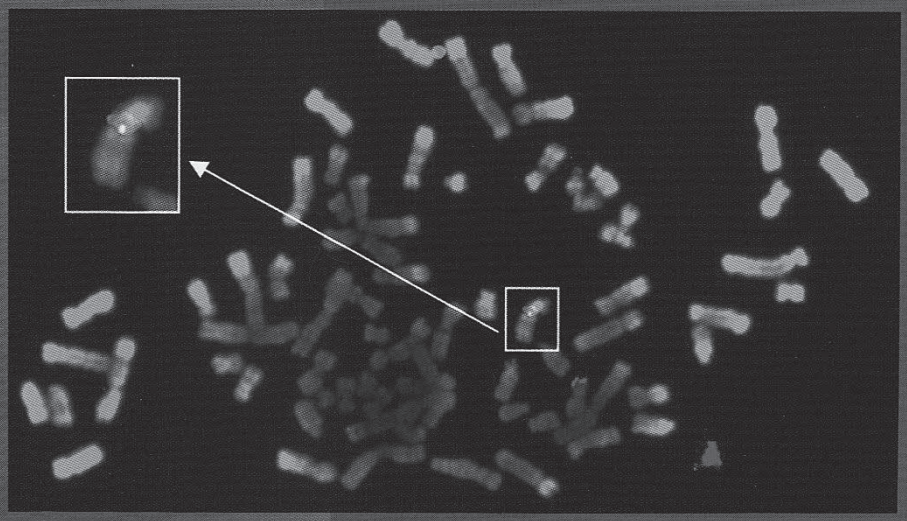
VOLUME 18, NUMBER 9, SEPTEMBER 2007

ISSN: 1043-0342

Human Gene Therapy

The Official Journal of the
European Society of Gene and Cell Therapy



Mary Ann Liebert, Inc. publishers

General Information

***Human Gene Therapy* answers the need for a central forum dealing with all aspects of gene transfer in mammals.**

Human Gene Therapy (ISSN: 1043-0342) is published 12 times a year (monthly) by Mary Ann Liebert, Inc., 140 Huguenot Street, 3rd Floor, New Rochelle, NY 10801-5215. Periodicals postage paid at New Rochelle, NY, and at additional mailing offices. **Postmaster:** Send address changes to *Human Gene Therapy,* c/o Subscription Department, Mary Ann Liebert, Inc., 140 Huguenot Street, 3rd Floor, New Rochelle, NY 10801-5215. Mailed in Canada under CPC IPM #40026674.

Subscriptions should be addressed to the Publisher and are payable in advance. Rates for subscriptions are for a volume of 12 issues: USA print $3,480, International print $3,930, USA print and online $4,165, International print and online $4,623, and online only (worldwide) $3,323. The student postdoctorate/fellow rate is $100. Subscriptions begin with the first issue of the current volume. Bulk subscriptions available upon request from the Publisher. No cancellations/refunds can be honored after publication of a volume's first issue. There are no refunds/returns on single issue purchases.

Telephone: (914) 740-2100; fax: (914) 740-2109; e-mail: info@liebertpub.com Online: www.liebertpub.com

Reprints, except special orders of 100 or more, are available from the authors. For permission to photocopy fewer than 25 copies of an article for internal purposes, please request permission and pay the appropriate fee by contacting the Customer Relations Dept. of the Copyright Clearance Center, Inc., 22 Rosewood Drive, Danvers, MA 01923; 978-750-8400, fax; 978-750-4470. If the number of copies of an article is 25 or more, contact Reprint Sales Manager, Karen Ballen, at 914-740-2194, directly for options.

Information for Manuscript Submission is given elsewhere in the publication and on our web site: www.liebertpub.com

Business Communications should be addressed to the Publisher; e-mail: hturnbull@impressmedia.com

Advertising Inquiries should be addressed to David Brownstein, Mary Ann Liebert, Inc., 140 Huguenot Street, 3rd Floor, New Rochelle, NY 10801-5215, (914) 740-2175. For Europe/Outside the U.S., contact: Hilary Turnbull, imPRESS International Media Ltd., Carrington Kirk, Carrington, Midlothian, EH23 4LR, UK. Telephone: +44-1875-825700; fax: +44-1875-825701; e-mail: hturnbull@impressmedia.com All advertisements are subject to approval by the Publisher.

Manuscripts should be submitted online using the following URL: **http://mc.manuscriptcentral.com/hum**

All papers, news, comments, opinions, findings, conclusions, or recommendations in *Human Gene Therapy* are those of the author(s), and do not constitute opinions, findings, conclusions, or recommendations of the Journal, its Publisher, and its editorial staff.

Human Gene Therapy* is indexed in *Index Medicus, MEDLINE, Current Contents/Life Science, EMBASE/Excerpta Medica, Cambridge Scientific Abstracts, Reference Update,* and *BIOSIS Previews.

Human Gene Therapy is a **Journal Club** selection.

Please visit us on the web: www.liebertpub.com

Human Gene Therapy

The Official Journal of the
European Society of Gene and Cell Therapy

Mary Ann Liebert, Inc. publishers
www.liebertpub.com

Human Gene Therapy

VOLUME 18 NUMBER 9 SEPTEMBER 2007

SCIENTIFIC PAPERS

NEWS AND COMMENTS

Instructions for Authors can be found at the back of the issue on our website at 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.

HUMAN GENE THERAPY 18:798–810 (September 2007)

DOI: 10.1089/hum.2006.061

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

V. ARECHAVALA-GOMEZA,[1,*] I.R. GRAHAM,[2,*] L.J. POPPLEWELL,[2] A.M. ADAMS,[3] A. AARTSMA-RUS,[4] M. KINALI,[1] J.E. MORGAN,[1] J.C. VAN DEUTEKOM,[4,5] S.D. WILTON,[3] G. DICKSON,[2] and F. MUNTONI[1]

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

DUCHENNE MUSCULAR DYSTOPHY (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

[1]Department of Paediatrics, Imperial College, London W12ONN, United Kingdom.
[2]School of Biological Sciences, Royal Holloway-University of London, Egham TW20 0EX, United Kingdom.
[3]Centre for Neurological and Neuromuscular Disorders, Australian Neuromuscular Research Institute, University of Western Australia, Perth WA6009, Australia.
[4]Center for Human and Clinical Genetics, Leiden University Medical Center, 2300RC Leiden, The Netherlands.
[5]Prosensa, 2333AL Leiden, The Netherlands.
*V.A.-G. and I.R.G. contributed equally to this paper.

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 Prosensa [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 (Bremmer-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 ESEfinder 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°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 (Gebski *et al.*, 2003), and stored at 4°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

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

Canine model MD (GRMD)	Myoblast culture	McClorey *et al.* (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
		McClorey *et al.* (2006b)	PMO-Pep	Transfection	PMO-Pep chemistry induced high and sustained levels of dystrophin expression with no apparent adverse effects on cells
Human	Myoblast culture	van Deutekom *et al.* (2001)	2-OMe	Transfection	Exon skip (RT-PCR); dystrophin in cultured cells (immunostaining)
	Myoblast culture	de Angelis *et al.* (2002)	Retroviral construct	Transfection	Exon skip (RT-PCR); dystrophin in Western blot
	Myoblast culture	Aartsma-Rus *et al.* (2003)	2-OMe	Transfection	Exon skip (RT-PCR); dystrophin in cultured cells (immunostaining); dystrophin in Western blot
	Myoblast culture	Aartsma-Rus *et al.* (2004a)	2-OMe	Transfection	Exon skip (RT-PCR); dystrophin in cultured cells (immunostaining); dystrophin in Western blot
	Myoblast culture	Aartsma-Rus *et al.* (2004b)	2-OMe, PMO, LNA	Transfection	Exon skip (RT-PCR); comparison of AO analogues
	Myoblast culture	Surono *et al.* (2004)	2-OMe plus ENA	Transfection	Exon skip (RT-PCR); dystrophin in cultured cells (immunostaining)
	In vivo	Takeshima *et al.* (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.

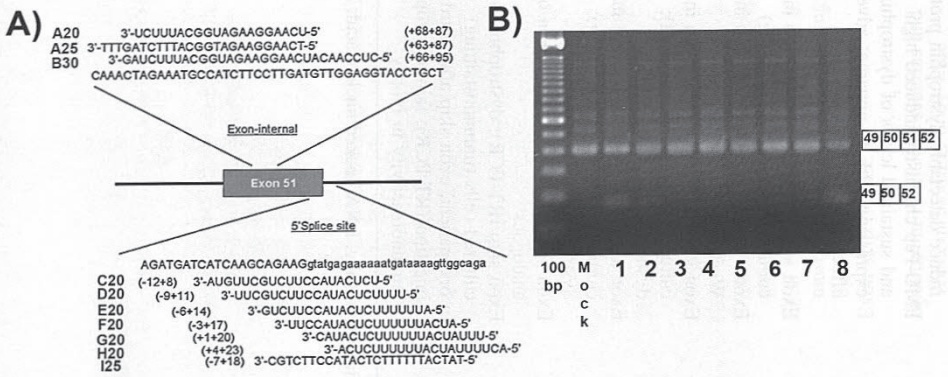


FIG. 1. (**A**) Sequences and localization of the various antisense oligonucleotides (AOs) compared in this project. AOs A25 and I25 were phosphorodiamidate morpholino oligomers (PMOs), whereas B30 corresponds to the sequences of both the 2′-*O*-methyl (2-OMe) version and the PMO. All remaining sequences were compared as 2-OMe AOs. (**B**) Eight different 2-OMe 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 μl of Lipofectin to 1 μg of 2′-OMe AO, or 4 μl of Lipofectin to 1 μg 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 n*M* were used for experiments exploring dose–response correlations, whereas time course experiments were undertaken at concentrations of 100 and 300 n*M*.

Explants

Muscle was cut into fragments approximately 2–3 mm^3 in size and transferred to a 24-well plate containing a 400 n*M* concentration of the AOs diluted in a final volume of 500 μl of OptiMEM (Invitrogen) plus penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Forty-eight hours after the infusion, 500 μl 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 system (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-μl 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 μl 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 μl of loading buffer (75 m*M* 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 × *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 μl 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 μl 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 n*M* 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[a]

Institution	*AO name*	*Sequence*	*Compared in this study*	*Chemistry*
Leiden	h51AON1	UCAAGGAAGAUGGCAUUUCU	A20	2OMe
University	h51AON24	GAAAGCCAGUCGGUAAGUUC		
Medical Center,	h51AON27	CACCCACCAUCACCC		
The	h51AON2e	CCUCUGUGAUUUAUAACUUGAU		
Netherlands	h51AON29	UGAUAUCCUCAAGGUCACCC		
University of	H51A (−01+25)	ACCAGAGUAACAGUCUGAGUAGGAGC		
Western	H51A (+61+90)	ACAUCAAGGAAGAUGGCAUUUCUAGUUUGG		
Australia,	H51A (+66+90)	ACAUCAAGGAAGAUGGCAUUUCUAG		
Australia	H51A (+68+95)	CUCCAACAUCAAGGAAGAUGGCAUUUCUAG	B30	2OMe/PMO
	H51A (+111+134)	UUCUGUCCAAGCCCGGUUGAAAUC		
	H51A (+175+195)	CACCCACCAUCACCCUCUGUG		
	H51A (+199+220)	AUCAUCUCGUUGAUAUCCUCAA		
	H51A (+08−17)	AUCAUUUUUUCUCAUACCUUCUGCU		
	H51A (+16−07)	CUCAUACCUUCUGCUUGAUGAUC		
Royal Holloway	DMD51 (−12+8)	UAGUUCGUCUUCCAUACUCU	C20	2OMe
University of	DMD51 (−9+11)	UUCGUCUUCCAUACUCUUUU	D20	2OMe
London, United	DMD51 (−6+14)	GUCUUCCAUACUCUUUUUUA	E20	2OMe
Kingdom	DMD51 (−3+17)	UUCCAUACUCUUUUUCUA	F20	2OMe
	DMD51 (+1+20)	CAUACUCUUUUUUACUAUUU	G20	2OMe
	DMD51 (+4+23)	ACUCUUUUUUACUAUUUUCA	H20	2OMe
	DMD51 (−7+18)	CGTCTTCCATACTCTTTTTTACTAT	I25	PMO
	DMD51 (+63+87)	TTTGATCTTTACGGTAGAAGGAACT	A25	PMO

[a]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 n*M* 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 n*M*, 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 (Δ48–50 and Δ50) 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 n*M*, 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 n*M*, 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,

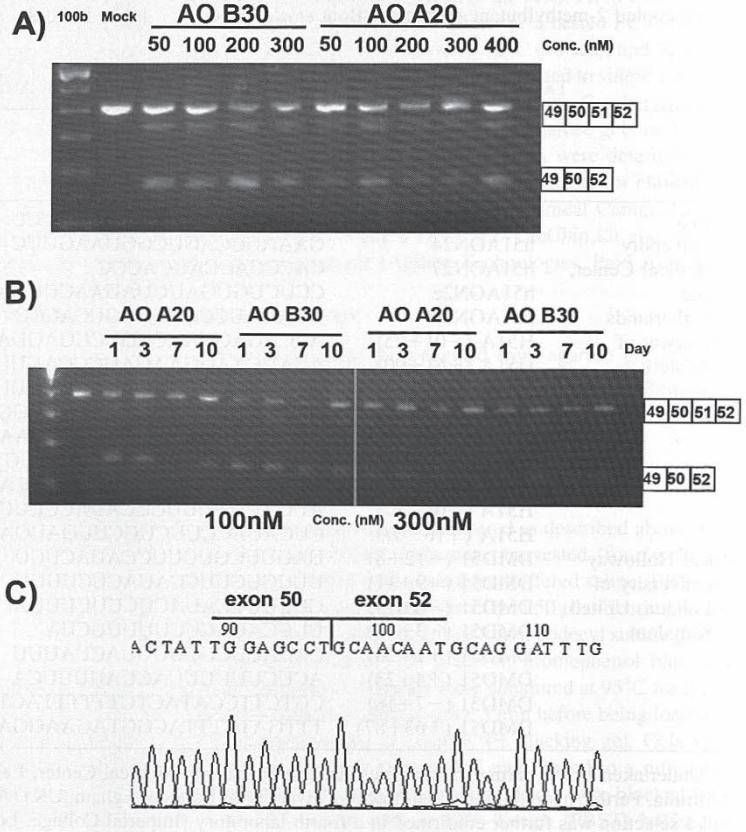


FIG. 2. (**A**) Normal hSkMCs were transfected with increasing concentrations of the two 2-OMe AOs. Both AOs achieved the correct skip at low concentrations, with AO B30 (+66+95), showing more consistent results. (**B**) Normal hSkMCs were transfected with two concentrations (100 and 300 n*M*) of the 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).

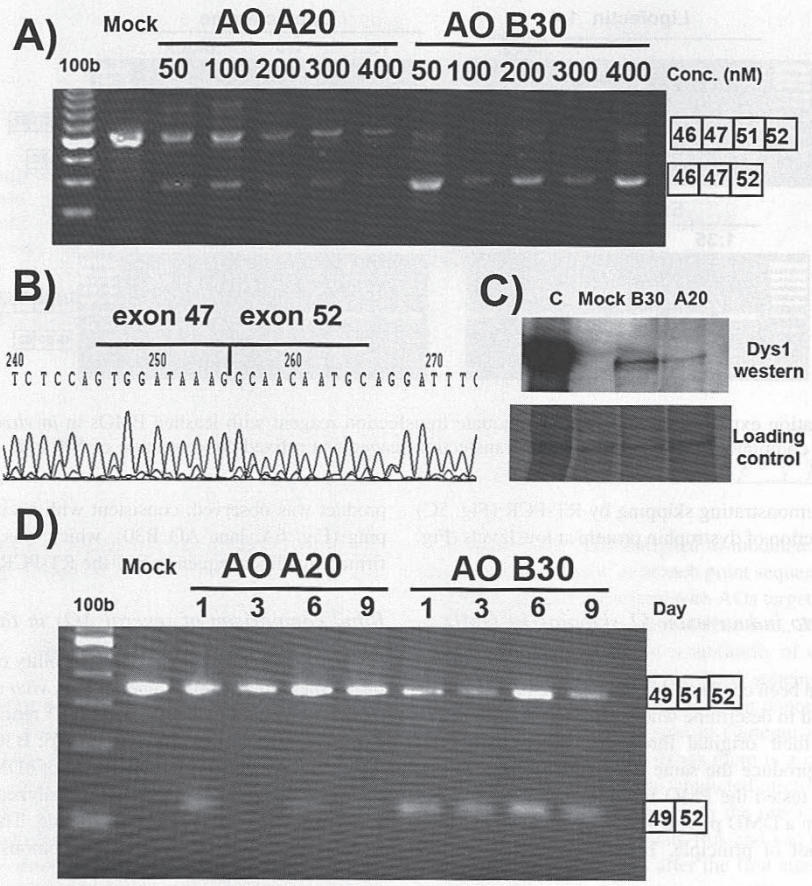


FIG. 3. (**A**) Cells from a DMD patient with a deletion in exons 48 to 50 were transfected with increasing concentrations of the two 2-OMe AOs and analyzed 9 days after transfection. Cells transfected with AO B30 showed exon skip (50–70%), even at a low dose (50 n*M*), whereas the skip achieved with 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 n*M* 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 n*M*. 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 n*M*) (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 n*M* (Fig. 5B). The leashed PMOs A25 and B30 were also transfected at IC into cells from the DMD patient with a dele-

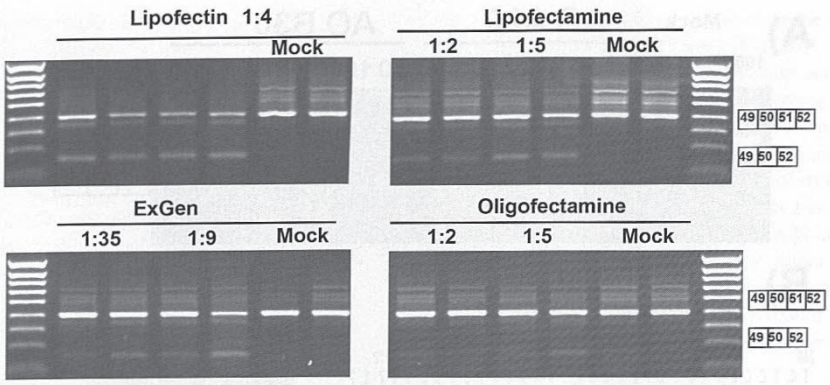


FIG. 4. Optimization experiments to select an adequate transfection reagent with leashed PMOs in *in vitro* experiments. AO B30 was tested in combination with four different transfection reagents at a fixed concentration of 500 n*M*.

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 (Δ3–11), 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

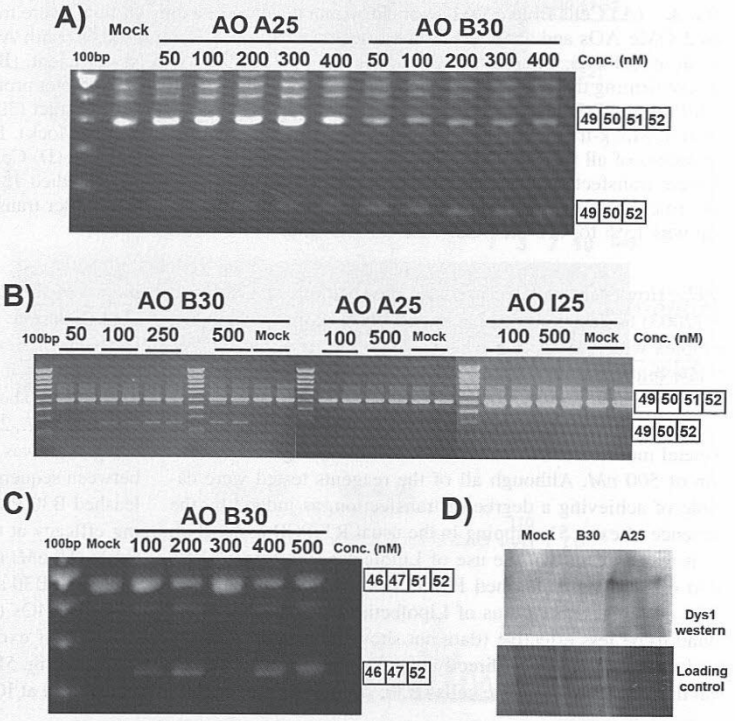


FIG. 5. (**A**) HFM cells were transfected at Imperial College (IC) with increasing concentrations of the PMO AOs A25 and B30. Both AOs achieved the correct skip at low concentrations. AO A25 had a skipping efficiency between 10 and 20% and AO B30, between 40 and 50%. (**B**) In a separate experiment 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 (Δ50) treated with the two PMOs shows the presence of dystrophin protein in this *in vitro* experiment.

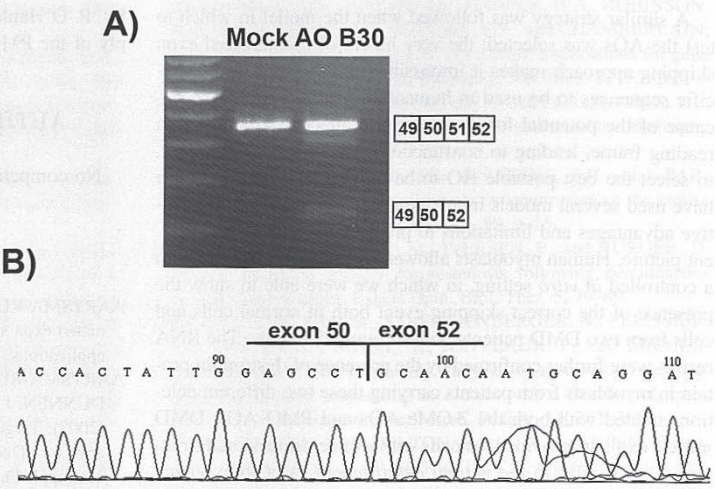


FIG. 6. (**A**) Muscle explants from a DMD patient (Δ3–11) were immersed in medium containing a 400 n*M* concentration of PMO AO B30. AO B30 was successful at achieving the skip in the muscle explant. (**B**) The skipped band was analyzed by sequencing analysis, showing the correct skip.

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 β-globin gene associated with β-thalassemia (Dominski and Kole, 1993). These splicing modulation approaches were later reproduced in human cell lines (HeLa and NIH 3T3) stably expressing the β-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.

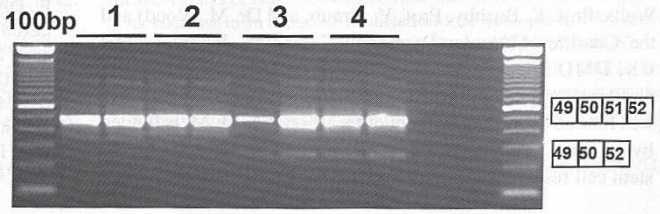


FIG. 7. Three different PMOs were injected, in a blinded experiment, into the gastrocnemius muscle of hDMD mice. Quantification of the PCR products, using a DNA LabChip, gave the following results: lane 1 (AO I25, −7+18), 1.6%; lane 2 (Invert control), 0%; lane 3 (AO A25, +63+87), 5.6%; lane 4 (AO B30,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

ACKNOWLEDGMENTS

The authors thank their coordinator, Jenny Versnel, and the remaining members of the UK Muscular Dystrophy EXon skipping (MDEX) consortium for helpful discussion (Prof. D. Wells, Prof. K. Bushby, Prof. V. Straub, and Dr. M. Wood) and the Charities Muscular Dystrophy Campaign, Parent Project UK, DMD Support Group, Muscular Dystrophy Ireland, and participating patients for the support to our study. This work was funded by the Department of Health (UK). J.E.M. is funded by an MRC collaborative career development fellowship in stem cell research. The authors also thank J.R. Christensen and Dr. P. O'Hanley for the support of AVI Biopharma in the supply of the PMO AOs.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

AARTSMA-RUS, A., and VAN OMMEN, G.J. (2007). Antisense-mediated exon skipping: A versatile tool with therapeutic and research applications. RNA **13,** 1–16.

AARTSMA-RUS, A., BREMMER-BOUT, M., JANSON, A.A., DEN DUNNEN, J.T., VAN OMMEN, G.J., and VAN DEUTEKOM, J.C. (2002). Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy. Neuromuscul. Disord. **12**(Suppl. 1), S71–S77.

AARTSMA-RUS, A., JANSON, A.A., KAMAN, W.E., BREMMER-BOUT, M., DEN DUNNEN, J.T., BAAS, F., VAN OMMEN, G.J., and VAN DEUTEKOM, J.C. (2003). Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients. Hum. Mol. Genet. **12,** 907–914.

AARTSMA-RUS, A., JANSON, A.A., KAMAN, W.E., BREMMER-BOUT, M., VAN OMMEN, G.J., DEN DUNNEN, J.T., and VAN DEUTEKOM, J.C. (2004a). Antisense-induced multiexon skipping for Duchenne muscular dystrophy makes more sense. Am. J. Hum. Genet. **74,** 83–92.

AARTSMA-RUS, A., KAMAN, W.E., BREMMER-BOUT, M., JANSON, A.A., DEN DUNNEN, J.T., VAN OMMEN, G.J., and VAN DEUTEKOM, J.C. (2004b). Comparative analysis of antisense oligonucleotide analogs for targeted DMD exon 46 skipping in muscle cells. Gene Ther. **11,** 1391–1398.

AARTSMA-RUS, A., DE WINTER, C.L., JANSON, A.A., KAMAN, W.E., VAN OMMEN, G.J., DEN DUNNEN, J.T., and VAN DEUTEKOM, J.C. (2005). Functional analysis of 114 exon-internal AONs for targeted DMD exon skipping: indication for steric hindrance of SR protein binding sites. Oligonucleotides **15,** 284–297.

ADAMS, A.M., HARDING, P.L., IVERSEN, P.L., COLEMAN, C., FLETCHER, S., and WILTON, S.D. (2007). Antisense oligonucleotide induced exon skipping and the dystrophin gene transcript: Cocktails and chemistries. BMC Mol. Biol. **8,** 57.

ALTER, J., LOU, F., RABINOWITZ, A., YIN, H., ROSENFELD, J., WILTON, S.D., PARTRIDGE, T.A., and LU, Q.L. (2006). Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. Nat. Med. **12,** 175–177.

BEGGS, A.H., HOFFMAN, E.P., SNYDER, J.R., ARAHATA, K., SPECHT, L., SHAPIRO, F., ANGELINI, C., SUGITA, H., and KUNKEL, L.M. (1991). Exploring the molecular basis for variability among patients with Becker muscular dystrophy: Dystrophin gene and protein studies. Am. J. Hum. Genet. **49,** 54–67.

BEROUD, C., TUFFERY-GIRAUD, S., MATSUO, M., HAMROUN, D., HUMBERTCLAUDE, V., MONNIER, N., MOIZARD, M.P., VOELCKEL, M.A., CALEMARD, L.M., BOISSEAU, P., BLAYAU, M., PHILIPPE, C., COSSEE, M., PAGES, M., RIVIER, F., DANOS, O., GARCIA, L., and CLAUSTRES, M. (2007). Multiexon skipping leading to an artificial DMD protein lacking amino acids from exons 45 through 55 could rescue up to 63% of patients with Duchenne muscular dystrophy. Hum. Mutat. **28,** 196–202.

BREMMER-BOUT, M., AARTSMA-RUS, A., DE MEIJER, E.J., KAMAN, W.E., JANSON, A.A., VOSSEN, R.H., VAN OMMEN, G.J., DEN DUNNEN, J.T., and VAN DEUTEKOM, J.C. (2004). Targeted exon skipping in transgenic hDMD mice: A model for direct pre-

clinical screening of human-specific antisense oligonucleotides. Mol. Ther. **10,** 232–240.

BURROW, K.L., COOVERT, D.D., KLEIN, C.J., BULMAN, D.E., KISSEL, J.T., RAMMOHAN, K.W., BURGHES, A.H., and MENDELL, J.R. (1991). Dystrophin expression and somatic reversion in prednisone-treated and untreated Duchenne dystrophy. CIDD Study Group. Neurology **41,** 661–666.

CARTEGNI, L., CHEW, S.L., and KRAINER, A.R. (2002). Listening to silence and understanding nonsense: Exonic mutations that affect splicing. Nat. Rev. Genet. **3,** 285–298.

DE ANGELIS, F.G., STHANDIER, O., BERARDUCCI, B., TOSO, S., GALLUZZI, G., RICCI, E., COSSU, G., and BOZZONI, I. (2002). Chimeric snRNA molecules carrying antisense sequences against the splice junctions of exon 51 of the dystrophin pre-mRNA induce exon skipping and restoration of a dystrophin synthesis in Delta 48-50 DMD cells. Proc. Natl. Acad. Sci. U.S.A. **99,** 9456–9461.

DOMINSKI, Z., and KOLE, R. (1993). Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides. Proc. Natl. Acad. Sci. U.S.A. **90,** 8673–8677.

DUNCKLEY, M.G., MANOHARAN, M., VILLIET, P., EPERON, I.C., and DICKSON, G. (1998). Modification of splicing in the dystrophin gene in cultured Mdx muscle cells by antisense oligoribonucleotides. Hum. Mol. Genet. **7,** 1083–1090.

FABB, S.A., WELLS, D.J., SERPENTE, P., and DICKSON, G. (2002). Adeno-associated virus vector gene transfer and sarcolemmal expression of a 144 kDa micro-dystrophin effectively restores the dystrophin-associated protein complex and inhibits myofibre degeneration in nude/mdx mice. Hum. Mol. Genet. **11,** 733–741.

FANIN, M., DANIELI, G.A., VITIELLO, L., SENTER, L., and ANGELINI, C. (1992). Prevalence of dystrophin-positive fibers in 85 Duchenne muscular dystrophy patients. Neuromuscul. Disord. **2,** 41–45.

FANIN, M., DANIELI, G.A., CADALDINI, M., MIORIN, M., VITIELLO, L., and ANGELINI, C. (1995). Dystrophin-positive fibers in Duchenne dystrophy: Origin and correlation to clinical course. Muscle Nerve **18,** 1115–1120.

FLETCHER, S., HONEYMAN, K., FALL, A.M., HARDING, P.L., JOHNSEN, R.D., and WILTON, S.D. (2006). Dystrophin expression in the *mdx* mouse after localised and systemic administration of a morpholino antisense oligonucleotide. J. Gene Med. **8,** 207–216.

FRIEDMAN, K.J., KOLE, J., COHN, J.A., KNOWLES, M.R., SILVERMAN, L.M., and KOLE, R. (1999). Correction of aberrant splicing of the cystic fibrosis transmembrane conductance regulator (CFTR) gene by antisense oligonucleotides. J. Biol. Chem. **274,** 36193–36199.

GEBSKI, B.L., MANN, C.J., FLETCHER, S., and WILTON, S.D. (2003). Morpholino antisense oligonucleotide induced dystrophin exon 23 skipping in mdx mouse muscle. Hum. Mol. Genet. **12,** 1801–1811.

GEBSKI, B.L., ERRINGTON, S.J., JOHNSEN, R.D., FLETCHER, S., and WILTON, S.D. (2005). Terminal antisense oligonucleotide modifications can enhance induced exon skipping. Neuromuscul. Disord. **15,** 622–629.

GRAHAM, I.R., HILL, V.J., MANOHARAN, M., INAMATI, G.B., and DICKSON, G. (2004). Towards a therapeutic inhibition of dystrophin exon 23 splicing in *mdx* mouse muscle induced by antisense oligoribonucleotides (splicomers): Target sequence optimisation using oligonucleotide arrays. J. Gene Med. **6,** 1149–1158.

GREGOREVIC, P., ALLEN, J.M., MINAMI, E., BLANKINSHIP, M.J., HARAGUCHI, M., MEUSE, L., FINN, E., ADAMS, M.E., FROEHNER, S.C., MURRY, C.E., and CHAMBERLAIN, J.S. (2006). rAAV6-microdystrophin preserves muscle function and extends lifespan in severely dystrophic mice. Nat. Med. **12,** 787–789.

HARDING, P.L., FALL, A.M., HONEYMAN, K., FLETCHER, S., and WILTON, S.D. (2007). The influence of antisense oligonucleotide length on dystrophin exon skipping. Mol. Ther. **15,** 157–166.

HARPER, S.Q., HAUSER, M.A., DELLORUSSO, C., DUAN, D., CRAWFORD, R.W., PHELPS, S.F., HARPER, H.A., ROBINSON, A.S., ENGELHARDT, J.F., BROOKS, S.V., and CHAMBERLAIN, J.S. (2002). Modular flexibility of dystrophin: Implications for gene therapy of Duchenne muscular dystrophy. Nat. Med. **8,** 253–261.

HOFFMAN, E.P., BROWN, R.H., Jr., and KUNKEL, L.M. (1987). Dystrophin: The protein product of the Duchenne muscular dystrophy locus. Cell **51,** 919–928.

HOFFMAN, E.P., MORGAN, J.E., WATKINS, S.C., and PARTRIDGE, T.A. (1990). Somatic reversion/suppression of the mouse mdx phenotype *in vivo*. J. Neurol. Sci. **99,** 9–25.

KIPSHIDZE, N., TSAPENKO, M., IVERSEN, P., and BURGER, D. (2005). Antisense therapy for restenosis following percutaneous coronary intervention. Expert Opin. Biol. Ther. **5,** 79–89.

LESCA, G., TESTARD, H., STREICHENBERGER, N., PELISSIER, J.F., LESTRA, C., BUREL, E., JONVEAUX, P., and MICHEL-CALEMARD, L. (2007). [Family study allows more optimistic prognosis and genetic counselling in a child with a deletion of exons 50–51 of the dystrophin gene.]. Arch. Pediatr. **14,** 262–265.

LU, Q.L., MORRIS, G.E., WILTON, S.D., LY, T., ARTEM'YEVA, O.V., STRONG, P., and PARTRIDGE, T.A. (2000). Massive idiosyncratic exon skipping corrects the nonsense mutation in dystrophic mouse muscle and produces functional revertant fibers by clonal expansion. J. Cell Biol. **148,** 985–996.

LU, Q.L., MANN, C.J., LOU, F., BOU-GHARIOS, G., MORRIS, G.E., XUE, S.A., FLETCHER, S., PARTRIDGE, T.A., and WILTON, S.D. (2003). Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse. Nat. Med. **9,** 1009–1014.

LU, Q.L., RABINOWITZ, A., CHEN, Y.C., YOKOTA, T., YIN, H., ALTER, J., JADOON, A., BOU-GHARIOS, G., and PARTRIDGE, T. (2005). Systemic delivery of antisense oligoribonucleotide restores dystrophin expression in body-wide skeletal muscles. Proc. Natl. Acad. Sci. U.S.A. **102,** 198–203.

MANN, C.J., HONEYMAN, K., CHENG, A.J., LY, T., LLOYD, F., FLETCHER, S., MORGAN, J.E., PARTRIDGE, T.A., and WILTON, S.D. (2001). Antisense-induced exon skipping and synthesis of dystrophin in the *mdx* mouse. Proc. Natl. Acad. Sci. U.S.A. **98,** 42–47.

MANN, C.J., HONEYMAN, K., McCLOREY, G., FLETCHER, S., and WILTON, S.D. (2002). Improved antisense oligonucleotide induced exon skipping in the *mdx* mouse model of muscular dystrophy. J. Gene Med. **4,** 644–654.

MATSUO, M., and TAKESHIMA, Y. (2005). Rescue of dystrophin mRNA of Duchenne muscular dystrophy by inducing exon skipping. Acta Myol. **24,** 110–114.

McCLOREY, G., FALL, A.M., MOULTON, H.M., IVERSEN, P.L., RASKO, J.E., RYAN, M., FLETCHER, S., and WILTON, S.D. (2006a). Induced dystrophin exon skipping in human muscle explants. Neuromuscul. Disord. **16,** 583–590.

McCLOREY, G., MOULTON, H.M., IVERSEN, P.L., FLETCHER, S., and WILTON, S.D. (2006b). Antisense oligonucleotide-induced exon skipping restores dystrophin expression *in vitro* in a canine model of DMD. Gene Ther. **13,** 1373–1381.

MELIS, M.A., CAU, M., MUNTONI, F., MATEDDU, A., GALANELLO, R., BOCCONE, L., DEIDDA, F., LOI, D., and CAO, A. (1998). Elevation of serum creatine kinase as the only manifestation of an intragenic deletion of the dystrophin gene in three unrelated families. Eur. J. Paediatr. Neurol. **2,** 255–261.

MERCATANTE, D.R., MOHLER, J.L., and KOLE, R. (2002). Cellular response to an antisense-mediated shift of Bcl-x pre-mRNA splicing and antineoplastic agents. J. Biol. Chem. **277,** 49374–49382.

MONACO, A.P., BERTELSON, C.J., LIECHTI-GALLATI, S., MOSER, H., and KUNKEL, L.M. (1988). An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. Genomics **2,** 90–95.

MORRONE, A., ZAMMARCHI, E., SCACHERI, P.C., DONATI,

M.A., HOOP, R.C., SERVIDEI, S., GALLUZZI, G., and HOFFMAN, E.P. (1997). Asymptomatic dystrophinopathy. Am. J. Med. Genet. **69,** 261–267.

MOSER, H. (1984). Duchenne muscular dystrophy: Pathogenetic aspects and genetic prevention. Hum. Genet. **66,** 17–40.

MUNTONI, F., BUSHBY, K., and VAN OMMEN, G. (2005). 128th ENMC International Workshop on "Preclinical Optimization and Phase I/II Clinical Trials Using Antisense Oligonucleotides in Duchenne Muscular Dystrophy," 22–24 October 2004, Naarden, The Netherlands. Neuromuscul. Disord. **15,** 450–457.

NICHOLSON, L.V., DAVISON, K., JOHNSON, M.A., SLATER, C.R., YOUNG, C., BHATTACHARYA, S., GARDNER-MEDWIN, D., and HARRIS, J.B. (1989). Dystrophin in skeletal muscle. II. Immunoreactivity in patients with Xp21 muscular dystrophy. J. Neurol. Sci. **94,** 137–146.

NIKRAVESH, A., DRYSELIUS, R., FARIDANI, O.R., GOH, S., SADEGHIZADEH, M., BEHMANESH, M., GANYU, A., KLOK, E.J., ZAIN, R., and GOOD, L. (2007). Antisense PNA accumulates in *Escherichia coli* and Mediates a Long Post-antibiotic Effect. Mol. Ther. **15,** 1537–1542.

PRAMONO, Z.A., TAKESHIMA, Y., ALIMSARDJONO, H., ISHII, A., TAKEDA, S., and MATSUO, M. (1996). Induction of exon skipping of the dystrophin transcript in lymphoblastoid cells by transfecting an antisense oligodeoxynucleotide complementary to an exon recognition sequence. Biochem. Biophys. Res. Commun. **226,** 445–449.

SAZANI, P., GEMIGNANI, F., KANG, S.H., MAIER, M.A., MANOHARAN, M., PERSMARK, M., BORTNER, D., and KOLE, R. (2002). Systemically delivered antisense oligomers upregulate gene expression in mouse tissues. Nat. Biotechnol. **20,** 1228–1233.

SHERRATT, T.G., VULLIAMY, T., DUBOWITZ, V., SEWRY, C.A., and STRONG, P.N. (1993). Exon skipping and translation in patients with frameshift deletions in the dystrophin gene. Am. J. Hum. Genet. **53,** 1007–1015.

SIERAKOWSKA, H., SAMBADE, M.J., AGRAWAL, S., and KOLE, R. (1996). Repair of thalassemic human β-globin mRNA in mammalian cells by antisense oligonucleotides. Proc. Natl. Acad. Sci. U.S.A. **93,** 12840–12844.

SMITH, P.J., ZHANG, C., WANG, J., CHEW, S.L., ZHANG, M.Q., and KRAINER, A.R. (2006). An increased specificity score matrix for the prediction of SF2/ASF-specific exonic splicing enhancers. Hum. Mol. Genet. **15,** 2490–2508.

SUMMERTON, J., and WELLER, D. (1997). Morpholino antisense oligomers: Design, preparation, and properties. Antisense Nucleic Acid Drug Dev. **7,** 187–195.

SURONO, A., VAN KHANH, T., TAKESHIMA, Y., WADA, H., YAGI, M., TAKAGI, M., KOIZUMI, M., and MATSUO, M. (2004). Chimeric RNA/ethylene-bridged nucleic acids promote dystrophin expression in myocytes of Duchenne muscular dystrophy by inducing skipping of the nonsense mutation-encoding exon. Hum. Gene Ther. **15,** 749–757.

TAKESHIMA, Y., NISHIO, H., SAKAMOTO, H., NAKAMURA, H., and MATSUO, M. (1995). Modulation of *in vitro* splicing of the upstream intron by modifying an intra-exon sequence which is deleted from the dystrophin gene in dystrophin Kobe. J. Clin. Invest. **95,** 515–520.

TAKESHIMA, Y., WADA, H., YAGI, M., ISHIKAWA, Y., ISHIKAWA, Y., MINAMI, R., NAKAMURA, H., and MATSUO, M. (2001). Oligonucleotides against a splicing en sequence led to dystrophin production in muscle cells from a Duchenne muscular dystrophy patient. Brain Dev. **23,** 788–790.

TAKESHIMA, Y., YAGI, M., WADA, H., ISHIBASHI, K., NISHIYAMA, A., KAKUMOTO, M., SAKAEDA, T., SAURA, R., OKUMURA, K., and MATSUO, M. (2006). Intravenous infusion of an antisense oligonucleotide results in exon skipping in muscle dystrophin mRNA of Duchenne muscular dystrophy. Pediatr. Res. **59,** 690–694.

THANH, L.T., NGUYEN, T.M., HELLIWELL, T.R., and MORRIS, G.E. (1995). Characterization of revertant muscle fibers in Duchenne muscular dystrophy, using exon-specific monoclonal antibodies against dystrophin. Am. J. Hum. Genet. **56,** 725–731.

VAN DEUTEKOM, J.C., and VAN OMMEN, G.J. (2003). Advances in Duchenne muscular dystrophy gene therapy. Nat. Rev. Genet. **4,** 774–783.

VAN DEUTEKOM, J.C., BREMMER-BOUT, M., JANSON, A.A., GINJAAR, I.B., BAAS, F., DEN DUNNEN, J.T., and VAN OMMEN, G.J. (2001). Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells. Hum. Mol. Genet. **10,** 1547–1554.

VAN DEUTEKOM, J.C., JASON, A.A., GINJAAR, I.B., FRANKHUIZEN, W.S., AARTSMA-RUS, A., BREMMER-BOUT, M., DEN DUNNEN, J.T., KOOP, K., VAN DER KOOI, A.J., GOEMANS, N.M., DE KIMPE, S.J., EKHART, P.F., VENNEKER, E.H., PLATENBURG, G.J., VERSCHUUREN, J.J., and VAN OMMEN, G.J. (2007). Antisense oligonucleotide PRO051 restores local dystrophin expression in DMD patients. N. Engl. J. Med. (in press).

WANG, B., LI, J., and XIAO, X. (2000). Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy in mdx mouse model. Proc. Natl. Acad. Sci. U.S.A. **97,** 13714–13719.

WELLS, K.E., FLETCHER, S., MANN, C.J., WILTON, S.D., and WELLS, D.J. (2003). Enhanced in vivo delivery of antisense oligonucleotides to restore dystrophin expression in adult mdx mouse muscle. FEBS Lett. **552,** 145–149.

WILTON, S.D., DYE, D.E., and LAING, N.G. (1997). Dystrophin gene transcripts skipping the *mdx* mutation. Muscle Nerve **20,** 728–734.

WILTON, S.D., LLOYD, F., CARVILLE, K., FLETCHER, S., HONEYMAN, K., AGRAWAL, S., and KOLE, R. (1999). Specific removal of the nonsense mutation from the *mdx* dystrophin mRNA using antisense oligonucleotides. Neuromuscul. Disord. **9,** 330–338.

WILTON, S.D., FALL, A.M., HARDING, P.L., McCLOREY, G., COLEMAN, C., and FLETCHER, S. (2007). Antisense oligonucleotide-induced exon skipping across the human dystrophin gene transcript. Mol. Ther. **15,** 1288–1296.

YOKOTA, T., LU, Q.L., MORGAN, J.E., DAVIES, K.E., FISHER, R., TAKEDA, S., and PARTRIDGE, T.A. (2006). Expansion of revertant fibers in dystrophic *mdx* muscles reflects activity of muscle precursor cells and serves as an index of muscle regeneration. J. Cell Sci. **119,** 2679–2687.

ZAMECNIK, P.C., and STEPHENSON, M.L. (1978). Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. Proc. Natl. Acad. Sci. U.S.A. **75,** 280–284.

Address reprint requests to:
Dr. Francesco Muntoni
Dubowitz Neuromuscular Centre
Department of Paediatrics
Imperial College London
Hammersmith Hospital
Du Cane Road
London W12ONN, UK

E-mail: f.muntoni@imperial.ac.uk

Received for publication May 24, 2007; accepted after revision August 9, 2007.

Published online: August 31, 2007.