

Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells

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Due to frame-shifting mutations in the DMD gene that cause dystrophin deficiency, Duchenne muscular dystrophy (DMD) patients suffer from lethal muscle degeneration. In contrast, mutations in the allelic Becker muscular dystrophy (BMD) do not disrupt the translational reading frame, resulting in a less severe phenotype. In this study, we explored a genetic therapy aimed at restoring the reading frame in muscle cells from DMD patients through targeted modulation of dystrophin pre-mRNA splicing. Considering that exon 45 is the single most frequently deleted exon in DMD, whereas exon (45+46) deletions cause only a mild form of BMD, we set up an antisense-based system to induce exon 46 skipping from the transcript in cultured myotubes of both mouse and human origin. In myotube cultures from two unrelated DMD patients carrying an exon 45 deletion, the induced skipping of exon 46 in only ~15% of the mRNA led to normal amounts of properly localized dystrophin in at least 75% of myotubes. Our results provide first evidence of highly effective restoration of dystrophin expression from the endogenous gene in DMD patient-derived muscle cells. This strategy may be applicable to not only >65% of DMD mutations, but also many other genetic diseases.

INTRODUCTION

The Duchenne and Becker muscular dystrophies (DMD/BMD) are X-linked muscle diseases typically caused by frame-shifting (DMD) or non-frame-shifting (BMD) mutations in the dystrophin-coding DMD gene (1–4). Dystrophin is an essential component of the dystrophin–glycoprotein complex (DGC) maintaining the membrane integrity of muscle fibers (5–8). In DMD, the absence of dystrophin induces a continuous loss of muscle fibers which eventually causes premature death in adolescence, while in BMD a partly to largely functional dystrophin results in a milder phenotype and a longer life expectancy (2,4).

Given the severity and frequency (1:3500 newborn males) of DMD, great effort has been put into the development of an effective (gene) therapy. Most DMD gene therapy strategies have been based on ‘gene addition’ through viral or non-viral delivery of dystrophin-encoding sequences to muscle tissue. Studies in mice have been encouraging in showing that expression of dystrophin gene constructs at 20–30% of normal levels is already sufficient to avert muscle degeneration (9). However, two major challenges have hampered clinical applications, the large size of the dystrophin-coding region (11 kb) and the efficient delivery and long-term expression of therapeutic gene constructs into skeletal muscle (reviewed in 10). Alternative DMD gene therapy approaches aiming at gene correction instead of gene addition are therefore gaining increased attention.

Oligonucleotide technology has evolved rapidly in the last decade, facilitating different applications in gene therapy studies. Promising results have been obtained with chimeric DNA/RNA oligonucleotides that are capable of inducing site-specific correction of small DNA mutations using the cellular DNA mismatch repair system (11–13). Such gene correction has been demonstrated in several *in vitro* and *in vivo* disease models, including the mouse (*mdx*) and canine (GRMD) models for DMD (14,15). Although this type of gene correction may be cumulative and potentially even permanent, its efficiency has been limited and depends crucially on DNA repair activity in the host cells of interest.

Antisense oligo(ribo)nucleotides (AONs) have been investigated to modulate gene expression through interference with RNA processing. AONs can block undesired gene expression through RNase H cleavage of RNA–AON duplexes, e.g. for the treatment of viral infections, cancers and inflammatory disorders (16–20), or modulate pre-mRNA splicing by inducing the skipping of specific exons, so that frame-shifting mutations are bypassed and protein synthesis is restored (21). That the latter application may have promising therapeutic potential for DMD has recently been demonstrated in muscle cells from the *mdx* mouse carrying a nonsense point mutation in dystrophin exon 23. Following transfection of AONs directed to the 3′ or 5′ splice sites of exon 23, the skipping of this exon from the transcript was induced, which corrected the translational reading frame and restored dystrophin expression at the sarcolemma both *in vitro* and *in vivo* (22–24). However, these studies have also indicated that antisense targeting of

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3' or 5' splice site sequences can cause less predictable skipping of additional adjacent exons. Therefore, in this study we aimed at more specific, internal exon sequences that are involved in the splicing of the upstream intron, such as the polypurine-rich exon recognition sequences (ERSs) or exonic splicing enhancers (ESEs) (25,26). Based on the presence of purine-rich sequences and suboptimal splice sites, several dystrophin exons (e.g. exon 46) may contain such splicing regulatory elements that are potential targets for AONs to induce exon skipping (27,28).

In patients carrying an exon 45 deletion, the single most frequently deleted exon causing DMD, exon 46 skipping would correct the reading frame and generate the shorter dystrophin protein found in the milder affected BMD patients with a deletion of both exons 45 and 46 (Fig. 1A). Here we report highly effective skipping of exon 46 from the transcript using AONs complementary to a putative ERS within this exon. This targeted conversion of a DMD into a BMD mutation induced the synthesis of normal amounts of properly localized dystrophin in cultured muscle cells from two unrelated exon 45 deletion DMD patients.

RESULTS

Exon 46 skipping in mouse muscle cells

We first tested the feasibility of exon 46 skipping in mouse muscle cells. A series of partly overlapping AONs was designed, directed to a polypurine-rich sequence (stretching from cDNA position 6883 to 6953) resembling an ERS-like splicing regulatory element (Fig. 1B). The efficacy of AONs is largely determined by their binding affinity for the target sequence. Due to base composition and pre-mRNA secondary or tertiary structure, it is difficult to predict which AONs are capable of binding the target sequence. Therefore, gel mobility shift assays were performed by hybridization of a 32 P-labeled exon 46 RNA fragment to the different deoxy-AONs (mAONs). Five out of the 12 mAONs designed (mAON 4, 6, 8, 9 and 11) (Fig. 1B and Table 1) induced a mobility shift (Fig. 2A), indicating their specific affinity for the target RNA. The binding was both time- and dose-dependent (data not shown). These five mAONs were then analyzed for their efficacy in inducing skipping of dystrophin exon 46 in cultured mouse myotubes. For that purpose, we applied 5'-fluorescein (FAM)-labeled 2'-O-methyl phosphorothioate modified oligoribonucleotide-AONs (2'OMe-PS-mAONs), which are resistant to endonucleases and RNaseH and bind to RNA with high affinity (29,30). Upon transfection into mouse myotubes using the cationic polymer polyethylenimine (PEI), typically 60–70% of cells showed specific nuclear uptake of the fluorescent 2'OMe-PS-mAONs (Fig. 2B). At 24 h post-transfection, RT-PCR analysis of myotubes treated with mAON 4, 6, 9 and 11 showed the presence of a novel shorter transcript fragment (324 bp), corresponding in size to exon 45 spliced to exon 47 (Fig. 2C). Sequence analysis of these shorter fragments confirmed the precise skipping of exon 46 (Fig. 2D). Despite its apparent binding affinity in gel mobility shift assays, mAON 8 did not reproducibly induce exon 46 skipping in myotubes. This may have been due to variable binding kinetics. No exon skipping was induced in myotubes following transfection with the non-complementary AON 19, demonstrating

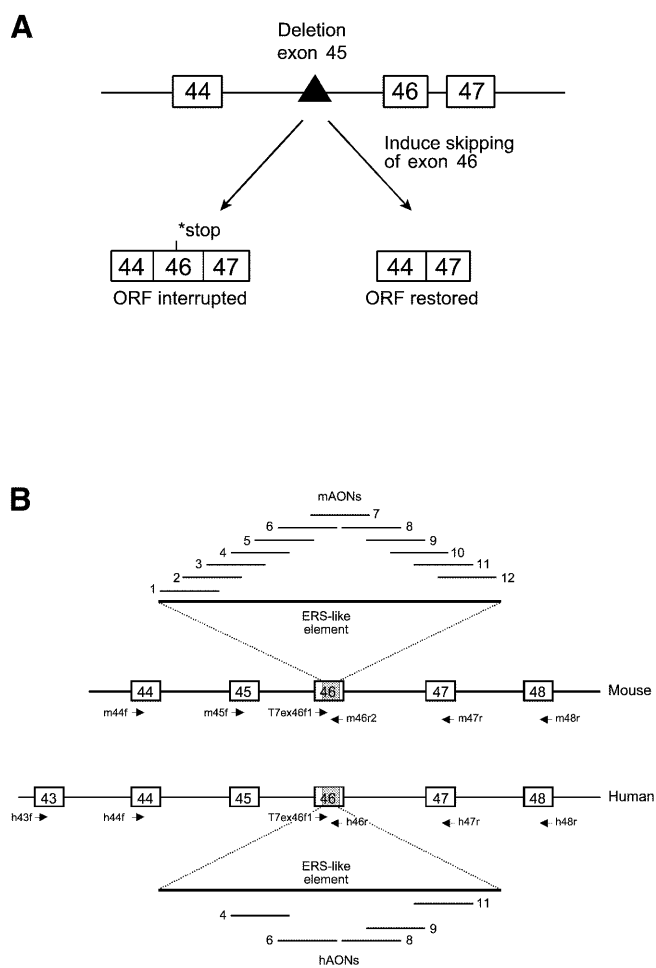


Figure 1. Schematic illustrations of the exon 46 skipping strategy. (A) In DMD patients affected by an exon 45 deletion, a stop codon occurs in exon 46, aborting dystrophin synthesis. Induced skipping of exon 46 would restore the open reading frame (ORF) and lead to a shorter, BMD-like protein. (B) Binding sites for the various mouse- and human-specific RT-PCR primers and AONs (Table 1). The putative splicing regulatory sequence, resembling an ERS and located between cDNA positions 6883 and 6953, is indicated by the gray box drawn in exon 46.

the sequence-specificity of the AON-mediated exon skipping. The skipping efficiencies of the different mAONs were estimated by visual comparison to a concentration series of control DNA (not shown), and found to range between 5 and 15% of total RT-PCR products.

Exon 46 skipping in human muscle cells

Following the initial set up in mouse cells, we focused on inducing the skipping of exon 46 from the human dystrophin transcript. Primary muscle cells were isolated from muscle biopsies from one healthy individual and two unrelated DMD patients with an exon 45 deletion (DL279.1 and DL272.2). Myotube cultures were prepared and, prior to AON-treatments, tested for their myogenic quality by immunohistochemical analyses with muscle-specific antibodies raised against desmin (stains all muscle cells) and myosin (stains only differentiated myotubes). The differentiating myogenic cell content was determined to be ~25% for the control sample, 25% for DL279.1 and 10% for DL272.2 (Fig. 3A). Staining with two

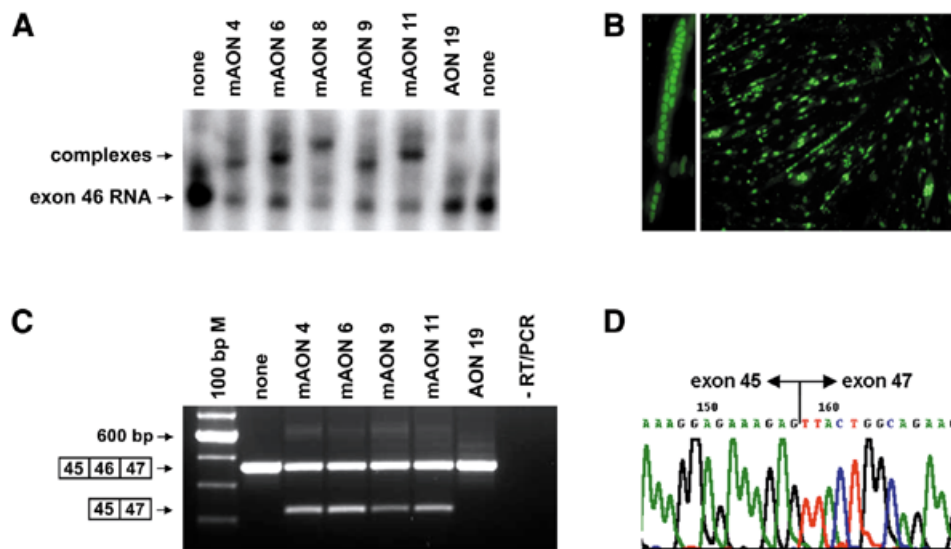


Figure 2. Exon 46 skipping in cultured mouse C2C12 muscle cells. (A) Gel mobility shift assay demonstrating the binding of mAON 4, 6, 8, 9 and 11 to the 32 P-labeled target exon 46 RNA. Complexes of RNA and AON exhibited a lower gel mobility when compared to free RNA (none). AON 19, a non-complementary control oligoribonucleotide derived from sequences elsewhere in the DMD gene, did not bind to exon 46. (B) 5'-Fluorescein-labeled 2'OMe-PS-mAONs were transfected into mouse myotubes. By 24 h post-transfection, 60–70% of myotubes showed multiple fluorescent nuclei indicating successful delivery of the mAONs. The left panel shows an enlargement of one myotube containing more than 20 mAON-positive nuclei. (C) RT-PCR analysis of dystrophin mRNA around exon 46. A shorter transcript fragment of 324 bp, corresponding to exon 45 spliced to exon 47, appeared upon treatment with mAON 4, 6, 9 and 11. AON 19 is a non-complementary control. M, 100 bp size marker; -RT/PCR, negative control. (D) Sequence analysis of the shorter RT-PCR products confirmed the precise skipping of exon 46.

different monoclonal antibodies against epitopes corresponding to dystrophin exons 31–32 (MANDYS1) and exon 77–79 (Dys2), confirmed the presence of dystrophin at the membranes of the control myotubes, whereas the patient-derived myotubes were dystrophin-negative (Fig. 3A). Five human-specific AONs were designed (hAON 4, 6, 8, 9 and 11) (Fig. 1B and Table 1), corresponding to the mouse-specific AONs that induced the skipping of exon 46 in mouse muscle cells. Gel mobility shift assays with these hAONs showed high affinity of hAON 4, 6, 8 and 9 to labeled human exon 46 RNA (data not shown). Control and patient-derived myotube cultures were then transfected with these hAONs (2'OMe-PS-hAON 4, 6, 8 and 9). Based on the nuclear fluorescence observed, the transfection efficiencies were at least 50%. At 24 h post-transfection, RNA was isolated from the myotubes and analyzed by RT-PCR. Upon treatment with hAON 4, 6 and 8, the control showed a shorter RT-PCR product of 471 bp, corresponding to exons 44 and 45 being directly spliced to exon 47. In the DMD patients, a novel shorter transcript fragment of 295 bp, suggesting a direct splicing of exon 44 to exon 47, appeared in response to the same hAONs (Fig. 3B). The skipping efficiency was reproducibly highest with hAON 8, and estimated (by visual comparison to a concentration series of control DNA) to be up to 15% of total RT-PCR products. Sequence analysis of the RT-PCR products revealed that the shorter fragments were products of the exact skipping of exon 46 from the human dystrophin transcript (Fig. 3C). The AON treatments did not induce any other aberrant dystrophin splicing patterns (data not shown), indicating the high specificity of the strategy.

To assess whether skipping of exon 46 restored the reading frame and dystrophin synthesis in DL279.1 and DL272.2, hAON8-treated myotubes from both patients were fixed at

different time points post-transfection, and analyzed with dystrophin antibodies MANDYS1 and Dys2. By 24 h post-transfection, strong dystrophin expression was observed at a level not distinguishable from control myotubes, but still located mostly in the cytoplasm (Fig. 3D). By 48 h, the dystrophin signal had markedly moved to the myotube membrane (Fig. 3D). The levels of dystrophin were still high at day 3, but reduced at days 4 and 5 (data not shown). To determine the percentage of myotubes in which dystrophin expression was restored, the transfected cultures were double-stained with antibodies against myosin (identifies sufficiently differentiated myotubes) and dystrophin. For DL279.1, 42 out of 57 myosin-positive myotubes analyzed (74%) showed dystrophin expression with MANDYS1, and 37 out of 45 (82%) with Dys2. Similar percentages were obtained for DL272.2.

DISCUSSION

Alternative splicing has been observed throughout the DMD gene in numerous BMD/DMD patients, modifying the translational reading frame and adjusting the clinical phenotype (28,31,32). Furthermore, in muscle from DMD patients dystrophin-positive (revertant) fibers can be found, originating from secondary somatic mutations and/or alternative splicing events that restore the translational reading frame (33–35). These findings suggest the potential utility of gene therapy strategies that are based on the targeted manipulation of dystrophin pre-mRNA splicing to restore the reading frame and thus the dystrophin production in muscle cells from DMD patients.

One of the most frequent mutations causing DMD is a deletion of exon 45. The much milder phenotype of BMD patients

Table 1. Sequences of AONs and PCR primers

Name	Sequence (5'→3')	Remarks
mAON 4	FAM-cugcuuccagccc	2'OMe-PS-RNA
mAON 6	FAM-guuauucguuccagccc	2'OMe-PS-RNA
mAON 8	FAM-guuguucuuuagcugcugc	2'OMe-PS-RNA
mAON 9	FAM-uuagcugcugcucuu	2'OMe-PS-RNA
mAON 11	FAM-cugcugcucucucc	2'OMe-PS-RNA
AON 19	FAM-ucugcugcucucucc	Mouse/human AON directed at dystrophin exon 19
T7ex46f1	<i>GGATCCTAATACGACTCACTATAGGAACAGACCACCATGGTCTTGTCAGAATTTCAAAGAG^a</i>	Mouse/human exon 46 forward primer including 5' T7 promoter sequence
m46r2	ACTTGTTCAAGTTGTTCTTTTAG	Mouse exon 46 reverse primer
m44f	GCGATTTCGACAGATCAGTTG	Mouse exon 44 forward primer
m45f	GAACTCCAGGATGGCATTGG	Mouse exon 45 forward primer
m47r	TTATCCACTGGAGATTTGTCTG	Mouse exon 47 reverse primer
m48r	CTTIATGTCAAACGGTCCTGC	Mouse exon 48 reverse primer
hAON 4	FAM-cugcuuccucaacc	2'OMe-PS-RNA
hAON 6	FAM-guuauucguuccucaacc	2'OMe-PS-RNA
hAON 8	FAM-gcuuuucuuuaguugcugc	2'OMe-PS-RNA
hAON 9	FAM-uuaguugcugcucuu	2'OMe-PS-RNA
hAON 11	FAM-uugcugcucuuuucc	2'OMe-PS-RNA
h46r	ACTTGCTCAAGCTTTTCTTTTAG	Human exon 46 reverse primer
h43f	TCTCTCCAGCTTGATTCC	Human exon 43 forward primer
h44f	GCGATTTGACAGATCTGTTG	Human exon 44 forward primer
h47r	TTATCCACTGGAGATTTGTCTG	Human exon 47 reverse primer
h48r	CTGAACGTCAAATGGTCCTTC	Human exon 48 reverse primer

^aThe T7 promoter sequence is shown in italic typeface.

carrying an exon 45–46 deletion implies that a dystrophin truncated in this way (lacking amino acids 2147–2254) is still largely functional. We therefore hypothesize that DMD patients with a deletion of exon 45 may benefit significantly from exon 46 skipping therapy. In this study, we show that AONs directed to a putative splicing regulatory sequence in exon 46 induced substantial, specific skipping of this exon from the dystrophin transcript in muscle cells of both mouse and human origin. Exon 46 skipping in muscle cells from patients with an exon 45 deletion restored dystrophin synthesis to control levels and with a correct localization in at least 75% of myotubes. By 24 h post-transfection, the dystrophin was still predominantly located in the cytoplasm. This was to be expected as complete transcription of the 2.4 Mb DMD gene requires 16 h and most splicing occurs co-transcriptionally (36). The expression at 24 h therefore resulted from early translation of matured in-frame transcripts. The dystrophin signals that were detected at the myotube membranes after 48 h indicate that the novel protein was properly routed and accumulated, suggesting at least partial functionality. The dystrophin expression at the membrane was sustained for 3 days post-transfection, followed by a gradual loss after 4 and 5 days. While this may suggest a different half life or stability of the BMD-type dystrophin, it may be at least partly due to the

experimental setting. At this phase, myotubes lose viability and are lost from the culture. Moreover, after a few days the concentration of the AONs is expected to be significantly reduced due to limited stability.

Our results demonstrate that AON-based DMD gene therapy can be specific and highly efficient, two important factors to be addressed prior to future clinical applications. Upon AON-treatment of the mouse and human muscle cells, the full-length dystrophin cDNA was checked by RT-PCR analysis using 10 different primer sets (data not shown). Unlike earlier studies in *mdx* mice showing the simultaneous skipping of other exons in addition to exon 23 in muscle cells transfected with different AONs directed to the 3' or 5' splice sites of exon 23 (22,24), we did not observe any other aberrant splicing patterns. Although non-consecutive exon splicing may cause co-skipping of additional exons in response to AONs, we believe that by directing the AONs not to splice site consensus sequences but to internal exon sequences such as ERSs, a higher level of skipping specificity will be obtained. In addition, a BLAST search of the human EST-database for sequences homologous to the AONs applied in our experiments did not reveal any perfect hits, suggesting a low probability of unintentional exon-skipping through non-specific interactions. The binding of the AONs to exon 46 induced the specific skipping of exon 46 from the

Table 2. Overview of potential target exons for AON-treatment

Exon to skip	Therapeutic for DMD deletions (exons)	Frequency in DMD–Leiden Database (%)
2	3–7	2
8	3–7, 4–7, 5–7, 6–7	4
43	44, 44–47	5
44	35–43, 45, 45–54	8
45	18–44, 44, 46–47, 46–48, 46–49, 46–51, 46–53	13
46	45	7
50	51, 51–55	5
51	50, 45–50, 48–50, 49–50, 52, 52–63	15
52	51, 53, 53–55	3
53	45–52, 48–52, 49–52, 50–52, 52	9

Skipping of these exons would restore the reading frame in series of patients affected by different deletions of one or more exons. Their total frequency in the DMD–Leiden Database (<http://www.dmd.nl>) is indicated.

dystrophin transcripts. Although we may not exclude the possibility that the binding itself disturbs the RNA secondary structure which inhibits exon 46 splicing, our data strongly suggests that the sequence targeted in these studies indeed acts as a splicing regulatory, ERS-like, element.

The efficiency of the exon 46 skipping therapy was determined through immunohistochemical analyses. In transfected myotube cultures derived from two unrelated DMD patients, the percentages of myosin-positive myotubes that also expressed dystrophin were 74 and 82%. These are remarkably high efficiencies since the AON transfection efficiencies were qualitatively estimated to be ~50%, and exon 46 skipping only occurred in ~15% of DMD transcripts (of all nuclei). Apparently, not all nuclei within one myotube need to be transfected, and a relatively low amount of corrected in-frame transcripts in the transfected nuclei is sufficient to accumulate almost normal quantities of dystrophin throughout the muscle fiber. This is consistent with studies in mice showing that restoration of dystrophin expression to 20–30% of normal levels is sufficient to avert muscle degeneration (9).

Our results provide promising proof of principle for anti-sense-based therapy for DMD. Due to their synthetic and sequence-specific nature, the application of AONs for therapeutic purposes is relatively safe in comparison with gene therapy based on viral vectors. Moreover, the targeted modulation of dystrophin pre-mRNA splicing offers important advantages, such as the simultaneous correction of most or all affected dystrophin isoforms and, most likely, the maintenance of the original (tissue)-specific gene regulation. This strategy may be applicable to a variety of DMD mutations. As most DMD-causing deletions are clustered in two mutation hot spots, the targeted skipping of one particular exon may restore the reading frame in a series of patients with different mutations. A survey of the Leiden DMD Database (<http://www.dmd.nl>) reveals that through skipping of only 10 different exons, >65% of DMD deletion mutations may already be convertible into their nearest adjacent BMD counterpart (Table 2). On-going studies aim to target these exons for AON-induced skipping in human muscle cells *in vitro*. In addition, to facilitate *in vivo* applications, we are currently

studying different strategies for the efficient delivery of AONs to mouse muscle tissue.

MATERIALS AND METHODS

Oligonucleotides

For the gel mobility shift assays, we used HPLC-purified oligodeoxynucleotides (EuroGentec, Belgium). The AONs with highest binding affinity for the target RNA were re-synthesized to contain a FAM group, a phosphorothioate modified backbone and 2'OMe-PS-RNA molecules, and were HPLC purified (EuroGentec, Belgium). To avoid interference in immunohistochemical analysis, we also used a 2'OMe-PS-RNA-hAON8 without a 5'-fluorescein label. All AONs and RT-PCR primers are depicted in Figure 1B and described in Table 1.

Gel mobility shift assay

Dystrophin exon 46 was amplified from mouse or human genomic DNA using primer combinations T7ex46f1 and m46r2 or T7ex46f1 and h46r (Table 1). The T7ex46f1 primer sequence is identical for mouse and human exon 46, and contains a T7 promoter sequence to facilitate T7 *in vitro* transcription. The PCR fragments (160 bp) were gel purified using the QIAquick Gel Extraction Kit (Qiagen). The *in vitro* transcription was performed using the Riboprobe System-T7 (Promega) according to the manufacturer's instructions, and in the presence of 60 μ Ci [32 P]CTP. The radiolabeled transcript was isolated from a 6% denaturing polyacrylamide gel through overnight incubation in a gel elution buffer (0.5 M ammonium acetate, 2 mM EDTA, 0.2% SDS) at 37°C. The binding affinity of the individual AONs (0.5 pmol) was determined by hybridization at 37°C for 30 min in a hybridization buffer (1 mM Tris-HCl pH 7.4, 50 mM NaCl, 5 mM MgCl₂), followed by 8% PAGE and visualization using a PhosphorImager (Molecular Dynamics).

Cell culture and transfection

Mouse myoblast cells derived from the C2C12 cell line (CRL-1772) were obtained from, and grown as recommended by ATCC. Primary human myoblasts were isolated by enzymatic dissociation and cultured as described previously (37). Both mouse and human myoblast cultures were seeded in collagen pre-coated flasks and plates. Myotubes were obtained from confluent myoblast cultures following 10–14 days of serum-deprivation. For transfection of myotubes, we used polyethyl- enimine (PEI) according to the manufacturer's instructions (ExGen500; MBI Fermentas). The cultures were transfected for 3 h in low-serum medium with 1 μ M of each AON linked

to PEI at a ratio-equivalent of 3 (mouse myotubes) or 3.5 (human myotubes).

RNA isolation and RT-PCR analysis

At 24 h post-transfection, total RNA was isolated from the myotube cell cultures (RNAzol B; Campro Scientific). An aliquot of 1 μ g of RNA was used for RT-PCR analysis using C. therm polymerase (Roche Diagnostics) in a 20 μ l reaction at 62°C for 30 min, primed with m48r or h48r. A first amplification was carried out with the outer primers m44f-m48r or h43f-h48r, for 20 cycles of 94°C for 45 s, 60°C for 45 s and 72°C for 1 min. A sample of 1 μ l of this reaction was then re-amplified using the nested primer combinations m45f-m47r or h44f-h47r for 32 cycles of 94°C for 45 s, 60°C for 45 s and 72°C for 1 min. PCR products were analyzed on 2% agarose gels.

Sequence analysis

RT-PCR products were isolated from 2% agarose gels using the QIAquick Gel Extraction Kit (Qiagen). Direct DNA sequencing was carried out by the Leiden Genome Technology Center (LGTC) using the BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) and analyzed on an ABI 3700 Sequencer (PE Applied Biosystems).

Immunohistochemical analysis

For immunohistochemical analyses, cells were grown and transfected on cover slips. At different time-points post-transfection (24, 48, 72, 96 or 120 h), cells were fixed in -20°C methanol. Prior to staining with the different antibodies, the cells were incubated for 1 h in a blocking solution containing 5% horse serum (Gibco BRL) and 0.05% Tween-20 (Sigma) in PBS (Gibco BRL). Antibodies were diluted in this blocking solution. The following primary antibodies were used: desmin polyclonal antibody (ICN Biomedicals) diluted 1:100, myosin

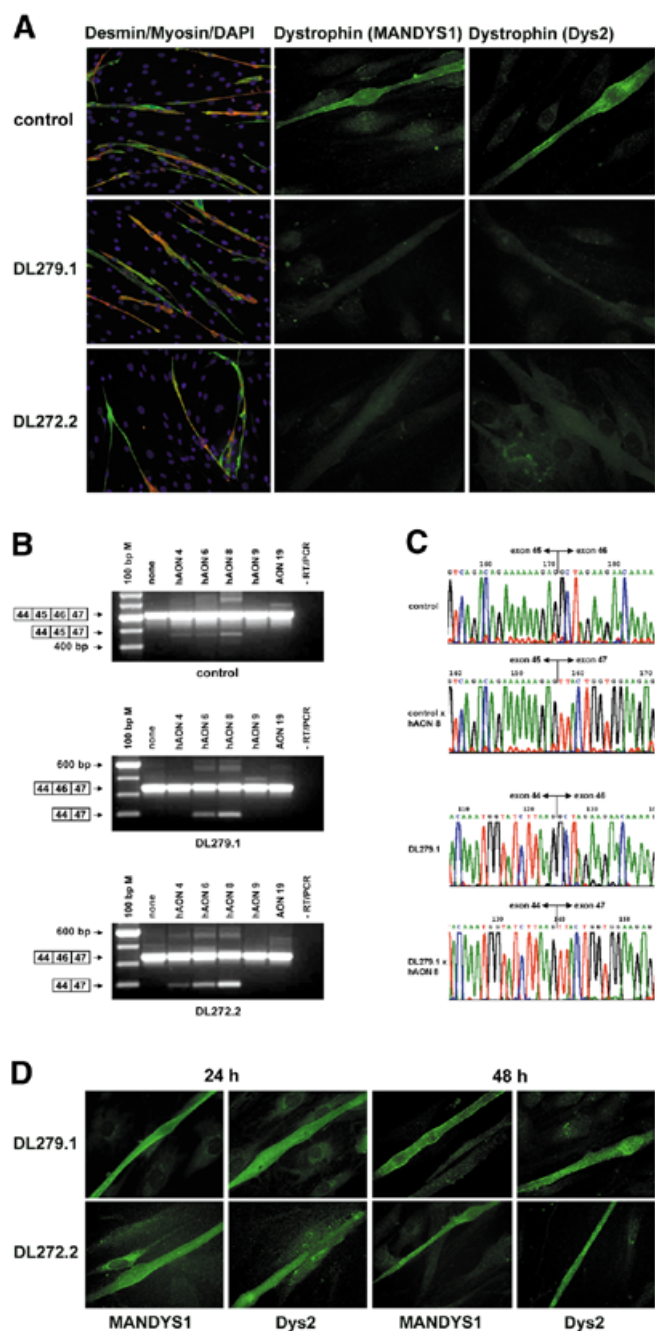


Figure 3. Exon 46 skipping in cultured human primary muscle cells. (A) Immunohistochemical analysis of control and DMD-patient (DL279.1 and DL272.2) myotube cultures prior to hAON-treatment. Cells were stained for DAPI (blue) to determine the number of nuclei, desmin (green fluorescence) to identify muscle cells, and myosin (red fluorescence) to differentiate matured myotubes. The myogenic quality (i.e. the percentage of myotubes) of the cultures was 25% for the control sample, 25% for DL279.1 and 10% for DL272.2. Using monoclonal antibodies MANDYS1 (middle panel) and Dys2 (right panel) positive dystrophin signals were detected at the membranes of control myotubes, whereas the patient-derived myotubes were negative for both antibodies. Magnifications: left panel 16 \times ; middle and right panel 63 \times . (B) RT-PCR analysis at 24 h post-transfection. In response to treatment with hAON 4, 6 and 8, novel shorter transcript fragments were detected in the control (471 bp) and in the patients DL279.1 and DL272.2 (295 bp). It is noteworthy that the intensity of the shorter transcript in the control was reproducibly lower than that observed in the patients. This may be explained by preferential degradation of this out-of-frame transcript by nonsense-mediated mRNA decay. AON 19 is the non-complementary control (none, non-transfected myotubes; M, 100 bp size marker; -RT/PCR, negative control). (C) Sequence analysis of RT-PCR products indicating that hAON 8 induces the precise skipping of exon 46, generating shorter transcripts containing exon 45 spliced to exon 47 in the control, and exon 44 spliced to exon 47 in DL279.1. (D) Immunohistochemical analysis (using MANDYS1 and Dys2) of DL279.1 and DL272.2 myotubes fixed at 24 and 48 h after treatment with hAON 8. In both patients, exon 46 skipping restored dystrophin synthesis. By 24 h, the dystrophin expression was mainly located in the cytoplasm, whereas by 48 h dystrophin signals were also detected at the myotube membranes. Magnification: 63 \times .

monoclonal antibody (MF20; Developmental Studies Hybridoma Bank, University of Iowa) or polyclonal antibody [L53 (38), a gift from Dr M. van den Hoff, AMC, The Netherlands], both diluted 1:100, and the dystrophin monoclonal antibodies NCL-dys2 (Novocastra Laboratories Ltd) and MANDYS1 (a gift from Dr G. Morris) diluted 1:10. After rinsing, the secondary antibodies Alexa Fluor 594 goat anti-rabbit conjugate (diluted 1:1000) and Alexa Fluor 488 goat anti-mouse conjugate (diluted 1:250) (Molecular Probes Inc.) were applied for 1 h. The coverslips were finally inverted and mounted on glass slides in a Gelvatol solution (39) and analyzed using a Leica confocal microscope equipped with epifluorescence optics. Digital images were captured using a CCD camera (Photometrics).

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