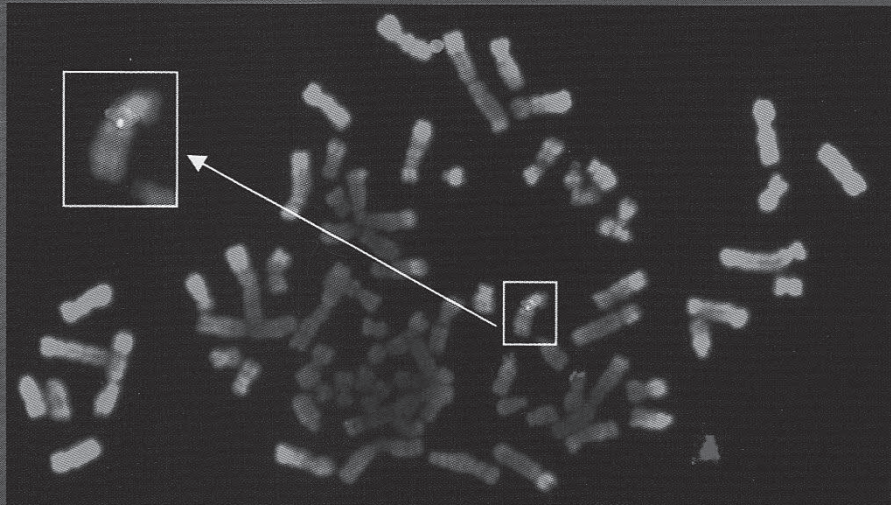


# Human Gene Therapy



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## SCIENTIFIC PAPERS

- E1A- and E1B-Double Mutant Replicating Adenovirus Elicits Enhanced Oncolytic and Antitumor Effects** 773  
*J. Kim, J.-H. Kim, K.-J. Choi, P.-H. Kim, and C.-O. Yun*
- Characterization of a Bipartite Recombinant Adeno-Associated Viral Vector for Site-Specific Integration** 787  
*C. Zhang, N.G. Cortez, and K.I. Berns*
- Comparative Analysis of Antisense Oligonucleotide Sequences for Targeted Skipping of Exon 51 During Dystrophin Pre-mRNA Splicing in Human Muscle** 798  
*V. Arechavala-Gomez, I.R. Graham, L.J. Popplewell, A.M. Adams, A. Aartsma-Rus, M. Kinali, J.E. Morgan, J.C. van Deutekom, S.D. Wilton, G. Dickson, and F. Muntoni*
- Transduction of Human Hematopoietic Stem Cells by Lentiviral Vectors Pseudotyped with the RD114-TR Chimeric Envelope Glycoprotein** 811  
*F. Di Nunzio, B. Piovani, F.-L. Cosset, F. Mavilio, and A. Stornaiuolo*
- Safety of Arylsulfatase A Overexpression for Gene Therapy of Metachromatic Leukodystrophy** 821  
*A. Capotondo, M. Cesani, S. Pepe, S. Fasano, S. Gregori, L. Tononi, M.A. Venneri, R. Brambilla, A. Quattrini, A. Ballabio, M.P. Cosma, L. Naldini, and A. Biffi*
- Polyethylene Glycol Modification of Adenovirus Reduces Platelet Activation, Endothelial Cell Activation, and Thrombocytopenia** 837  
*S.E. Hofherr, H. Mok, F.C. Gushiken, J.A. Lopez, and M.A. Barry*
- Promoter Choice for Retroviral Vectors: Transcriptional Strength Versus Trans-Activation Potential** 849  
*E.L. Weber and P.M. Cannon*
- Prolonged *In Vivo* Gene Silencing by Electroporation-Mediated Plasmid Delivery of Small Interfering RNA** 861  
*D. Eefting, J.M. Grimbergen, M.R. de Vries, V. van Weel, E.L. Kaijzel, I. Que, R.T. Moon, C.W. Löwik, J.H. van Bockel, and P.H.A. Quax*

## NEWS AND COMMENTS

*OBA Documents*

- Future Meeting Dates and Protocol Submission Deadlines for the RAC** 870

*Instructions for Authors can be found at the back of the issue on our website at [www.liebertpub.com](http://www.liebertpub.com)*

**Cover:** Fluorescent *in situ* hybridization demonstrating site-specific integration by an AAV vector. For further details, see the paper by Zhang *et al.* on page 787.



## Comparative Analysis of Antisense Oligonucleotide Sequences for Targeted Skipping of Exon 51 During Dystrophin Pre-mRNA Splicing in Human Muscle

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### ABSTRACT

Duchenne muscular dystrophy (DMD) is caused by mutations in the dystrophin gene that result in the absence of functional protein. In the majority of cases these are out-of-frame deletions that disrupt the reading frame. Several attempts have been made to restore the dystrophin mRNA reading frame by modulation of pre-mRNA splicing with antisense oligonucleotides (AOs), demonstrating success in cultured cells, muscle explants, and animal models. We are preparing for a phase I/IIa clinical trial aimed at assessing the safety and effect of locally administered AOs designed to inhibit inclusion of exon 51 into the mature mRNA by the splicing machinery, a process known as exon skipping. Here, we describe a series of systematic experiments to validate the sequence and chemistry of the exon 51 AO reagent selected to go forward into the clinical trial planned in the United Kingdom. Eight specific AO sequences targeting exon 51 were tested in two different chemical forms and in three different preclinical models: cultured human muscle cells and explants (wild type and DMD), and local *in vivo* administration in transgenic mice harboring the entire human DMD locus. Data have been validated independently in the different model systems used, and the studies describe a rational collaborative path for the preclinical selection of AOs for evaluation in future clinical trials.

### INTRODUCTION

**D**UCHENNE MUSCULAR DYSTROPHY (DMD) is caused by nonsense or frame-shifting mutations in the *DMD* gene, which results in nonfunctional dystrophin proteins (Hoffman *et al.*, 1987). At the same time, interstitial mutations at the DMD locus that maintain the dystrophin mRNA open reading frame give rise to internally deleted but semifunctional dystrophins and the milder Becker muscular dystrophy (BMD) (Hoffman *et al.*, 1987; Monaco *et al.*, 1988). Internally truncated but at least partially functional dystrophins are also expressed in so-called revertant fibers, individual dystrophin-positive fibers found in 50% of DMD patients and in *mdx* mice (Nicholson *et al.*, 1989; Hoffman *et al.*, 1990; Burrow *et al.*, 1991; Fanin *et al.*, 1992; Sherratt *et al.*, 1993; Yokota *et al.*, 2006). Revertant fibers arise

via some alternative splicing mechanism occurring within dystrophin pre-mRNAs, skipping of frame-shifting exons to remove protein-truncating mutations, and restoration of the dystrophin open reading frame (Lu *et al.*, 2000). These revertant dystrophins lack exon domains flanking the gene lesion in DMD patients (Fanin *et al.*, 1995; Thanh *et al.*, 1995) and the mutated exon 23 in the *mdx* mouse (Lu *et al.*, 2000). Despite being internally truncated, dystrophin molecules found in BMD patients can be functional, as demonstrated by several families with in-frame deletions in the DMD gene, associated with elevated serum creatine kinase but displaying no clinical myopathy (e.g., deletions of exons 32–44, 48–51, or 48–53 [Melis *et al.*, 1998], exon 48 [Morrone *et al.*, 1997], exons 48–51 or 50–53 [Beggs *et al.*, 1991], exons 45–55 [Beroud *et al.*, 2007], or exons 50–51 [Lesca *et al.*, 2007]). The efficacy of internally

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truncated dystrophins lacking an appreciable portion of the rod domain has also been demonstrated in transgenic *mdx* mice, and exploited to design so-called microdystrophins compatible with delivery by AAV vectors. These engineered recombinant microdystrophins have been shown to restore normal expression of the dystrophin-associated protein complex (DPC), improve sarcolemmal stability, and prevent myofiber degeneration in the *mdx* mouse model (Wang *et al.*, 2000; Fabb *et al.*, 2002; Harper *et al.*, 2002; Gregorevic *et al.*, 2006).

The first suggestion that a functional, truncated dystrophin molecule could be created in DMD patients originally came from Takeshima *et al.* after skipping exon 19 *in vitro* in control cells (Takeshima *et al.*, 1995; Pramono *et al.*, 1996). Shortly after, restoration of the reading frame in dystrophic cells was shown by Dunckley *et al.* (1998). These authors, and others immediately afterward, proposed the use of antisense oligonucleotides to modulate dystrophin mRNA splicing to enlarge out-of-frame DMD mutations into the nearest in-frame BMD-like mutation and produce an internally deleted functional dystrophin protein (Dunckley *et al.*, 1998; Wilton *et al.*, 1999; Takeshima *et al.*, 2001; van Deutekom *et al.*, 2001).

The mechanism of antisense oligonucleotide (AO) modulation of dystrophin pre-mRNA splicing involves hybridization to specific motifs involved in splicing and exon recognition in the pre-mRNA. This prevents normal spliceosome assembly and results in the failure of the splicing machinery to recognize and include the target exon(s) in the mature gene transcript (Mann *et al.*, 2001; Aartsma-Rus *et al.*, 2003).

In this way one or more exons and their flanking introns are removed during splicing of the pre-mRNA. In the case of the dystrophin gene deletions, selective removal of specific flanking exons should result in in-frame mRNA transcripts that may be translated into an internally deleted, BMD-like and functionally active dystrophin protein with predictable therapeutic activity (Wilton *et al.*, 1999; van Deutekom *et al.*, 2001).

In DMD research, the potential clinical use of AOs has evolved from studies *in vitro* on cultured *mdx* mouse muscle cells (Dunckley *et al.*, 1998; Mann *et al.*, 2001, 2002) and human DMD muscle cells (van Deutekom *et al.*, 2001; Aartsma-Rus *et al.*, 2002, 2003, 2004a) to *in vivo* studies in *mdx* and GRMD (golden retriever muscular dystrophy) animal models (Lu *et al.*, 2003, 2005; Fletcher *et al.*, 2006; McClorey *et al.*, 2006b) (Table 1). Direct *in vivo* AO-induced exon skipping in humans, however, has yet to be demonstrated and this proof of principle, along with clinical safety, is the crucial aspect of initial clinical studies. Preparation has been reported for two parallel clinical trials of AO therapeutics in DMD patients that will target dystrophin exon 51. Exclusion of exon 51 is predicted to allow the restoration of the dystrophin open reading frame (ORF) in ~17% of DMD deletion patients (deletions of exons 45–50, 47–50, 48–50, 49 and 50, 50, 52, and 52–63; van Deutekom and van Ommen, 2003). Although these two clinical trials will both target exon 51, each will evaluate different AO chemistries: 2'-O-methyl-modified ribose moieties on a phosphorothioate backbone (2-OMe AOs) (completed by Leiden University Medical Center [LUMC] and Prosenza [Leiden, The Netherlands] [van Deutekom *et al.*, 2007]) and phosphorodiamidate morpholino oligomers (PMOs) (<http://clinicaltrials.gov/ct/show/NCT00159250>; in progress in the United Kingdom [Muntoni *et al.*, 2005]).

The majority of the AO work undertaken on cultured cells

has been performed with 2-OMe AOs, as this chemistry is readily available and, more importantly, allows efficient cell transfection as a cationic lipoplex preparation (Summerton and Weller, 1997; Fletcher *et al.*, 2006). Although PMOs appear to be more efficient after direct administration to tissues in the *mdx* mouse than the equivalent 2-OMe AO (Fletcher *et al.*, 2006), their poor uptake *in vitro* limits the approaches that can be used to refine AO design.

In this study, we have compared, in a blinded fashion, eight different sequences targeting dystrophin exon 51. The two most efficient sequences were finally compared in their PMO version. Dose–response experiments were used to evaluate the lowest concentration capable of inducing skipping and time course experiments were performed to study the persistence of the exon skipping after transfection. All preliminary experiments were undertaken in normal human skeletal muscle cells (hSkMCs) because these cells are more readily available than patient-derived cell lines. All the key experiments were repeated on the cells of DMD patients with different but relevant deletions. As a proof of principle, the AOs were also tested on muscle explants from DMD patients to study their effect in an *ex vivo* structure, as such explants have been described as a putative model in which to test gene therapy models (Fletcher *et al.*, 2006; McClorey *et al.*, 2006a). These experiments were complemented by the intramuscular administration of PMO versions of the two most promising sequences into the gastrocnemius muscle of a mouse model transgenic for the entire human dystrophin locus (Bremner-Bout *et al.*, 2004). All these approaches suggested that one of the AOs (AO B30, +66+95) more robustly induced exon skipping, and this sequence has now been chosen for the phase I/IIa trial which is underway in the United Kingdom.

## MATERIALS AND METHODS

### AO design

Eight different 2'-O-methyl AOs to human dystrophin exon 51 (A20, B30, and C20–H20) were designed on the basis of ES-Efinder analysis (Smith *et al.*, 2006), and relative to previously published sequences (Fig. 1A). Splice site AOs (C20 to H20) were designed on the basis of work performed in our laboratory on the *mdx* mouse, published previously (Graham *et al.*, 2004). To avoid differences in synthesis conditions and concentrations all AOs were purchased from Eurogentec (Seraing, Belgium), diluted to the same concentration in water by the same operator, aliquoted, and stored at  $-80^{\circ}\text{C}$ . Three further AOs (A25, B30, and I25; Fig. 1A) were synthesized as phosphorodiamidate morpholino oligomers (PMOs) by Gene Tools (Philomath, OR). To facilitate introduction into cultured cells, the uncharged PMOs were hybridized to phosphorothioate-capped DNA leashes, based on a previous design (Gebbski *et al.*, 2003), and stored at  $4^{\circ}\text{C}$ .

### Cell culture and AO transfection

Transfections of AOs were performed at two separate institutions, Imperial College (IC, London, UK) and Royal Holloway (RH, London, UK), using normal primary human skeletal muscle cells from different sources: human fetal muscle cells were obtained from the Medical Research Council (MRC) Tissue Bank (London, UK) and human primary skeletal muscle cultures were



TABLE 1. DUCHENNE MUSCULAR DYSTROPHY ANTISENSE OLIGONUCLEOTIDE-BASED STUDIES PUBLISHED TO DATE

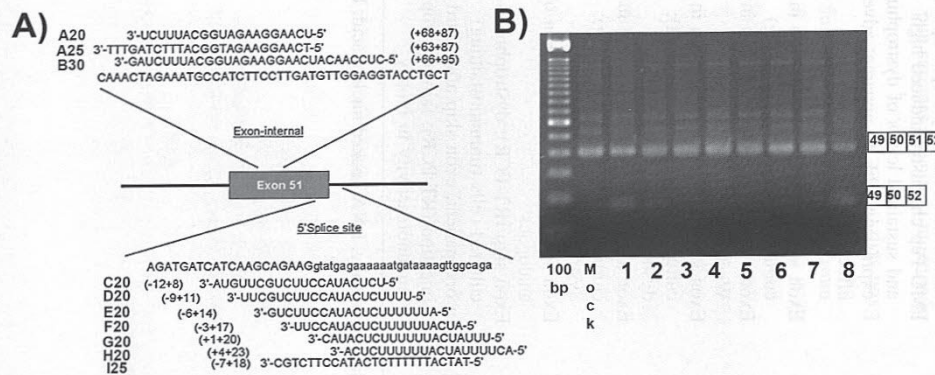
<i>Model</i>	<i>Tissue</i>	<i>Study</i>	<i>AO</i>	<i>Administration</i>	<i>Result</i>
<i>mdx</i> mouse	Myoblast culture	Dunckley <i>et al.</i> (1998)	2-OMe	Transfection	Exon skip (RT-PCR): dystrophin in cultured cells (immunostaining)
	Myoblast culture	Wilton <i>et al.</i> (1999)	2-OMe	Transfection	Exon skip (RT-PCR)
	Myoblast culture	Mann <i>et al.</i> (2001, 2002)	2-OMe	Transfection	Exon skip (RT-PCR)
	Myoblast culture	Gebski <i>et al.</i> (2003)	PMO plus leashes	Transfection	Exon skip (RT-PCR); dystrophin in Western blot
	Myoblast culture	Graham <i>et al.</i> (2004)	2-OMe	Transfection	Exon skip (RT-PCR), dystrophin in Western blot
	<i>In vivo</i>	Mann <i>et al.</i> (2001)	2-OMe	Intramuscular	Exon skip (RT-PCR): dystrophin in muscle sections (immunostaining and Western blotting)
	<i>In vivo</i>	Gebski <i>et al.</i> (2003)	PMO plus leashes	Intramuscular	Exon skip (RT-PCR): dystrophin in muscle sections (immunostaining)
	<i>In vivo</i>	Graham <i>et al.</i> (2004)	2-OMe	Intramuscular	Exon skip (RT-PCR): dystrophin in muscle sections (immunostaining); dystrophin in Western blots
	<i>In vivo</i>	Wells <i>et al.</i> (2003)	2-OMe	Intramuscular	Dystrophin in muscle sections (immunostaining and Western blotting)
	<i>In vivo</i>	Adjei <i>et al.</i> (2003)	2-OMe	Systemic	Dystrophin in muscle sections (immunostaining); dystrophin in Western blots
	<i>In vivo</i>	Gebski <i>et al.</i> (2005)	2-OMe and PMO	Systemic and intramuscular	Exon skip (RT-PCR): dystrophin in muscle sections (immunostaining); dystrophin in Western blots
	<i>In vivo</i>	Aartsma-Rus <i>et al.</i> (2005)	2-OMe	Systemic	Exon skip (RT-PCR): dystrophin in muscle sections (immunostaining); in dystrophin Western blots
	<i>In vivo</i>	Matsuo and Takeshima (2005)	2-OMe	Systemic	Exon skip (RT-PCR): dystrophin in muscle sections (immunostaining); in dystrophin Western blots
	<i>In vivo</i>	Lu <i>et al.</i> (2005)	2-OMe	Systemic	Dystrophin in muscle sections and Western blotting; body-wide functional levels of dystrophin
	<i>In vivo</i>	Alter <i>et al.</i> (2006)	PMO	Systemic	Dystrophin in muscle sections and by Western blotting; body-wide functional levels of dystrophin



Canine model MD (GRMD)	Myoblast culture	McCloy <i>et al.</i> (2006b)	2-OMe	Transfection	Exon skip (RT-PCR); dystrophin in Western blot. 2-OMe chemistry was effective only for short-term induction of corrected transcript and could not induce detectable dystrophin protein PMO-Pep chemistry induced high and sustained levels of dystrophin expression with no apparent adverse effects on cells
Human	Myoblast culture	McCloy <i>et al.</i> (2006b)	PMO-Pep	Transfection	Exon skip (RT-PCR); dystrophin in cultured cells (immunostaining)
	Myoblast culture	van Deutekom <i>et al.</i> (2001)	2-OMe	Transfection	Exon skip (RT-PCR); dystrophin in Western blot
	Myoblast culture	de Angelis <i>et al.</i> (2002)	Retroviral construct	Transfection	Exon skip (RT-PCR); dystrophin in cultured cells (immunostaining); dystrophin in Western blot
	Myoblast culture	Aartsma-Rus <i>et al.</i> (2003)	2-OMe	Transfection	Exon skip (RT-PCR); dystrophin in cultured cells (immunostaining); dystrophin in Western blot
	Myoblast culture	Aartsma-Rus <i>et al.</i> (2004a)	2-OMe	Transfection	Exon skip (RT-PCR); dystrophin in cultured cells (immunostaining); dystrophin in Western blot
	Myoblast culture	Aartsma-Rus <i>et al.</i> (2004b)	2-OMe, PMO, LNA	Transfection	Exon skip (RT-PCR); dystrophin in cultured cells (immunostaining)
	Myoblast culture	Surono <i>et al.</i> (2004)	2-OMe plus ENA	Transfection	Exon skip (RT-PCR); dystrophin in cultured cells (immunostaining)
	<i>In vivo</i>	Takeshima <i>et al.</i> (2006)	2-OMe	Systemic	In one patient, exon skip after third injection (RT-PCR); faint dystrophin immunoreactivity in biopsy

*Abbreviations:* 2-OMe, 2'-O-methyl; AO, antisense oligonucleotide; ENA, ethylene-bridged nucleic acid; GRMD, golden retriever muscular dystrophy; LNA, locked nucleic acid; PMO, phosphorodiamidate morpholino oligomer; PMO-Pep, peptide-linked PMO; RT-PCR, reverse transcription-polymerase chain reaction.





**FIG. 1.** (A) Sequences and localization of the various antisense oligonucleotides (AOs) compared in this project. AOs A25 and I25 were phosphorodiamidate morpholino oligomers (PMOs), whereas B30 corresponds to the sequences of both the 2'-*O*-methyl (2-*O*-Me) version and the PMO. All remaining sequences were compared as 2-*O*-Me AOs. (B) Eight different 2-*O*-Me AOs were compared in a blinded fashion to evaluate their capacity to produce correct exon skipping in normal human skeletal muscle cells (hSkMCs). Once the code was revealed, the two most successful AOs (lanes 1 and 8) were studied in terms of dose and time course. Lane 1, AO A20; lane 8, AO B30.

obtained from muscle biopsies obtained at the Dubowitz Neuromuscular Unit, Hammersmith Hospital (London, UK), both with the approval of the institutional ethics committee, and using slightly different protocols. The comparable results obtained in this way reinforce the findings presented here. Myoblasts were seeded on Matrigel (0.1 mg/ml)-precoated 6-well plates, cultured in differentiation medium (Dulbecco's modified Eagle's medium [DMEM] plus 5% horse serum) (IC), or in supplemented muscle cell differentiation medium (Promocell, Heidelberg, Germany) (RH), and transfected when myoblasts fused to form visible myotubes (elongated cells containing multiple nuclei and myofibrils), usually after 2 days. Transfection reagent Lipofectin (Invitrogen, Paisley, UK) was added to the AOs at a ratio of 2  $\mu$ l of Lipofectin to 1  $\mu$ g of 2'-*O*-Me AO, or 4  $\mu$ l of Lipofectin to 1  $\mu$ g of PMO, optimized previously (data not shown), and myotube cultures were incubated with the mixture for 4–5 hr, according to the manufacturer's instructions. AO concentrations ranging from 50 to 500 nM were used for experiments exploring dose–response correlations, whereas time course experiments were undertaken at concentrations of 100 and 300 nM.

#### Explants

Muscle was cut into fragments approximately 2–3 mm<sup>3</sup> in size and transferred to a 24-well plate containing a 400 nM concentration of the AOs diluted in a final volume of 500  $\mu$ l of OptiMEM (Invitrogen) plus penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Forty-eight hours after the infusion, 500  $\mu$ l of DMEM plus 10% horse serum was added to each well. RNA was isolated 7 days after the infusion.

#### RNA isolation and reverse transcription-polymerase chain reaction analysis

Again, slightly different methods were employed in the different institutions for RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis, thereby confirming the validity of the results obtained. Twenty-four hours after transfection (or later in time course experiments), RNA was extracted with TRIzol (Invitrogen) (IC), or with the RNeasy sys-

tem (Qiagen, Crawley, UK) (RH). Aliquots of 400 ng of total RNA were used for RT-PCR analysis (55°C for 35 min) in a 20- $\mu$ l reaction using Transcriptor reverse transcriptase (Roche) (IC) and a specific primer (primer sequences available on request). Three microliters of this reaction was used as a template for a primary PCR consisting of 20 cycles of 94°C (40 sec), 60°C (40 sec), and 72°C (80 sec), using specific primers depending on the deletions present in the cells. From this reaction 1.5  $\mu$ l was later used as a template for a nested PCR, consisting of 30 cycles of 94°C (40 sec), 60°C (40 sec), and 72°C (80 sec). Alternatively (RH), RNA was subjected to single-tube RT-PCR using a GeneScript system (Genesys, Camberley, UK). PCR products were analyzed on 1.5% agarose gels in Tris-acetate/EDTA buffer. Skipping efficiencies were determined by quantification of the PCR products at the Center for Human and Clinical Genetics (Leiden University Clinical Center, Leiden, The Netherlands), using a DNA 100 LabChip kit and an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA).

#### Sequence analysis

RT-PCR products were excised from agarose gels and extracted with a QIAquick gel extraction kit (Qiagen). Direct DNA sequencing was carried out by the MRC Genomics Core Facility.

#### Western blot analysis of dystrophin protein

Cells were cultured and transfected as described above. One week after transfection, cells were harvested (four wells per sample) and washed with phosphate-buffered saline (PBS) and protein extracts were isolated directly in 50  $\mu$ l of loading buffer (75 mM Tris-HCl [pH 6.8], 15% sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, 2% glycerol, bromophenol blue, and protease inhibitors). Samples were denatured at 95°C for 5 min and centrifuged at 18,000  $\times$  g for 5 min before being loaded in a 6% polyacrylamide gel with a 4% stacking gel. Gels were electrophoresed for 4 hr at 100 V and blotted to a nitrocellulose membrane overnight at 200 mA. Blots were blocked for 1 hr with 10% nonfat milk in PBS–Tween (PBST) buffer and



dystrophin protein was detected by probing the membrane with NCL-DYS1 primary antibody (Vision BioSystems, Newcastle upon Tyne, UK) diluted 1:40 in 3% milk. A biotinylated secondary anti-mouse IgG antibody (diluted 1:1000; GE Healthcare, Little Chalfont, UK) and a streptavidin/horseradish peroxidase (HRP)-conjugated antibody (diluted 1:5000; Dako, Carpinteria, CA) allowed visualization in a luminol-HRP chemiluminescence reaction (ECL-Plus; GE Healthcare) on film (Hyperfilm; GE Healthcare) exposed at 20-sec intervals (20 sec to 2 min, with occasional longer exposures).

*Transgenic human DMD mice*

Work with transgenic human (h)DMD mice was performed at the LUMC, where a transgenic mouse expressing a complete copy of the human DMD gene was generated (Bremmer-Bout *et al.*, 2004). The experiments were authorized by the Animal Experimental Commission (UDEC) of the Medical Faculty of Leiden University. Mice (approximately 5 weeks of age) were anesthetized by intraperitoneal injection of a 1:1 (v/v) solution of Hypnorm (fentanyl and fluanisone; Janssen Pharmaceutica, Berchem, Belgium) and Dormicum (midazolam; Roche, Mijdrecht, The Netherlands). The muscles were pretreated to induce muscle degeneration and regeneration by injection of 3.6  $\mu$ l of polyethylenimine (PEI) in 5% (v/w) glucose on days -1 and -2. A total of 2.9 nmol of each PMO per injection in a final volume of 40  $\mu$ l was injected intramuscularly in both gastrocnemius muscles, using a Hamilton syringe with a 22-gauge needle. Mice received two injections at 24-hr intervals and were then killed. This muscle was isolated and frozen in liquid nitrogen-cooled 2-methylbutane (Bremmer-Bout *et al.*, 2004).

RESULTS

*Exon-internal 2-OMe AOs are more efficient than those targeting the 5' splice site*

AO sequences targeting exon 51 were designed using different strategies. One strategy, based on previous results obtained in mouse dystrophin exon 23 (Graham *et al.*, 2004), was that of designing an overlapping stepped array of 2-OMe AOs complementary to the 5' (donor) splice site of intron 51. The other strategies examined other potential splice motifs across exon 51, including the 3' (acceptor) splice site and the branch point, as well as exon-internal sequences (Aartsma-Rus *et al.*, 2004b; Wilton *et al.*, 2007). After initial characterization in each partner laboratory (Table 2) (Aartsma-Rus *et al.*, 2002, 2005; Harding *et al.*, 2007), eight AOs, six targeting the 5' splice site designed in the first partner laboratory (RH) (C20 to H20; Fig. 1A) plus two overlapping AOs (A20 and B30; Fig. 1A), designed independently in the other two partner laboratories (LUMC and the University of Western Australia [UWA], Perth, Australia), were then all compared in parallel in a fourth laboratory (IC), at a concentration of 100 nM on cultured differentiated human skeletal muscle cells. RNA from these cultures was analyzed blindly by RT-PCR, the results of which are shown in Fig. 1B. Whereas the AOs targeting the 5' splice site (C20 to H20; Fig. 1A) were confirmed, surprisingly, as being largely ineffective at inducing skipping of exon 51 (Fig. 1B, lanes 2 to 7), those against the exon-internal sequences, AOs A20 and B30 (Fig. 1A), were capable of inducing exon 51 skipping at a level approaching 50% (Fig. 1B, lanes 1 and 8).

TABLE 2. PRELIMINARY COMPARISON BETWEEN CANDIDATE ANTISENSE OLIGONUCLEOTIDES<sup>a</sup>

Institution	AO name	Sequence	Compared in this study	Chemistry
Leiden University Medical Center, The Netherlands	h51AON1	UCAAGGAAGAUGGCAUUUCU	A20	2OMe
	h51AON24	GAAAGCCAGUCGGUAAGUUC		
	h51AON27	CACCCACCAUCACCC		
	h51AON2e	CCUCUGUGAUUUUAACUUGAU		
University of Western Australia, Australia	h51AON29	UGAUAUCCUCAAGGUCACCC	B30	2OMe/PMO
	H51A (-01+25)	ACCAGAGUAACAGUCUGAGUAGGAGC		
	H51A (+61+90)	ACAUCAAGGAAGAUGGCAUUUCUAGUUUGG		
	H51A (+66+90)	ACAUCAAGGAAGAUGGCAUUUCUAG		
	H51A (+68+95)	CUCCAACAUCAAGGAAGAUGGCAUUUCUAG		
	H51A (+111+134)	UUCUGUCCAAGCCGGUUGAAAUC		
	H51A (+175+195)	CACCCACCAUCACCCUCUGUG		
	H51A (+199+220)	AUCAUCUCGUUGAUUCCUCAAA		
	H51A (+08-17)	AUCAUUUUUCUCAUACCUUCUGCU		
	H51A (+16-07)	CUCAUACCUUCUGUUGAUGAUC		
Royal Holloway University of London, United Kingdom	DMD51 (-12+8)	UAGUUCGUCUCCAUACUCU	C20	2OMe
	DMD51 (-9+11)	UUCGUCUCCAUACUCUUUU	D20	2OMe
	DMD51 (-6+14)	GUCUCCAUACUCUUUUUUA	E20	2OMe
	DMD51 (-3+17)	UUCCAUACUCUUUUUCA	F20	2OMe
	DMD51 (+1+20)	CAUACUCUUUUUACUAUUU	G20	2OMe
	DMD51 (+4+23)	ACUCUUUUUACUAUUUCA	H20	2OMe
	DMD51 (-7+18)	CGTCTTCCATACTCTTTTACTAT	I25	PMO
	DMD51 (+63+87)	TTTGATCTTACGGTAGAAGGAAC	A25	PMO

<sup>a</sup>Undertaken in the partner laboratories (Leiden University Medical Center, Leiden, The Netherlands; University of Western Australia, Perth, Australia; Royal Holloway-University of London, Egham, UK) (Aartsma-Rus *et al.*, 2004b; Harding *et al.*, 2007) and a selection was further confirmed in a fourth laboratory (Imperial College, London, UK).



*Exon-internal 2-OMe AOs induce efficient and persistent exon 51 skipping*

The two most efficient AOs (A20 and B30) were compared in terms of dose response in normal human skeletal muscle cells, to determine the concentration necessary to induce exon skipping, and in a time course study, to determine how long after transfection the effect lasted in culture. AO concentrations ranging from 50 to 400 nM were used, and the results shown in Fig. 2A demonstrate that skipping of exon 51 was achieved at the lowest dose used for both AOs, as judged by the size difference (212 bp) between the skipped and full-length bands. Precise exon 51 skipping was confirmed by direct sequencing of the lower band (Fig. 2C). Transfection of AOs A20 and B30 into normal human skeletal muscle cultures at concentrations of 100 and 300 nM, followed by harvesting of RNA at various time points, demonstrated that skipped products were still detectable by RT-PCR 10 days after transfection (Fig. 2B).

The same experiments were repeated in differentiated muscle cultures derived from DMD patients carrying deletions, which could be amenable to exon 51 skipping to restore the reading frame. Two patients carrying different deletions ( $\Delta 48-50$  and  $\Delta 50$ ) were used for these experiments. Cultured myogenic cells from the DMD patient with a deletion of exons 48 to 50 were used to perform a dose-response study of the two 2-OMe AOs. This demonstrated that, whereas both AOs were able to induce exon skipping at doses as low as 50 nM, AO B30 was much more efficient at inducing exon 51 skipping, even at low

concentrations, as can be appreciated by the relative intensities of the full and the skipped bands (Fig. 3A). Direct sequencing of the lower RT-PCR product showed an accurate junction between exons 47 and 52 (Fig. 3B), confirming exon 51 skipping.

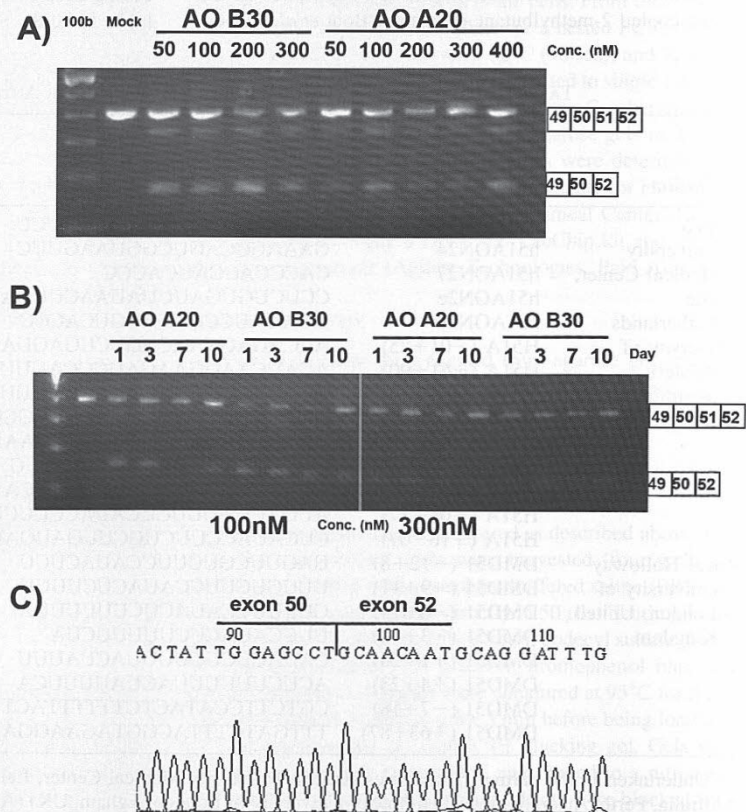
Furthermore, protein samples isolated from similarly transfected cultures from this patient were analyzed by Western blotting, and demonstrated the restoration of dystrophin protein at levels that correlate with the relative degree of exon skipping observed by RT-PCR (compare lanes A20 and B30; Fig. 3C). The levels of dystrophin are, however, rather low, as compared with an equivalent loading of protein extract from normal human cells (Fig. 3C, lane C). No dystrophin is detectable in a protein extract of nontransfected cells from this patient (Fig. 3C, lane Mock).

Cells derived from the DMD patient with a deletion of exon 50 were used to study the persistence of the skip over time. At concentrations of 100 nM, it was evident that AO B30 (+66+95) was substantially more effective at inducing the skipping of exon 51 for as long as 9 days after transfection (Fig. 3D).

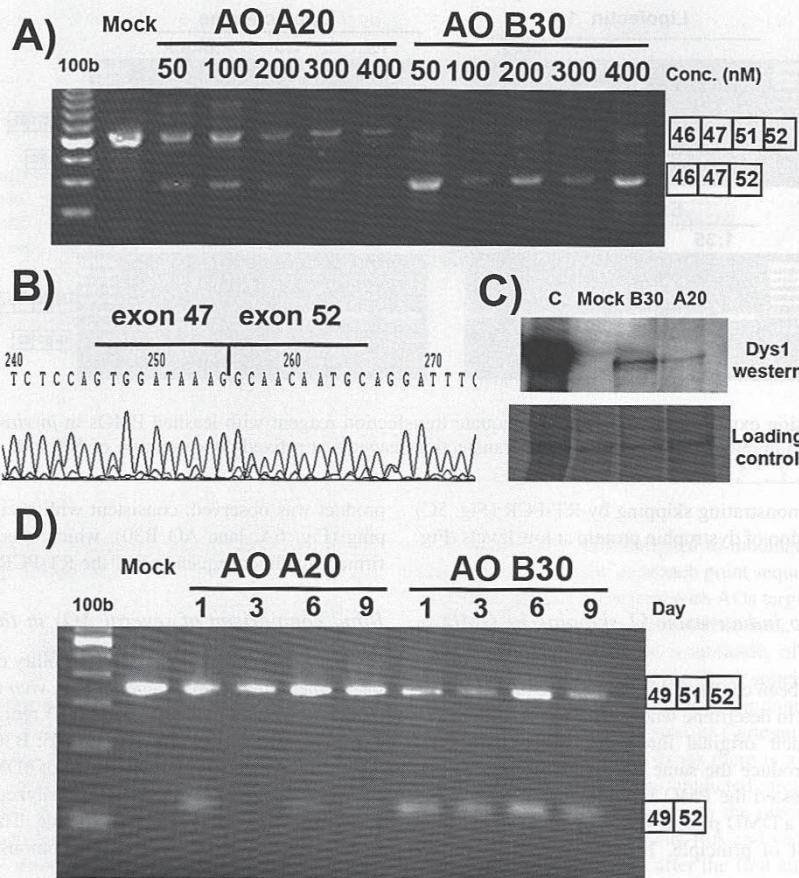
*Exon skipping in response to low doses of PMO in human fetal muscle cells and DMD cells*

The PMO backbone presents certain advantages for uptake by skeletal muscle after systemic injection *in vivo* (Sazani *et al.*, 2002) but its uptake *in vitro* is relatively poor unless either high concentrations are used, or scrape loading is employed to enhance cellular uptake (Summerton and Weller,

**FIG. 2.** (A) Normal hSkMCs were transfected with increasing concentrations of the two 2-OMe AOs. Both AOs achieved the correct skip at low concentrations, with AO B30 (+66+95), showing more consistent results. (B) Normal hSkMCs were transfected with two concentrations (100 and 300 nM) of the two AOs and studied 1, 3, 7, and 10 days after transfection. It was possible to confirm the presence of a fragment of the expected size 10 days after transfection in each case. (C) The bands were analyzed by sequencing, confirming that the correct exon skip had been achieved. In both experiments, whereas the skip achieved with AO A20 was approximately 40% of the total, the skipped band present when cells were transfected with AO B30 was approximately 60%. Note that additional fragments, slightly shorter than the wild-type products, are visible in some analyses. This is due to heteroduplex formation and has been described previously (Aartsma-Rus *et al.*, 2003).





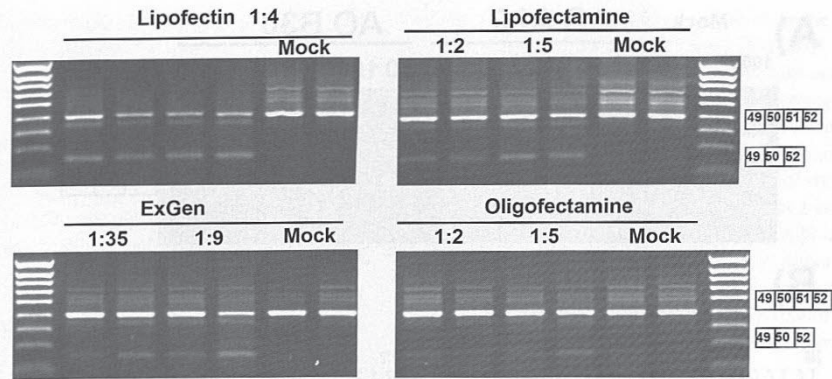


**FIG. 3.** (A) Cells from a DMD patient with a deletion in exons 48 to 50 were transfected with increasing concentrations of the two 2-OMe AOs and analyzed 9 days after transfection. Cells transfected with AO B30 showed exon skip (50–70%), even at a low dose (50 nM), whereas the skip achieved with AO A20 was less efficient. (B) The lower bands were analyzed by sequencing, confirming that the correct exon skip had been achieved. (C) Western blot probed with dystrophin and loading control stained with Protogold (BBInternational, Cardiff, UK). Normal muscle protein extract (30 μg) was used as a full-length dystrophin control (C). Mock-transfected DMD myoblasts acted as a negative control (Mock). Bands of the correct size can be appreciated in the lysates of all treated cells (A20 and B30) but not in the untreated cells. (D) Cells from a DMD patient with a deletion in exon 50 were transfected with a 100 nM concentration of the two AOs and studied 1, 3, 6, and 9 days after transfection. It was possible to confirm the presence of a fragment of the expected size 9 days after transfection of both AOs, but the percentage of the skip was 13% for AO A20 and 73% for AO B30.

1997). However, we have used the observations of GebSKI *et al.* (2003) to design mixed backbone DNA leashes, which can complex with transfection reagents and thus act as carriers for PMOs into cultured cells. We tested four different, commercially available transfection reagents for their ability to introduce a leashed PMO variant of AO B30 into normal human skeletal muscle cells in culture, at a relatively high concentration of 500 nM. Although all of the reagents tested were capable of achieving a degree of transfection, as judged by the presence of exon 51 skipping in the usual RT-PCR assay (Fig. 4), it was evident that the use of Lipofectin (Invitrogen), at a ratio of 4 μl/μg of leashed PMO, was the most effective in these cultures. Other ratios of Lipofectin were also tested but found to be less effective (data not shown). We subsequently used these conditions to directly compare three PMOs in normal human skeletal muscle cells (B30, A25, and I25; Fig. 1A)

in two of the research laboratories (IC and RH). I25 was included, despite the fact that the 2-OMe tested had not been effective at achieving exon skipping when targeting the 5' splice site, because it targeted the 5' splice site, which was shown previously to be effective in mouse exon 23 (Mann *et al.*, 2002; Graham *et al.*, 2004). In addition, when these experiments were designed it was not clear whether one could extrapolate results between sequences when different chemistries were used. The leashed B30 PMO was clearly the most efficient, demonstrating efficacy at the lowest concentration tested in both laboratories (50 nM) (Fig. 5A and B): A25 showed less efficient results than B30 at IC (Fig. 5A), whereas at RH, the two leashed 25-mer PMOs (A25 and I25) appeared incapable of inducing skipping of exon 51 to any substantial level at either 100 or 500 nM (Fig. 5B). The leashed PMOs A25 and B30 were also transfected at IC into cells from the DMD patient with a dele-





**FIG. 4.** Optimization experiments to select an adequate transfection reagent with leashed PMOs in *in vitro* experiments. AO B30 was tested in combination with four different transfection reagents at a fixed concentration of 500 nM.

tion of exon 50, demonstrating skipping by RT-PCR (Fig. 5C) and *de novo* production of dystrophin protein at low levels (Fig. 5C and D).

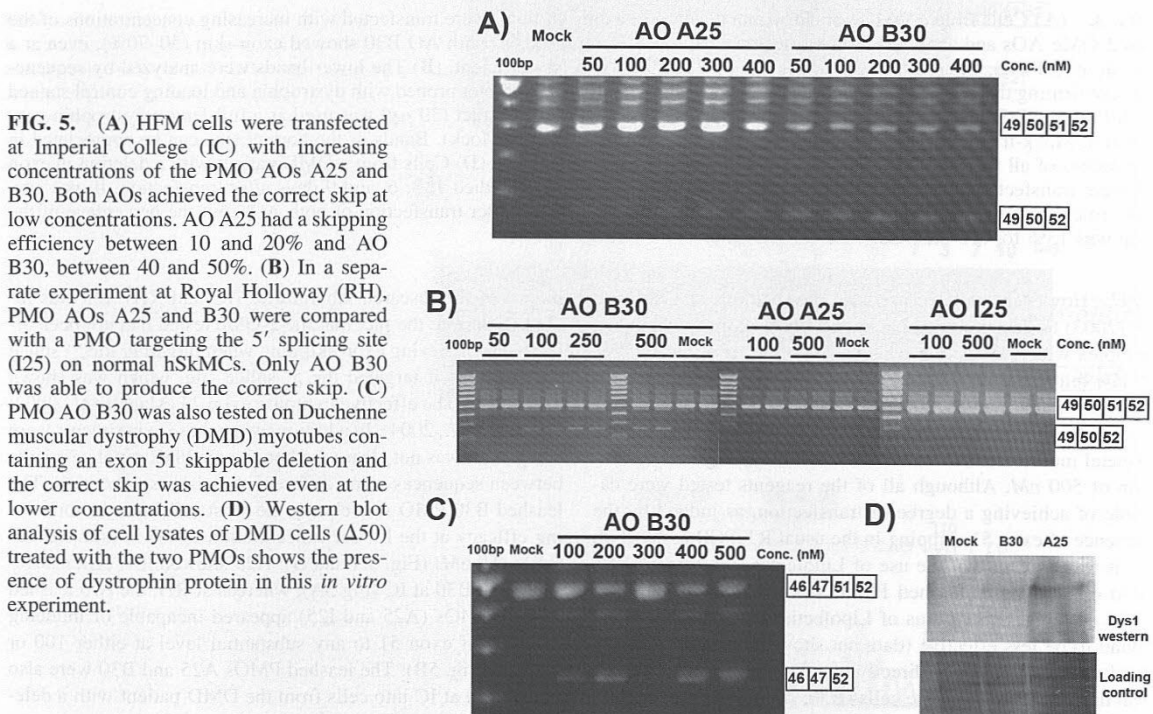
#### AO B30 is able to induce exon 51 skipping in DMD muscle explants

Once results had been confirmed in cultured cells from DMD patients, we wished to determine whether muscle fragments retaining some of their original three-dimensional properties would be able to produce the same exon skipping when AOs were applied. We tested the PMO B30 on several muscle explants derived from a DMD patient with a nonrelevant deletion ( $\Delta 3-11$ ), as a proof of principle. The correct-sized RT-PCR

product was observed, consistent with accurate exon 51 skipping (Fig. 6A, lane AO B30), which was subsequently confirmed by direct sequencing of the RT-PCR product (Fig. 6B).

#### Blind comparison of several AOs in the hDMD mouse

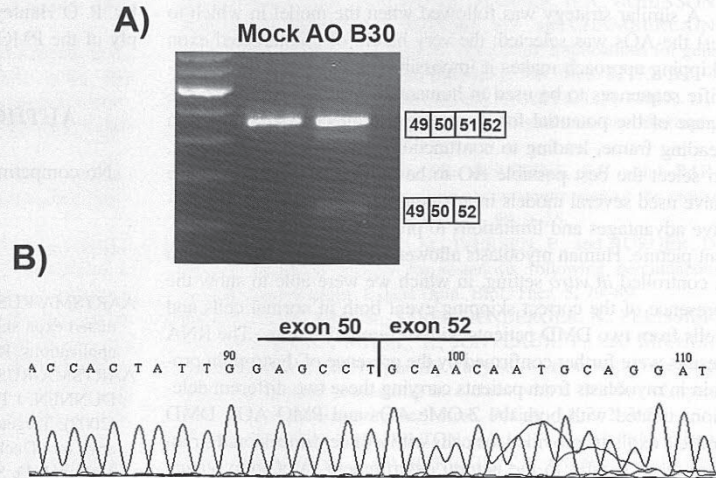
The hDMD mouse offers the possibility of studying processing of the human DMD gene in an *in vivo* setting, providing a model to test the behavior of the AO reagents *in vivo* before clinical testing in patients. PMOs A25, B30, and I25 were injected into the gastrocnemius muscle of hDMD mice, and RNA extracts from treated muscles were analyzed, in a blinded fashion, 2 weeks later for exon 51 skipping. The hierarchy of efficacy of PMOs was the same as that demonstrated *in vitro*, with



**FIG. 5.** (A) HFM cells were transfected at Imperial College (IC) with increasing concentrations of the PMO AOs A25 and B30. Both AOs achieved the correct skip at low concentrations. AO A25 had a skipping efficiency between 10 and 20% and AO B30, between 40 and 50%. (B) In a separate experiment at Royal Holloway (RH), PMO AOs A25 and B30 were compared with a PMO targeting the 5' splicing site (I25) on normal hSkMCs. Only AO B30 was able to produce the correct skip. (C) PMO AO B30 was also tested on Duchenne muscular dystrophy (DMD) myotubes containing an exon 51 skippable deletion and the correct skip was achieved even at the lower concentrations. (D) Western blot analysis of cell lysates of DMD cells ( $\Delta 50$ ) treated with the two PMOs shows the presence of dystrophin protein in this *in vitro* experiment.



**FIG. 6.** (A) Muscle explants from a DMD patient ( $\Delta 3-11$ ) were immersed in medium containing a 400 nM concentration of PMO AO B30. AO B30 was successful at achieving the skip in the muscle explant. (B) The skipped band was analyzed by sequencing analysis, showing the correct skip.



the highest percentage of skipping being achieved with PMO B30, +66+95 (Fig. 7, lane 4).

**DISCUSSION**

DMD is a fatal neuromuscular disorder that affects 1 in 3500 male newborn boys (Moser, 1984). There is no cure for DMD, but a putative treatment may lie in the correction of the reading frame to generate semifunctional dystrophins (Wilton *et al.*, 1997). The possibility of inducing exon skipping by blocking recognition sites required for the correct splicing of RNA has advanced enormously since it was first tested in lymphoblastoid DMD cells (Pramono *et al.*, 1996) and *mdx* muscle cells (Dunckley *et al.*, 1998), with promising results in human muscle cultures and more recently in the *mdx* mouse model *in vivo* (Table 1).

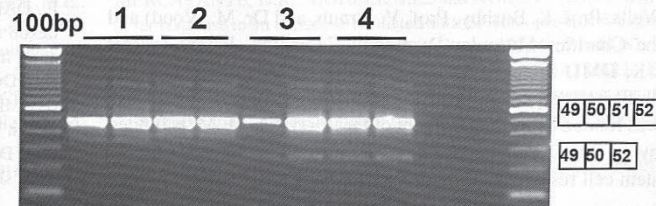
The therapeutic use of AOs has advanced enormously: previously, DNA AOs had been used to downregulate the expression of specific genes involved in cancer and inflammatory diseases in cell cultures (Zamecnik and Stephenson, 1978) and were used in an *in vitro* assay to modulate splicing by targeting splice sites in a mutated  $\beta$ -globin gene associated with  $\beta$ -thalassemia (Dominski and Kole, 1993). These splicing modulation approaches were later reproduced in human cell lines (HeLa and NIH 3T3) stably expressing the  $\beta$ -globin gene carrying the thalassemic mutation IVS2-654 (Sierakowska *et al.*, 1996). The method was also tested in human and mouse cell lines stably expressing mutated genes associated with cystic fibrosis (Friedman *et al.*, 1999). The modulation of splicing with AOs has also been shown to be useful in changing the ratio of alternative spliced transcripts, for example, from an antiapoptotic to a proapoptotic isoform (Dominski and Kole, 1993; Mercatante *et al.*, 2002).

In most cases, AOs designed to modulate splicing target the splice donor, acceptor, or branch point sequence. However, exon skipping can also be induced with AOs targeting exonic splicing enhancer (ESE) motifs (Aartsma-Rus *et al.*, 2005). These weakly defined motifs are bound by a subfamily of splicing factors, the so-called SR proteins, which facilitate splicing by recruitment of splicing effectors U1 and U2AF to the donor splice site and the polypyrimidine tract (reviewed in Cartegni *et al.*, 2002). Interestingly, for human DMD exons there is a correlation between exon “skippability” and the predicted strength of the acceptor splice site and ESEs but not with the predicted strength of the donor (5’) splice site (Aartsma-Rus and van Ommen, 2007).

However, several years after the first attempts at dystrophin exon skipping with AOs, there are still no clear rules to guide investigators in their design, and in mouse and human muscle cells *in vitro* there is great variability for different targets and exons. The consensus sequences at the intron–exon boundaries that are involved in splice site selection are only poorly conserved, and the ESEs that are involved in exon definition are themselves of multiple motifs and their identification is complex. Until these key elements are better understood only length and target region seem to be important when designing exon-skipping AOs for the *DMD* gene (Adams *et al.*, 2007).

As different approaches in AO design lead to different results, it was clear to us that it was necessary to test a wide range of AOs to select the best possible sequence for the trial planned in the United Kingdom. The independent design of AOs by three research groups (at RH, UWA, and LUMC) guaranteed as many different approaches to AO design as groups and, while an independent group (IC) validated the efficacy of the best sequences; the final results were eventually cross-checked by experiments performed by each group.

**FIG. 7.** Three different PMOs were injected, in a blinded experiment, into the gastrocnemius muscle of hDMD mice. Quantification of the PCR products, using a DNA LabChip, gave the following results: lane 1 (AO I25, -7+18), 1.6%; lane 2 (Invert control), 0%; lane 3 (AO A25, +63+87), 5.6%; lane 4 (AO B30, +66+95), 22.1%.





A similar strategy was followed when the model in which to test the AOs was selected: the very nature of this targeted exon skipping approach makes it impossible to test many of the specific sequences to be used in humans in healthy volunteers, because of the potential for disrupting the intact dystrophin open reading frame, leading to nonfunctional dystrophins. Therefore, to select the best possible AO to be used in a clinical trial, we have used several models in an attempt to combine their respective advantages and limitations to produce a reliable and coherent picture. Human myoblasts allowed us to test the sequences in a controlled *in vitro* setting, in which we were able to show the presence of the correct skipping event both in normal cells and cells from two DMD patients with different deletions. The RNA results were further confirmed by the presence of dystrophin protein in myoblasts from patients carrying these two different deletions treated with both the 2-OMe AOs and PMO AOs. DMD muscle explants provided the cells with a three-dimensional structure more similar to the natural environment (McCloy *et al.*, 2006a) and again we were able to show exon 51 skipping in this situation. To assess the results in an *in vivo* model before testing the AOs in patients, we used a mouse encoding the human DMD gene. Again, we were able to show the correct exon exclusion.

The comparison has been systematic and the results have been validated by different laboratories within the collaborating institutions. The strength of this collaborative approach derives both from the unbiased and blinded analysis of different sequences and from the multiple independent approaches used, which took advantage of patient samples with the appropriate deletions, but also *in vivo* assessment of efficacy in the hDMD mouse model. A similar collaborative approach could be used to validate future target sequences in DMD.

As the results of our study all indicated the superiority of the AO targeting the sequence (+66+95) in exon 51, this has been selected as the reagent that will be used in the first U.K. clinical trial. This trial will evaluate the efficacy of this sequence on a PMO backbone. Although both 2-OMe and PMO AOs show promising results *in vitro* and *in vivo*, PMOs have the advantage of having been tested for other purposes in human patients and are currently in use in clinical trials for restenosis, cancer, and viral infections (Kipshidze *et al.*, 2005; Nikravesh *et al.*, 2007). The U.K. trial will be a phase I/IIa study on the effect of the intramuscular injection of this AO in DMD patients with relevant deletions. If this first study is successful, a second clinical trial is planned in which the same AOs will be administered systemically, as this approach holds promises of functional benefit for DMD patients.

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#### AUTHOR DISCLOSURE STATEMENT

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