GFPsg25 [39] deleted for the *Stu*I site at the 3′ end of the SV40 promoter and with its CMV-GFP cassette removed. In some constructs, the 340-bp *Stu*I/*Sac*I U7 promoter fragment was replaced by a 280-bp *Pvu*II/*Sac*I promoter fragment of the mouse U1b gene [38]. Additionally, a 300-bp *Bgl*II/

*Bam*HI enhancer element (E1) of the MCK promoter [40] was inserted in front of the U7 or the U1 promoter in either orientation. Details of the constructions are available upon request. The sequences of all constructs were confirmed by automated sequencing (Microsynth, Balgach, Switzerland).

Cell culture and transfection

H-2K^b-tsA58 *mdx* myoblasts were generated as previously described [41], with minor modifications. In brief, myoblasts were isolated from a mouse generated by cross-breeding of an *mdx* and an H-2K^b-tsA58 transgenic mouse (Charles River, Margate, UK) expressing the SV40 large T antigen gene with the tsA58 mutation. The cells were proliferated at 33 °C in high-glucose DMEM (Sigma, St. Louis, Mo.) supplemented with 20 units/ml of recombinant mouse interferon-γ (all from Life Technologies, Basel, Switzerland). C_2C_{12} cells (CRL-1772, ATCC) were grown in high-glucose DMEM at 37 °C. The *mdx* and C_2C_{12} cells were transfected at the myoblast stage by calcium phosphate precipitation with 3.5 µg of U7-snRNA-expressing plasmid and 1.5 µg of λDNA. This transfection procedure yielded better results than methods based on liposomal preparations including lipofectamine. The next day, the cells were fed with DMEM containing 2% horse serum to induce myogenic differentiation.

For stable transfection, cells were put under selection using 600 µg/ml G-418 sulfate (Promega, Madison, Wis.) for at least 4 weeks, replacing the selection medium twice a week and passaging cells before they became confluent.

Gene expression studies

Total RNA was isolated using TRI-Reagent (Molecular Research Center, Cincinnati, Ohio). To detect dystrophin mRNA, nested reverse transcriptase (RT-)PCR was performed as described elsewhere [24] with some modifications. One microgram of total RNA was used for the RT reaction performed with M-MLV RT (Promega) in a 30-µl reaction at 42 °C for 90 min, primed with Ex26Ro, a reverse primer located in exon 26 of the dystrophin mRNA (Ex26Ro, 5′-ttcttcagcttgtgtcatcc-3′). One-twentieth of this reaction was used to carry out the first amplification by the Taq polymerase (Promega) with the outer primers located in exon 20 and 26 (Ex26Ro and Ex20Fo, 5′-agaattctgccaattgctgag-3′) for 30 cycles of 94 °C for 30 s, 55 °C for 1 min and 74 °C for 2 min. A sample of 1 µl of this first PCR was then reamplified using the nested (inner) primer pair (Ex26Ri, 5′-cctgcctttaaggcttccctt-3′ and Ex20Fi, 5′-cccagtctaccaccctatcagagc-3′) for 20 cycles of 94 °C for 30 s, 55 °C for 1 min and 74 °C for 2 min. PCR products were analyzed on 1.2% agarose (Promega) gels where individual bands were isolated from the gel using the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). After reamplification with the inner primer pair, the bands were subjected to automated sequencing (Microsynth).

The expression of the modified U7 snRNA was monitored by RNase protection assay, essentially performed as described previously [35, 38]. An aliquot of 7 µg of total RNA (from the same RNA sample used for RT-PCR experiments) was coprecipitated with 55 fmol ^{32}P-labeled riboprobe transcribed (in the presence of α-^{32}P-CTP) from the SP6 promoter located at the 3′end of the U7 gene in either pU7SmOPT or pU7BP22/5′ splice site 23. The pellet was resuspended in 30 µl hybridization buffer [40 mM piperazine-N, N′-bis(2-ethane-sulfonic acid) pH 6.4, 1 mM EDTA, 400 mM NaCl, 80% formamide], denaturated for 10 min at 85 °C, and allowed to form U7-specific hybrids by slowly cooling to 45 °C. Unpaired riboprobes were then subjected to digestion by adding 300 µl of RNase digestion mixture (300 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl pH 7.5, 20 µg/ml RNase A and 100 U/ml RNase T1) and incubating for 90 min at 37 °C. The reaction was stopped by proteinase K digestion and the protected fragments were purified by acid phenol extraction and ethanol precipitation, and finally separated on a 10% denaturing polyacrylamide gel. (The specificity of the protected sequence was controled with non-digested and digested hybrids of riboprobe and tRNA). Dried gels were exposed to storage phosphor screens and analyzed with the ImageQuant (both from Amersham Biosciences, Freiburg, Germany) program. To compare the amounts of antisense versus endogenous U7 snRNAs, the band intensities as determined by phosphor screens were divided by the number of cytosine residues present in each protected RNA fragment.

Western blotting

For detection of dystrophin, protein was extracted with lysis buffer (4% SDS, 125 mM Tris-HCl pH 6.4, 4 M urea, 10% β-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue) from 6 to 9-day-old myotubes, subjected to electrophoresis on 5.5% polyacrylamide/0.1% SDS gels for 3 h and electroblotted onto nitrocellulose membranes. After blocking with 3% skimmed milk in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20), membranes were probed with a rabbit polyclonal anti-dystrophin antiserum (1/200; kind gift of Dr. P. Holland, Montreal Neurological Institute, Montreal, Canada) and then incubated with a horseradish-peroxidase-conjugated secondary antibody (1/5000; Pierce, Rockford, Ill.). Signals were detected by chemiluminescence using the Super Signal substrate (Pierce). The blot was stripped and reprobed with a mouse monoclonal anti-myosin I antibody (1/10,000; Sigma) to show that similar amounts of protein were loaded in the lanes for the pU7BP22/5′ss23 transfected versus the pU7SmOPT control transfected *mdx* cells.

Immunocytochemistry

The day after transfection, cells were replated on collagen-coated round cover slips in 24-well plates, grown to confluence, induced to differentiate and kept in the starvation medium for 7–9 days. Immunodetection was performed essentially as described elsewhere [42]. Cells were fixed in ice-cold 100% ethanol for 10 min, rinsed three times with PBS and simultaneously permeabilized and blocked for 1 h in 0.3% Triton X100, 5% heat-inactivated horse serum, 2 mg/ml BSA in PBS at room temperature. The cells were then incubated with the first antibody (1/10 Dys2; Novocastra, Newcastle upon Tyne, UK) diluted in blocking solution without Triton for 2 h at room temperature. After washing with PBS, the secondary antibody (1/5000 Alexa Fluor 488; Molecular Probes, Leiden, The Netherlands) was applied for 1 h at room temperature. The cover slips were mounted with Dako Fluorescent Mounting Medium (Carpinteria, Calif.). Pictures were taken with a fluorescence microscope at × 63 magnification.

Results

Design of antisense U7 snRNA molecules

To explore the potential of antisense U7 snRNA molecules for DMD gene therapy, we tested their ability to induce the skipping of dystrophin exon 23 in mouse cells (fig. 1). We generated several antisense U7 snRNA genes that target functionally important sites in the vicinity of exon 23. Based on our previous results with thalassemic β-globin genes [34, 35], we chose a length of 24 nucleotides for the antisense sequences. Three single-target constructs were generated that were directed against the 3′ splice site of intron 22 (3′ss22), the 5′ splice site of intron 23 (5′ss23), and the region of the putative branch point in intron 22 (BP22). In addition, two double-target constructs combined antisense sequences against 3′ss22 and 5′ss23 or against BP22 and 5′ss23 (table 1).

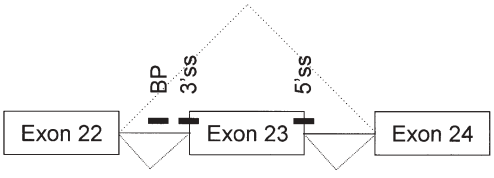

Figure 1. Blocking the 3′ or 5′ splice sites (ss) and/or the branch point (BP) upstream of exon 23 (black bars) should force the splicing machinery to skip the exon (upper dotted line) and retain the mouse dystrophin ORF.

As a first step to assess the ability of antisense U7 snRNAs to induce exon 23 skipping, we transiently transfected the U7-BP22/5′ss23 construct into C_2C_{12} myoblasts, which do not harbor a dystrophin mutation. This construct was chosen because a similar one had proven favorable in previous experiments designed to modify the splicing of β-globin mRNA [35]. Minor amounts of dystrophin mRNA were detected one day after induction of differentiation by serum starvation, 2 days after transfection. Full levels of dystrophin expression were reached at day 5 after transfection (fig. 2A). At this time point, not only was full-length dystrophin mRNA amplified, but an additional, faster-migrating band was present which was not observed in untransfected cells or in cells transfected with the original U7-SmOPT construct targeting the 3′ untranslated region of the histone pre-mRNA used as control (data not shown). Its size corresponded to an amplification product of exon-23-skipped dystrophin mRNA. Isolation of this band, PCR reamplification and sequencing confirmed that exon 23 was indeed skipped and exon 22 was precisely joined to exon 24, retaining the dystrophin ORF (fig. 2B). RNase protection analysis of the same samples confirmed strong expression of the therapeutic antisense U7 snRNAs immediately after transfection (fig. 2C). This strong expression was rapidly downregulated beginning at day 3 after transfection, probably for two reasons. First, there might have been loss of the transiently transfected plasmids, since some myoblasts could still divide for one or two cell cycles. Second, endogenous U7 snRNA expression was also downregulated, albeit less prominently, probably because of the cell cycle arrest that induced the decrease in ex-

Table 1. Antisense U7 snRNAs used.

Construct	Sequence
U7-3′ss22 (ST)	aactttgcagagc′ctcaaaattaaaaatttttggag
U7-5′ss23 (ST)	aacctcggcttac′ctgaaattttcgaatttttggag
U7-BP22 (ST)	aaaatagaagttcatttacactaacaatttttggag
U7-3′ss22/5′ss23 (DT)	aacctcggcttac′ctgaaattttcg/aactttgcagagc′ctcaaaattaaaaatttttggag
U7-BP22/5′ss23 (DT)	aacctcggcttac′ctgaaattttcg/aaatagaagttcatttacactaacaatttttggag
U7-SmOPT (control)	aagtgttacagctcttttagaatttttggag

The U7 constructs used in this work are derived from the parental mouse U7 gene. The Sm sequence has been modified to become an SmOPT sequence (underlined). The SmOPT sequence prevents the therapeutic U7 snRNA from interfering with histone pre-mRNA processing. The base-pairing region which normally targets histone pre-mRNA was altered to make it complementary to dystrophin pre-mRNA (non-underlined sequences). The apostrophes represent splice sites of the targeted RNA. The oblique (/) separates two different targets. ST, single target; DT, double target.

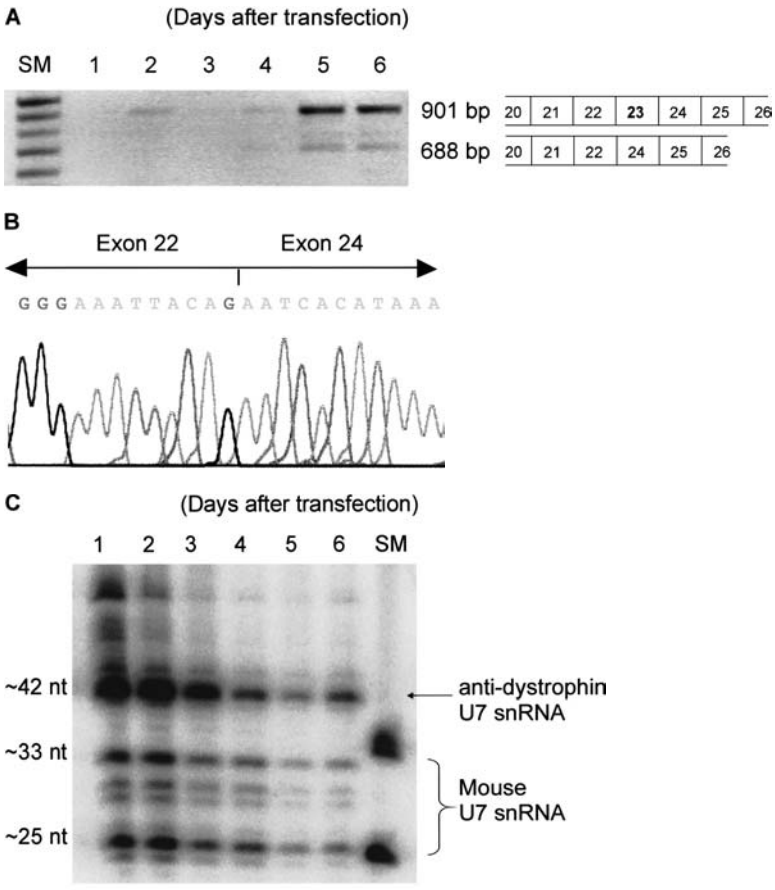

Figure 2. *(A)* Dystrophin mRNA expression kinetics of C_2C_{12} myoblasts transfected with U7-BP22/5′ss23 analyzed by nested RT-PCR. The starting point (day 1) corresponds to the day after transfection when growth medium was replaced by induction medium. Dystrophin mRNA amplification, using primer pairs in exon 20 and 26, was first observed after 1 day of differentiation, 2 days after transfection, as shown by the band at 901 bp. The 688-bp fragment indicating skipping of exon 23 first appeared at day 5. SM, size marker. *(B)* Chromatogram of the 688-bp fragment, gel extracted and reamplified by PCR. Exons 22 and 24 are correctly joined in frame. *(C)* RNase protection assay to analyze the expression of the therapeutic U7-BP22/5′ss23 construct. The riboprobe used for this assay was derived from the U7-SmOPT construct leading to the protected band at ~25 and ~33 nt bands for endogenous mouse U7 snRNA and ~42 nt for anti-dystrophin U7 snRNA. Both the endogenous and the transfected U7 snRNAs are downregulated during differentiation. Note that if the antisense and endogenous U7 snRNAs were present in equimolar amounts, the 42-nt band should have ~1.3 times the intensity of the 33-nt band.

pression of the replication-dependent histone genes. This cell-cycle-arrest-related decrease in U7 expression might also have contributed to the reduction in therapeutic U7 antisense snRNA expression. Nevertheless, the rather low level of antisense U7 snRNA at day 6 after transfection was sufficient to induce exon 23 skipping.

Exon 23 skipping in immortalized *mdx* cells

Similar experiments were then performed with *mdx* muscle cells immortalized by expression of SV40 large T antigen. These cells showed lower transfection efficiencies (5–25%) than C_2C_{12} cells (35–60%). To determine the efficacy and specificity of different antisense U7 constructs, we transfected the three single-target and two double-target constructs (table 1) in parallel. Again, cells were allowed to differentiate to myotubes for 6 days and nested dystrophin RT-PCR was performed on total RNA. Among the three single-target constructs, only the one blocking the 5′ss23 induced detectable exon 23 skipping (fig. 3, lane 3). In addition, both double-target constructs were effective (lanes 4 and 6). Here, combined binding of the putative BP22 and the 5′ss23 yielded the highest amounts of dystrophin mRNA lacking exon 23. As expected, transfection with a control U7 construct (lane 7) or mock transfection (lane 8) did not yield an exon 23-skipped RT-PCR amplification product. The fact that U7-BP22/5′ss23 was most effective in exon 23 skipping was consistent with our previous data on the correction of β-globin splicing [35]. However, in contrast to the β-globin system, the combined administration of two single-target anti-dystrophin constructs such as U7-5′ss23 + U7-3′ss22 did not show an additive effect on exon 23 skipping (data not shown).

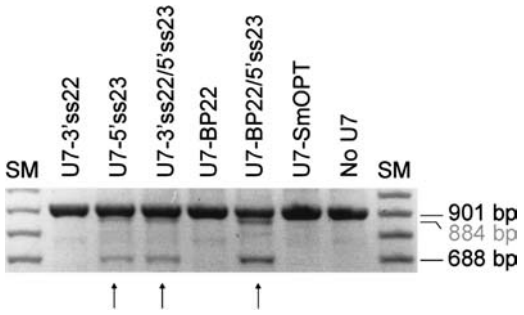

Figure 3. Exon 23 skipping in transiently transfected H-2K^b-tsA58 *mdx* myoblasts. The cells were transfected with different antisense U7 plasmids as indicated above each lane (see table 1). As controls, cells were transfected with U7-SmOPT directed against histone pre-mRNA or mock-transfected (lanes 7 and 8, respectively). Cells were induced for differentiation for 6 days. Total RNA was extracted and dystrophin nested RT-PCR was performed to assess exon 23 skipping. The 688-bp band in lanes 3, 4, and 6 indicates successful skipping of exon 23 and thus correction of the dystrophin mutation. The 884-bp amplification product just below the 901-bp band in lane 6 is derived from the first round of PCR and corresponds to exon-23-skipped dystrophin mRNA as determined by sequencing. SM, size marker.

Again, reamplification and sequencing of the shorter amplification product revealed precise joining of exon 22 to exon 24, in this case rejoining the interrupted dystrophin ORF (data not shown). An RNase protection assay further confirmed that all the different U7 antisense snRNAs were expressed at comparable levels (not shown). Thus, the lack of exon skipping by the U7-3′ss and U7-BP22 single-target constructs (fig. 3, lanes 2, 5) was not due to insufficient U7 snRNA expression.

Rescue of dystrophin protein synthesis

Knowing that the U7-BP22/5′ss23 antisense construct can effectively induce exon 23 skipping in immortalized *mdx* muscle cells, we analyzed whether this led to the restoration of dystrophin protein synthesis. Western blot analysis confirmed de novo expression of a dystrophin-immunoreactive band in *mdx* cells transiently transfected with the U7-BP22/5′ss23 antisense construct (fig. 4A). As expected, no difference in size was detectable compared to full-length dystrophin protein of the wild-type C_2C_{12} cells, indicating that, most likely, only exon 23 (71 amino acids) was skipped in the corrected mRNA. No immunoreactivity was observed for *mdx* cells transfected with the control construct U7-SmOPT.

To determine the localization of the protein, *mdx* myotubes transfected with the U7-BP22/5′ss23 antisense construct were immunostained with dystrophin antiserum. Sarcolemmal dystrophin immunoreactivity was detected in segments of these myotubes (fig. 4B), indicating that the dystrophin protein lacking exon 23 accumulated at the correct cellular location. No dystrophin immunoreactivity was detected in *mdx* myotubes transfected with the U7-SmOPT control (fig. 4C).

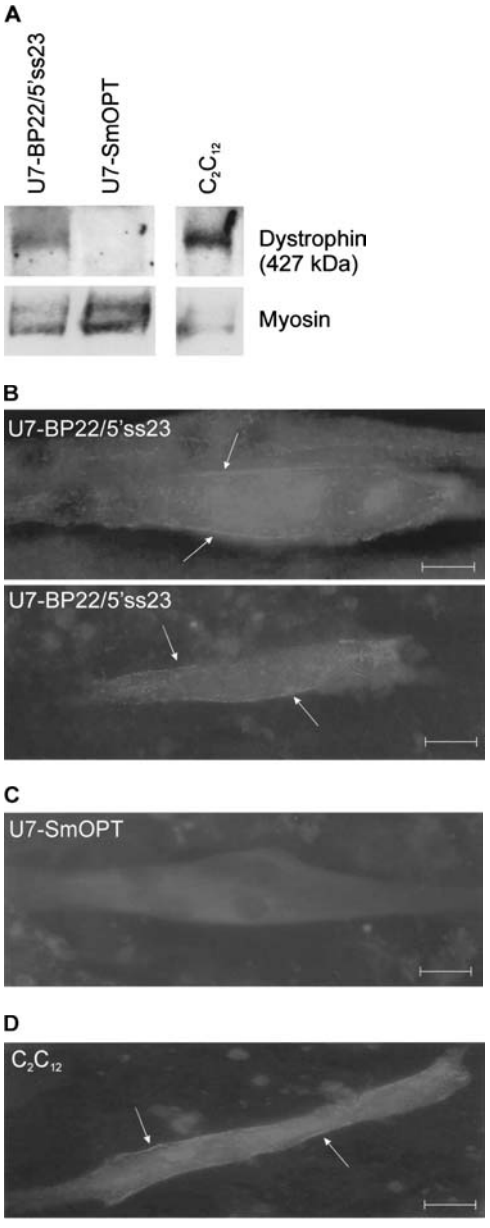

Figure 4. Restoration of dystrophin protein synthesis by the double-target construct U7-BP22/5′ss23 in H-2K^b-tsA58 *mdx* myotubes. *(A)* Western blotting of total protein extracted from 10-day-old *mdx* myotubes with rabbit polyclonal anti-dystrophin serum revealed the synthesis of approximately full-length dystrophin after transfection with U7-BP22/5′ss23, as compared with dystrophin extracted from C_2C_{12} myotubes. No dystrophin protein was detected in *mdx* myotubes transfected with U7-SmOPT. Similar amounts of *mdx* protein preparation were loaded as shown by staining with a myosin I antiserum. The protein concentration loaded on the C_2C_{12} myotubes was one-fifth of the *mdx* myotube lanes. *(B, C)* Immunostaining with Dys2 anti-dystrophin antibody revealed a focal sarcolemmal staining of dystrophin of *mdx* myotubes transfected with U7-BP22/5′ss23 (arrows) *(B)*, but an absence of the protein in cells transfected with U7-SmOPT *(C)* Scale bar, 8 µm. *(D)* Sarcolemmal dystrophin immunoreactivity (arrows) of C_2C_{12} myotubes (positive control). Scale bar 4 µm.

Stable expression of therapeutic antisense U7 snRNA

Given that sustained expression of U7 snRNAs is necessary to ensure long-term production of a corrected dystrophin protein, we assessed the exon skipping efficacy of stably transfected U7 constructs. Moreover, since U7 snRNA expression was strongly downregulated during differentiation in C_2C_{12} (fig. 2B) and *mdx* cells (data not shown), we also tried to connect the U7-BP22/5′ss23 gene to another, possibly more constitutive promoter or to a muscle-specific enhancer.

To select for stable integration, we used a vector carrying a neomycin resistance gene. In this vector background, we replaced the U7 promoter by the possibly more constitutive and stronger murine U1(b) promoter [43]. In addition, a 300-bp fragment of the MCK promoter previously characterized to enhance expression from heterologous promoters of non-muscle-specific genes [44] was positioned upstream of the U7 or U1 promoter to enhance muscle-specific U7 snRNA expression. In *mdx* cells stably transfected with the construct containing the U7 promoter, the ratio of antisense U7 snRNA to endogenous U7 snRNA (fig. 5A, lane pair 1) was lower than in the transiently transfected C_2C_{12} cells (fig. 2C). However, one must consider that the two plasmid backbones were different and that endogenous U7 snRNA levels were possibly different in the two cell lines. Moreover, the stably transfected cells probably integrated only few plasmid copies and fewer genes conceivably express fewer transcripts. Expression from the chimeric construct where the U1 promoter drove U7 snRNA transcription was ~2 times lower than with the original U7 promoter (fig. 5A, lane pair 2). In contrast, the MCK enhancer element positioned in front of the U7 promoter strongly increased antisense RNA expression independent of its orientation (lane pairs 3 and 4), and it also stimulated U1-promoter-driven antisense RNA expression, albeit to lower levels (lane pair 5). However, fusing the entire MCK promoter to the U7 gene did not result in detectable U7 snRNA expression (data not shown).

Despite the fact that putatively constitutive (U1 promoter) and/or muscle-specific (MCK enhancer) regulatory elements had been introduced to drive U7 snRNA expression, the levels were still downregulated in terminally differentiated myotubes, but the differences seen between the various promoter/enhancer fusions persisted (compare respective lanes B to T in fig. 5A). The expression level of the endogenous U7 snRNA was also downregulated, at least moderately, in differentiated myotubes. Most importantly, the relative levels of antisense snRNA expression were paralleled by the efficacies of exon 23 skipping (fig. 5B). However, the amount of 688-bp RT-PCR product corresponding to exon-23-skipped dystrophin mRNA was considerably lower than in the transiently transfected samples (figs. 2A, 3), consistent with the low antisense U7 snRNA expression. Moreover, additional amplification products of ~755 and ~542 bp were present. These bands were observed in some of our experiments, and their appearance seemed to correlate with advanced age of the culture. PCR amplification and sequencing revealed that the ~755-bp band which was present in all samples of a given culture corresponded to

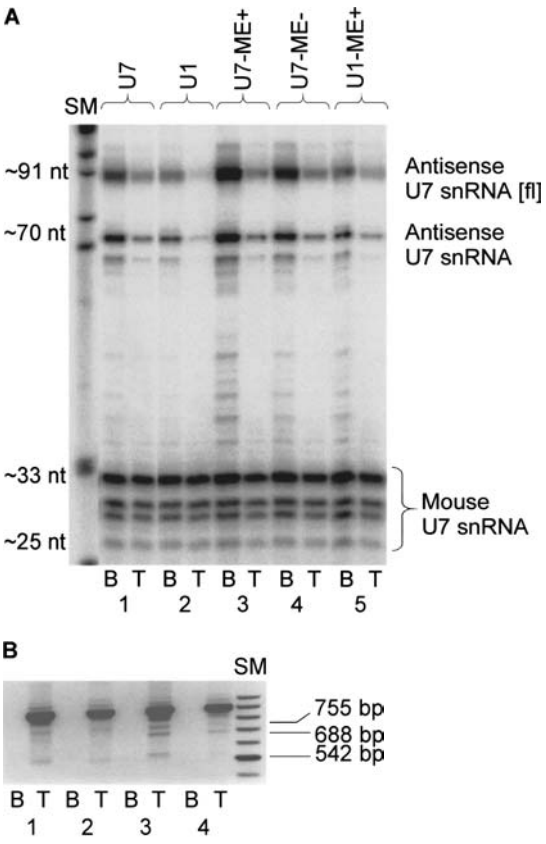

Figure 5. Antisense U7 snRNA expression and skipping of dystrophin exon 23 in stably transfected *mdx* myogenic cells. SM, size marker; U7, endogenous U7 promoter drives transcription of the antisense U7 snRNA BP22/5′ss23; U1, U1(b) promoter replaces the endogenous promoter of U7; U7-ME+, MCK enhancer is positioned in the normal orientation with regard to the MCK promoter upstream of U7; U7-ME–, same construct as U7-ME+ except for inversion of MCK enhancer sequence; U1-ME+, same construct as U7-ME+ except for replacement of U7 by U1 promoter. *(A)* RNase protection assay for U7-BP22/5′ss23 expression. Total RNAs from pools of *mdx* cells stably transfected with the constructs indicated above the lanes were isolated at the myoblast (B) or myotube (T) stage. RNase protection assays were performed with a ^{32}P-labeled riboprobe derived from U7-BP22/5′ss23. Signals of ~25 and ~33 nt correspond to endogenous mouse U7 snRNA; the ~91-nt band corresponds to full-length antisense U7-BP22/5′ss23 snRNA [fl]. An additional band of ~70 nt [70] originates from RNase cleavage within a run of seven consecutive AT pairs and also represents antisense U7-BP22/5′ss23 snRNA. *(B)* Detection of exon 23 skipping in dystrophin mRNA by nested RT-PCR as described in figure 2A and 3. Lane numbers correspond to those in panel *A*. The 688-bp band in lanes 1T, 3T, and 4T indicates successful skipping of exon 23 and thus correction of the dystrophin mutation. Additional bands of ~755 and ~542 bp correspond to mRNAs lacking exon 22 or both exons 22 and 23, respectively. These mRNAs are out of frame and should not yield any functionally active protein.

mRNA lacking exon 22. In contrast, the ~542-bp band was only observed when exon 23 skipping was induced by U7 snRNA expression, and its sequence lacked both exons 22 and 23. Skipping of exon 22 alone or of exon 22 and 23 leads to out-of-frame mRNAs. Whereas exon-23-skipped mRNA was produced in stably transfected cells, the level of correction was probably too low and dystrophin protein levels were below detection levels in both our immunocytochemistry and Western blotting assays.

Thus, in summary, antisense U7 snRNA expression and the skipping of exon 23 could also be observed when the U7-BP22/5′ss23 gene was stably integrated into the genome of *mdx* cells, but both its expression and the efficacy of exon 23 skipping were lower than in transiently transfected cells. Moreover, we observed a strong stimulation of antisense U7 snRNA expression by the MCK enhancer.

Discussion

Using antisense U7 snRNA expressed from modified U7 transgenes, we were able to induce the skipping of exon 23 in dystrophin pre-mRNA, first in the wild-type C_2C_{12} myoblast cell line (fig. 2), then in immortalized *mdx* myoblasts carrying a nonsense mutation in exon 23 (fig. 3). As intended, exons 22 and 24 were joined correctly, thereby reconnecting the dystrophin ORF. Because this treatment led to the restoration of dystrophin synthesis in differentiating *mdx* myotubes which normally lack dystrophin, and since the protein appeared to be properly located at the sarcolemma (fig. 4), these results strongly suggest that U7 snRNA derivatives could be used for a functional repair of DMD mutations.

Compared to synthetic antisense oligoribonucleotides that have been used previously to induce the skipping of a dystrophin exon [24–26], U7 snRNA antisense molecules offer a number of important advantages, such as resistance to degradation and decreased risk of RNA interference due to the formation of snRNP particles, specific accumulation in the nucleus where pre-mRNA splicing takes place, as well as continuous expression. In addition, the U7 gene is so small that it can be inserted into any transfer vector. Moreover, compared to approaches using dystrophin mini-transgenes, this mRNA correction approach offers the advantages that most of the dystrophin protein sequence is preserved and that dystrophin expression is governed by the endogenous gene regulatory elements.

Although the nested RT-PCR used for detection of dystrophin mRNA is not quantitative, our results still allow for a few semi-quantitative assessments. First, an increase in dystrophin mRNA during myotube formation was clearly evident (fig. 2A). Second, the differences in exon skipping observed between the various U7 constructs were substantial, and the U7-BP22/5′ss23 double-target construct was – in all experiments – the most efficient construct tested (fig. 3). What is more difficult to judge is the absolute efficiency of splicing correction. The intensity of the exon-23-skipped band in various transient transfections represents an estimated 5–20% of total RT-PCR products, but one has to consider that shorter products tend to be amplified more efficiently.

The results are similar to those previously obtained for thalassemic human β-globin genes expressed in HeLa cells [34, 35, 45] (see Introduction). However, in the thalassemia project, HeLa cells were stably transfected with the mutated β-globin gene. In the present project, skipping of an exon of an endogenous gene (dystrophin) was achieved using a U7 construct.

In the transiently transfected C_2C_{12} cells, U7-BP22/5′ss23 snRNA appeared to be produced at similar or slightly higher amounts as the endogenous U7 snRNA (fig. 2C), which corresponds to a few thousand copies in proliferating cells [38]. Since only about half of the cells took up the antisense U7 gene, these must have expressed large amounts of U7 snRNA. This high level decreased during myotube formation. Because the endogenous U7 snRNA showed a similar decline, this phenomenon was probably not only due to a loss of the transgene, but also due to physiological downregulation caused by the well-documented decrease in replication-dependent histone gene expression [46].

Together, the small proportion of cells transfected (5–25% for *mdx*), the partial downregulation of the antisense U7 snRNA gene, and the relatively low level of exon 23 skipping may have been responsible for the low level of dystrophin protein expression by the *mdx* cells (fig. 4). To improve expression, several approaches were conceivable, and we tested some of these in the present project. In stably transfected cells, the expression of U7-BP22/5′ss23 snRNA and the skipping of exon 23 seemed to be less efficient than in transient transfections (fig. 5). This may have been due to the particular vector used. Alternatively, the U7 transgene may have been present in fewer copies than in the transiently transfected cells. Thus, although stable transfection allowed persistent U7 gene expression, its level and hence its effect on dystrophin pre-mRNA splicing was low. Moreover, the expression of the U7 transgene, as well as expression of the endogenous U7 snRNA, was still downregulated during myotube differentiation.

We then tried to increase the level of U7 transgene expression and at the same time avoid its downregulation during myotube differentiation by altering the promoter. snRNAs have specific types of promoter and RNA 3′ end formation signals [47]. Replacing the U7 snRNA promoter by an mRNA promoter (from the murine MCK gene) did not result in detectable U7 snRNA expression (data not shown), most likely because the 3′ end formation signal was not recognized. We therefore replaced the U7 promoter by the mouse U1(b) gene promoter, but, in contrast to previous re-

sults where a similar insertion of a U1 promoter had led to a minor increase in U7-induced effects in HeLa cells [45], we found that substitution with the U1(b) promoter actually reduced U7 snRNA expression in our *mdx* cells (fig. 5). On the other hand, inserting the 300-bp MCK enhancer element [44] in front of the U7 promoter in either orientation strongly stimulated antisense U7 snRNA expression and led to a corresponding increase in the fraction of exon-23-skipped dystrophin mRNA in myotubes. This result indicates that the expression of a U snRNA gene can be stimulated by an mRNA enhancer; this is promising for our gene therapy approach and by itself deserves further investigation. Surprisingly, however, both the construct with the U1 promoter and those carrying the MCK enhancer still showed a reduction in antisense U7 snRNA proportional to the endogenous U7 snRNA. This downregulation may therefore be a property of the U7 snRNA itself and not one of the U7 promoter.

In conclusion, although various aspects (such as the transfer of the U7 transgene, the expression and persistence of its encoded antisense snRNA, and the efficiency with which dystrophin exons can be skipped) still need to be improved, the present results provide a proof of principle that splicing of mutated dystrophin pre-mRNA can be altered in *mdx* cells using custom-made U7 constructs. They set the stage for future studies using the *mdx* cells stably transfected with improved U7 constructs to further characterize the properties of the corrected dystrophin protein and the assembly of the complex of dystrophin-associated proteins.

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