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RNA

VOL. 13, NO. 10 OCTOBER 2007

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- Review of antisense-mediated exon skipping
- Review of the birth of new exons
- Improved amber and opal suppressor tRNAs
- Cloning and expression analysis of piRNA-like RNAs
- Modulation of group I intron catalysis by a peripheral metal ion

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Publishing and Advertising Offices: Cold Spring Harbor Laboratory Press, 1 Bungtown Road, Cold Spring Harbor, New York 11724-2203. For advertising rates and data, contact Marcie Siconolfi, Advertising Manager, at 516-422-4010 (telephone) or 516-422-4092 (fax).

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RNA (ISSN 1355-8382) is published monthly by Cold Spring Harbor Laboratory Press, 500 Sunnyside Blvd., Woodbury, NY 11797-2924. Periodicals paid at Woodbury, NY, and additional mailing offices.
POSTMASTER: Send address changes to Cold Spring Harbor Laboratory Press, Journals Fulfillment Dept., 500 Sunnyside Blvd., Woodbury, NY 11797-2924.

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Cover Illustration: *Crystal structure of a phage Twort group I ribozyme-product complex (PDB code: 1y0q; Golden, B.L., Kim, H., and Chase, E. 2005. Crystal structure of a phage Twort group I ribozyme product complex.* Nat. Struct. Mol. Biol. **12:** *82–89). Image details: ribozyme derived from the second group I intron in the* orf142 gene *(*orf142-I2*): ribbon–plate representation, transparent surface, P1-P2 domain—red, P3-P7 region—green, P4-P6 domain—blue, P9-P9.1 domain—purple, P7.1-P7.2 subdomain—yellow, nucleotides not included in these domains—white; oligonucleotide representing a 5′ exon: ball-and-stick representation, cyan. The image was generated with the Accelrys Discovery Studio Visualizer. Cover image provided by the Jena Library of Biological Macromolecules (JenaLib; www.fli-leibniz.de/IMAGE.html).*

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Antisense-mediated exon skipping: A versatile tool with therapeutic and research applications

ANNEMIEKE AARTSMA-RUS and GERT-JAN B. VAN OMMEN
DMD genetic therapy group, Department of Human Genetics, Leiden University Medical Center, 2300 RC, Leiden, The Netherlands

ABSTRACT

Antisense-mediated modulation of splicing is one of the few fields where antisense oligonucleotides (AONs) have been able to live up to their expectations. In this approach, AONs are implemented to restore cryptic splicing, to change levels of alternatively spliced genes, or, in case of Duchenne muscular dystrophy (DMD), to skip an exon in order to restore a disrupted reading frame. The latter allows the generation of internally deleted, but largely functional, dystrophin proteins and would convert a severe DMD into a milder Becker muscular dystrophy phenotype. In fact, exon skipping is currently one of the most promising therapeutic tools for DMD, and a successful first-in-man trial has recently been completed. In this review the applicability of exon skipping for DMD and other diseases is described. For DMD AONs have been designed for numerous exons, which has given us insight into their mode of action, splicing in general, and splicing of the *DMD* gene in particular. In addition, retrospective analysis resulted in guidelines for AON design for *DMD* and most likely other genes as well. This knowledge allows us to optimize therapeutic exon skipping, but also opens up a range of other applications for the exon skipping approach.

Keywords: exon skipping; splicing; Duchenne muscular dystrophy; antisense oligonucleotides; therapy

INTRODUCTION

Antisense oligonucleotides (AONs) are mostly known for their ability to hybridize to a sense target sequence, which leads to RNase H cleaving of the RNA:DNA hybrid and results in specific gene expression knockdown (Hausen and Stein 1970; Zamecnik and Stephenson 1978). This approach offered useful opportunities to study development because it allowed timed gene knockdown in early or later stages of development, as well as therapeutic opportunities to knockdown genes involved in cancer, inflammatory diseases, and viral infections. Currently, an AON to treat CMV-induced retinitis (Vitravene) has been registered as a drug, and other AONs to treat cancer and inflammatory diseases are in phase II and III clinical trails (Marwick 1998; Kurreck 2003). However, with the emergence of RNAi, which turned out to be a more efficient and more predictable tool for expression knockdown, the field of AON-induced knockdown has gone in decline (Elbashir et al. 2001). A notable exception is the modulation of pre-mRNA splicing to induce exon skipping, where RNase H-independent AONs are employed to block splicing signals (Kole and Sazani 2001). This approach has gained increasing interest over the past decade (van Deutekom and van Ommen 2003). Actually, antisense-mediated exon skipping is currently one of the most promising therapeutic approaches for Duchenne muscular dystrophy (DMD). A first-in-man trial has recently been completed successfully in our institute (J.C.T. van Deutekom, A.A.M. Janson, I.B. Ginjaar, W.S. Frankhuizen, A. Aartsma-Rus, M. Bremmwe-Bout, J.T. den Dunnen, K. Koop, A.J. van der Kooi, N.M. Goemans, et al., in prep.) and a second trial is about to start in the United Kingdom (Muntoni et al. 2005; F. Muntoni, pers. comm.). This review describes the mechanism of antisense-mediated exon skipping for DMD and gives an overview of other exon skipping applications reported thus far. It discusses how the numerous AONs designed for DMD exon skipping give us insight into splicing of the *DMD* gene in particular, but splicing in general as well. Finally, ways to implement exon skipping in future applications will be discussed.

Reprint requests to: Annemieke Aartsma-Rus, DMD genetic therapy group, Department of Human Genetics, Leiden University Medical Center, P.O. Box 9600, 2300 RC, Leiden, The Netherlands; email: a.m.rus@lumc.nl; fax: 31-71-5268285.
Article published online ahead of print. Article and publication date are at http://www.rnajournal.org/cgi/doi/10.1261/rna.653607.

DUCHENNE MUSCULAR DYSTROPHY

Duchenne muscular dystrophy is a severely invalidating, progressive neuromuscular disorder (Emery 2002). Patients

are wheelchair bound before the age of twelve, often require assisted ventilation later in life, and generally die in their early twenties. The disease is caused by mutations in the *DMD* gene that abolish the production of functional dystrophin (Hoffman et al. 1987). This protein consists of two essential functional domains connected by a central rod domain that is partly dispensable (Hoffman et al. 1988; Koenig et al. 1988). Dystrophin links the cytoskeleton to the extracellular matrix and is thought to be required to maintain muscle fiber stability during contraction (Matsumura and Campbell 1994). Mutations that disrupt the open reading frame result in prematurely truncated proteins unable to fulfill their bridge function (Fig. 1). Ultimately, this leads to muscle fiber damage and the continuous loss of muscle fibers, replacement of muscle tissue by fat and fibrotic tissue, impaired muscle function, and eventually the severe phenotype observed for DMD patients.

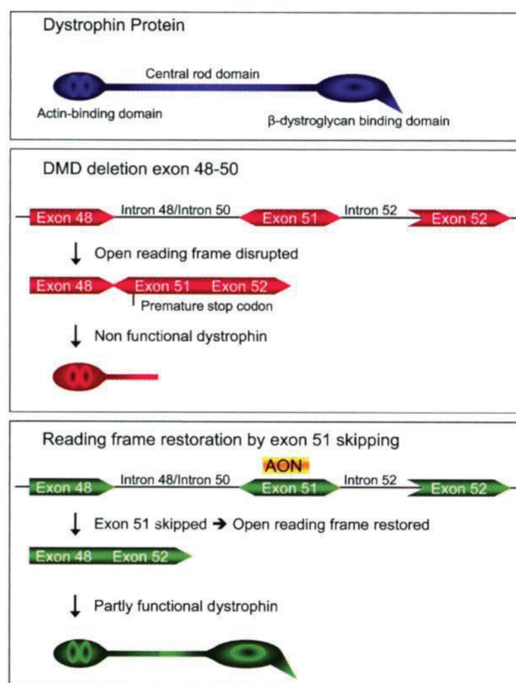

FIGURE 1. Antisense-mediated exon skipping for Duchenne muscular dystrophy. The dystrophin protein (*upper* panel) contains an N-terminal actin-binding domain connected to a β-dystroglycan binding domain by the central rod domain. Dystroglycan is a transmembrane protein that is bound to the extracellular protein laminin-2. Dystrophin thus fulfills a bridge function in muscle fibers by linking the cytoskeletal actin to the extracellular matrix. In Duchenne muscular dystrophy (*middle* panel), the open reading frame is disrupted (in this example by a deletion of exons 48–50, the most common mutation in DMD patients), resulting in a premature stop codon and a truncated dystrophin, which is unable to fulfill its bridge function. Antisense oligoribonucleotides (AONs) can be employed to restore the open reading frame (*lower* panel). Specific AONs hybridize to exon 51 and hide this exon from the splicing machinery, resulting in the splicing of exon 51 with its flanking intron. This restores the open reading frame, allowing the generation of an internally deleted dystrophin, that contains both the actin- and dystroglycan binding domains and therefore is partially to largely functional.

In contrast, mutations that maintain the open reading frame allow for the generation of internally deleted, but partially functional dystrophins (Monaco et al. 1988). These mutations are associated with Becker muscular dystrophy (BMD), a much milder disease when compared to DMD. Patients generally remain ambulant until later in life and have near normal expectancies, although more severely affected patients have been reported as well (Emery 2002).

The *DMD* gene is the largest known human gene and its 79 exons span an astonishing 2.4 Mb (Roberts et al. 1993, 1994). Over 70% of all DMD and BMD patients suffer from deletions of one or multiple exons (Aartsma-Rus et al. 2006c). Mildly affected BMD patients carrying deletions that involve over two thirds of the central rod domain have been described, suggesting that this domain is largely dispensable. Dystrophin can be largely functional as long as the N- and C-terminal domains are present to convey the link between the cytoskeleton and the extracellular matrix (England et al. 1990; Mirabella et al. 1998). Due to this rather unique feature, the counterintuitive skipping of additional internal exons can be employed to enlarge a deletion, but at the same time restore the open reading frame and thus convert a severe DMD into a milder BMD phenotype (Fig. 1; van Deutekom et al. 2001).

THE EXON SKIPPING APPROACH

Antisense-mediated modulation of pre-mRNA splicing has been pioneered by Ryszard Kole (Dominski and Kole 1993). In the first experiments, AONs were aimed at activated cryptic splice sites in the β-globin (*HBB*) and cystic fibrosis transmembrane conductance regulator (*CFTR*) genes in order to restore normal splicing in β-thalassemia and cystic fibrosis patients (Dominski and Kole 1993; Sierakowska et al. 1996; Friedman et al. 1999). Even though this approach does not technically qualify as exon skipping (but rather the redirection of normal splicing), it does offer therapeutic potential for diseases where mutations often induce cryptic splice sites such as the Hutchinson–Gilford progeria syndrome (Scaffidi and Misteli 2005). In fact, for most genetic disorders an estimated 5%–10% of mutations induce abnormal splicing (Krawczak et al. 1992; Cartegni et al. 2002), part of which can, in principle, be corrected.

A finding of the group of Matsuo eventually alerted the DMD field to a potential therapeutic application of exon skipping for DMD. Matsuo and colleagues observed that a 52-base pair (bp) deletion within exon 19 resulted in the skipping of this exon in the so-called DMD Kobe patient (Matsuo et al. 1990, 1991). This hinted at the presence of a motif within this 52-bp deletion required for proper inclusion of exon 19 in the mRNA. Indeed, AONs targeting

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part of this deletion induced exon 19 skipping in vitro and in human control lymphoblastoma cells (Takeshima et al. 1995; Pramono et al. 1996).

The feasibility of the approach was then studied in parallel in patient-derived cell lines and in cells from the *mdx* mouse model. This mouse carries a nonsense point mutation in the in-frame exon 23 (Sicinski et al. 1989). Thus, by skipping exon 23 the nonsense mutation is bypassed while the reading frame is maintained. Proof of principle on RNA level was obtained first in cultured muscle cells from the *mdx* mouse by two groups independently (Dunckley et al. 1998; Wilton et al. 1999). In both cases, the reading frame was restored on RNA level as analyzed by RT-PCR analysis. Our group was the first to show restoration of dystrophin on protein level after targeted exon 46 skipping in cultured muscle cells from two DMD patients with an exon 45 deletion (van Deutekom et al. 2001). The wide therapeutic applicability was then confirmed by others and us in numerous patient-derived cell cultures (Takeshima et al. 2001; Aartsma-Rus et al. 2003, 2004a; Surono et al. 2004; Aartsma-Rus et al. 2007). The majority of these mutations involved deletions of one or more exons, but reading frame restoration for nonsense point mutations and single exon duplications has been reported as well. Notably, for single exon duplications, skipping either one of the duplicated exons will restore the wild-type transcript and dystrophin protein (Aartsma-Rus et al. 2007). Some mutations require the skipping of two exons in order to restore the reading frame. We confirmed that this so-called double exon skipping is indeed feasible using a combination of individual AONs targeting the two different exons (Aartsma-Rus et al. 2004a). Remarkably, the efficiency of this double exon skipping approach was only slightly lower than that of single exon skipping (~70%–75% versus 75%–80% dystrophin-positive myotubes, respectively). In parallel, results in *mdx* mouse underlined the therapeutic promise of exon skipping (Mann et al. 2001, 2002; Lu et al. 2003). Local intramuscular injections of an optimized AON resulted in ~20% of wild-type dystrophin levels accompanied by improvement in muscle histology and function (Lu et al. 2003). Dystrophin protein was detectable by Western blot analysis for at least 3 months after a single intramuscular injection.

In theory, exon skipping would be applicable to the majority of DMD patients. Exceptions are mutations located between exon 64 and exon 70, which are essential for protein function, deletions that abolish all actin-binding sites in the N-terminal region or involve the first or the last exon, and large chromosomal rearrangements such as translocations. These mutations are uncommon and make up less than 10% of all mutations (Aartsma-Rus et al. 2006c). Thus exon skipping can theoretically be applicable for up to 90% of DMD patients (Aartsma-Rus et al. 2004a).

A disadvantage of the AON approach is that it is mutation specific in that different mutations require the skipping of different exons to restore the open reading frame. Fortunately, DMD deletions and duplications mainly occur in two hot spot regions, i.e., the major hot spot region (involving exon 45 to exon 53) and the minor hot spot region (located between exon 2 and exon 20) (Liechti-Gallati et al. 1989; Beggs et al. 1990; White et al. 2006). Therefore, by strategically choosing target exons, through the skipping of eight different exons, this strategy would be therapeutic for over 50% of all patients (van Deutekom and van Ommen 2003; Aartsma-Rus and van Deutekom 2007). The most notable example is exon 51 skipping, which is applicable to almost 25% of DMD patients with a deletion, or 16% of all DMD patients (Aartsma-Rus and van Deutekom 2007).

To obtain proof of concept in humans, a "first-in-man study" on exon skipping was undertaken by our center in collaboration with Prosensa B.V. using 2′-*O*-methyl phosphorothioate AONs (chemistries will be discussed in more detail later) (J.C.T. van Deutekom, A.A.M. Janson, I.B. Ginjaar, W.S. Frankhuizen, A. Aartsma-Rus, M. Bremmwe-Bout, J.T. den Dunnen, K. Koop, A.J. van der Kooi, N.M. Goemans, et al., in prep.). Four DMD patients received a single, local intramuscular injection with AONs targeting exon 51 and a biopsy was taken one month later. Preliminary results are very promising and no serious adverse effects were observed or reported by the patients as a result of AON injection. Another local study using morpholino AONs is to start soon in the United Kingdom. These first-in-man studies are an important step toward the clinical application of antisense-mediated exon skipping for DMD.

A systemic pilot study has been performed by Takeshima and colleagues in a single DMD patient at a very low dosage using phosphorothioate RNA (0.5 mg/kg) (Takeshima et al. 2006).

AON DESIGN AND MODE OF ACTION

The first targets to induce exon skipping are the donor and acceptor splice sites and the branch point sequence. These sites have indeed been successfully targeted in the majority of the exon skip applications, including exon skipping for DMD (Table 1; Dunckley et al. 1998; Mann et al. 2002; Wilton and Fletcher 2005). However, they consist of consensus sequences shared with many different genes and consequently targeting them involves the risk of mistargeting splice sites of other genes. Alternatively, it has now been shown that exon skipping can be induced by targeting exon-internal sites, which has been successful in the *DMD* and *WT1* genes (van Deutekom et al. 2001; Renshaw et al. 2004; Aartsma-Rus et al. 2005; Wilton and Fletcher 2005). Proper recognition by the splicing machinery and inclusion into the mRNA is thought to depend on exonic splicing enhancer (ESE) motifs for the majority of exons (Cartegni et al. 2002). These sites are involved in exon recognition through the binding of members of a subfamily of splicing

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TABLE 1. Overview of exon skipping applications

Target gene	Protein	Target[a]	Goal	Application	Reference[b]
APOB	Apolipoprotein B	3′ SS and BP exon 27	Knockdown of APOB100 isoform	Retard atherosclerosis	Khoo et al. (2007)
Bcl-X	Bcl-xS and Bcl-xL	5′ SS Bcl-xL exon	Isoform switching from anti- to pro-apototic Bcl-x	Cancer therapy	Mercatante et al. (2001, 2002)
COL7A1	Collagen type 7	EI Exon 70	Allele specific knockdown	Dystrophic epidermolysis bullosa therapy	Goto et al. (2006)
DMD	Dystrophin	3′ SS, 5′ SS, EI numerous DMD exons	Reading frame restoration leading to partially functional dystrophins	DMD therapy	van Deutekom et al. (2001) Aartsma-Rus et al. (2003) Lu et al. (2003) Aartsma-Rus et al. (2004a) Alter et al. (2006)
FOLH1	Prostate-specific membrane antigen	5′ SS exon 1, exon 6, or exon 18	Isoform switching from transmembrane to cytoplasmatic form	Prostate cancer therapy	Williams and Kole (2006)
IL-5Ralpha	IL-5 receptor-α	3′ SS or 5′ SS exon 9	Isoform switching from transmembrane to soluble form	Asthma therapy	Karras et al. (2000, 2001)
MyD88	MyD88	5′ SS exon 2	Isoform switching	Anti-inflammatory	Vickers et al. (2006)
Tau	Tau	5′ SS or 3; SS exon 10	Restore normal ratio 3R/4R tau isoform	FTDP-17[c] therapy	Kalbfuss et al. (2001)
TNFRSF1B	TNFα 2 receptor	Exon 7 and 8	Isoform switching from transmembrane to soluble form	Rheumatoid arthritis therapy	P. Sazani (pers. comm.)
Ttn	Titin	5′ SS exon 45, 79, 37, 47	Isoform specific knockdown	Functional analysis of isoforms	Seeley et al. (2007)
WT1	WT1	IE Exon 5	Isoform switching to pro-apoptotic form	Leukemia therapy	Renshaw et al. (2004)

[a]SS, splice site; IE, intra-exonic; BP, branch point site.
[b]An overview of the most important publications for each application.
[c]Frontotemporal dementia and parkinsonism linked to chromosome 17.

factors, known as serine and arginine rich proteins (SR proteins) (Stojdl and Bell 1999). These SR proteins have one or several RNA domains able to bind to loosely defined sequence motifs that make up ESEs. SR proteins then recruit the essential U2AF and U1 snRNP splicing factors to the 3′ polypyrimidine tract and 5′ splice sites, respectively, and thus facilitate splicing. The importance of ESEs is underlined by the finding that intraexonic point mutations often result in exon skipping on the RNA level, rather than yielding no or missense amino acid changes as deduced from DNA analysis (Cartegni et al. 2002). Famous examples are the *neurofibromatosis type 1* gene and the *ataxia telangiectasia mutated* gene, where a significant number of mutations lead to exon skipping (Teraoka et al. 1999; Ars et al. 2000; Wimmer et al. 2007). In addition, predicted nonsense mutations in in-frame exons of the DMD gene occasionally turn out to actually induce exon skipping and a BMD phenotype, indicative that these nonsense mutations disrupt ESE sites (Shiga et al. 1997; Ginjaar et al. 2000; Tuffery-Giraud et al. 2004; Disset et al. 2006). As SR protein binding to ESEs is essential for exon inclusion, blocking ESEs with AONs would be expected to result in exon skipping. Matsuo and colleagues indeed showed that blocking the ESE they had identified in exon 19 resulted in exon skipping (Pramono et al. 1996). ESE motifs are only loosely defined because, even though inclusion of the exon in mRNA is essential, strict motifs would interfere with the main task of an exon, i.e., to encode protein information. Therefore, targeting ESEs reduces the chance of mistargeting. Software packages, such as RESCUE-ESE, ESEfinder, and the PESX server, predict putative ESE sites (Fairbrother et al. 2002; Cartegni et al. 2003; Zhang and Chasin 2004; Smith et al. 2006), which facilitates the design of exon-internal AONs. We have now designed almost 150 exon-internal AONs, of which nearly 70% are effective in inducing the skipping of 39 different DMD exons (2, 8, 17, 19, 29, 33, 40–64, 71–78) (A. Aartsma-Rus and J.C.T. van Deutekom, unpubl.; Aartsma-Rus et al. 2005). Initially,

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not much was known about ESE sites besides that they were thought to be purine rich, probably due to the fact that the most abundant SR proteins, SF2/ASF and SC35, can bind these motifs (Tacke and Manley 1995). We reasoned that, in order to be able to be bound by SR proteins, ESE sites had to be open regions in the secondary RNA structure. Therefore, our initial 114 exon-internal were mainly directed against purine-rich sequences located in open regions in the secondary RNA structure as predicted by the Mfold server (Zuker 2003). We found that two out of three (78 out of 114) of these AONs were effective in inducing the skipping of 36 exons in control muscle cell cultures (Aartsma-Rus et al. 2005).

No difference was observed between the "openness" of the target sequence in the predicted secondary structure of effective versus ineffective AONs (Aartsma-Rus et al. 2005). Because of its extreme size it was difficult to predict the secondary structure of the entire DMD gene or even of a target exon with its flanking introns before splicing. Thus, AON design was based on the secondary structure of an exon and 100 bp of flanking intron sequences. Analysis of larger regions revealed that often the local secondary structure of the exon and its immediate surroundings were present within the larger secondary structure. For each exon there were numerous predicted structures that were often more or less equally energetically stable. Our AON design was based on the most likely secondary structure. However, it is likely that a certain pre-mRNA exists in more than one secondary structure in the nucleus. Therefore, it may be better to analyze the number of times each nucleotide in the target sequence is present in an open structure in all predicted structures or, in other words, the propensity of the nucleotide to be single stranded (SS) in each of the predicted structures (also known as the SS count) (Zuker 2003). Nevertheless, when we calculated this propensity for target sequences of effective and ineffective AONs, no significant differences were observed (M. Hirshi and A. Aartsma-Rus, unpubl.). At first sight, this would suggest that the level of openness is irrelevant for AON efficacy. However, as our AONs were designed to target open structures in the first place, this finding is biased. In addition, the longest stretch of nucleotides predicted never to be single stranded in any of the predicted structures was eight, and our AONs were 17–21 nucleotides (nt) long. This made it hard to verify whether AONs targeting completely closed structures are indeed ineffective. The closest one can come to a successful comparison is by noting that our empirical approach of selecting partly open structures has a 2 out of 3 success rate, while in several other approaches typically more AONs need to be designed to achieve proper exon skipping (Mann et al. 2002).

The availability of ESE predicting software allowed retrospective analysis of our set of 114 AONs for the presence or absence of putative ESE sites. Interestingly, compared to ineffective AONs, effective AONs targeted significantly more RESCUE ESE hexamers and significantly higher values for SF2/ASF- and SC35-binding sites as predicted by ESEfinder v2.0 (Aartsma-Rus et al. 2005, 2006a). When we compared the highest value for any of the four SR proteins for which ESEfinder has an algorithm (i.e., the most likely ESE), the difference between effective and ineffective AONs became even more significant (Aartsma-Rus et al. 2005). This suggests that exon-internal AONs indeed act by steric blocking of SR protein binding. This finding was further underlined by the fact that effective AONs were located significantly closer to the acceptor splice sites (Aartsma-Rus et al. 2005), and ESEs located within 70 nt of the acceptor splice sites have been reported to be more active than ESEs beyond this distance (Wu and Maniatis 1993; Fairbrother et al. 2004). Using ESE-predicting software to fine tune our AON design improved our success rate from 70% to ~75% (A. Aartsma-Rus, unpubl.). On comparison, GC content and AON length were similar for effective and ineffective AONs (Aartsma-Rus et al. 2005). Nevertheless it was recently reported that, for some AONs, increasing the length enhanced exon skipping efficiency (Harding et al. 2007). Our own recent studies indicate that the efficiency of some AONs that induce very low levels of exon skipping can be enhanced by increasing AON length, whereas increasing the length of an already efficient AON did not enhance AON efficiency and occasionally even reduced exon skipping levels (H.A. Heemskerk, G.-J.B. van Ommen, S. de Kimpe, P. van Kuik, J.C.T. van Deutekom, and A. Aartsma-Rus, in prep.). This phenomenon was also occasionally observed by Harding and colleagues (Harding et al. 2007).

Recently, the Wilton group described AON sequences to induce the skipping of each DMD exon except the first and last (Wilton et al. 2007). AON design was based on targeting the donor or acceptor splice sites or putative ESE sites as predicted by ESEfinder v3.0 (Smith et al. 2006). AONs that proved effective were then further optimized by designing overlapping AONs specific for the same target site; 470 AONs were screened in this manner. Of the optimal AON for each exon, 56 target exon-internal sequences, 16 the 3′ (acceptor) splice sites, and one the 5′ (donor) splice site. For 26 of the 56 exons skipped by exon-internal AONs, we had already identified effective AONs (Aartsma-Rus et al. 2005). In 25 out of 26 cases the optimal target sequences overlapped partially or completely with the target sites we determined earlier. Interestingly, the percentage of exon-internal AONs is highest in the most efficiently skipped group (83%), lower in the medium efficient group (73%), and lowest (64%) in the group of exons that can barely be skipped. The finding that exon-internal AONs appear to be more efficient than blocking the splice sites themselves contrasts with the fact that mutations abolishing a splice site result in exon skipping for virtually all cases, while mutations abolishing an ESE site often lead to partial exon skipping (Ginjaar et al. 2000;

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Deburgrave et al. 2007). A possible explanation is that the splice site motifs are more sharply defined, while ESEs are more of a pattern. Thus, a single mutation in an ESE will have a less pronounced effect than one affecting a splice site. In contrast, binding of U1 snRNP and U2 snRNP to the donor and acceptor splice sites, respectively, may occur with a higher affinity than SR protein binding to ESE sites. This would imply that U1 and U2 may be better able to compete with AON binding than SR proteins. Moreover, as SR proteins recognize motifs rather than consensus sequences, it is not unlikely that the binding site is partly determined by a specific secondary structure. AON binding to ESEs is likely to disrupt the local secondary structure, and would so further prevent SR protein binding.

Given the enormous length of DMD introns, it is likely that DMD exons are more dependent on ESEs for recognition by the splicing machinery than exons of other genes. Therefore, we hypothesized that some exons might have two or more mutually exclusive ESE sites (Aartsma-Rus et al. 2006b). This would render them insensitive to steric hindrance of SR protein binding to one of those sites, while blocking both should induce exon skipping. We indeed recently reported efficient and reproducible skipping of three thus far unskippable exons (exons 47, 57, and 64) and of the poorly skippable exon 45 using a combination of exon-internal AONs (Aartsma-Rus et al. 2006b). The potential of double targeting was confirmed by Wilton and colleagues, who reported that for exons 10, 20, 34, and 65, which were poorly or not skippable with individual AONs, skipping at high levels could be induced using a mix of two or even three (exon 65) AONs (Wilton et al. 2007).

AONS TO STUDY SPLICING

Regardless of whether exon-internal or splice site AONs are used, the aim of antisense-mediated exon skipping has thus far been to disrupt the splicing of the targeted exon. To determine if exon characteristics such as exon length, length of the flanking introns, and/or strength of the predicted splice sites affected the levels of exon skipping (or the "skippability" of an exon), we compared said characteristics for exons that could be skipped at either high, medium, or low levels as reported by Wilton and colleagues (Wilton et al. 2007). We observed that the predicted acceptor splice sites of poorly skippable exons were significantly higher than those of exons that could be skipped at medium or high efficiency ($P = 0.04$, Kruskal–Wallis signed rank sum test) (Fig. 2). It makes sense that exons with poorly defined splice sites, which critically depend on ESEs, are easier to skip. This finding explains why for exons, which are skipped at low levels, the percentage of AONs targeting the acceptor splice sites was much higher than for efficiently skipped exons (83% versus 64%). This implies that when one is free to choose a target exon within a transcript for antisense-mediated exon skipping, it is probably best to choose exons with low predicted values for acceptor splice sites. Interestingly, no significant difference was observed for the strengths of donor splice sites between the different groups (Fig. 2). Combined with the finding that for only one single exon out of the 77 tested the donor splice site was the optimal target, this must imply that donor splice sites are of lesser importance for exon definition during DMD splicing. This is in contradiction with the current view on exon definition, which states that exons are defined by binding of splicing factors first to the donor and then to the acceptor splice site of an exon (Robberson et al. 1990). It is possible that this view needs revisiting, or that DMD splicing is atypically complex, with its intron sizes varying from 107 bp to 248 kb, thus DMD exons may not behave as other exons. Indeed, for several other genes, the donor (5′) splice site has been targeted successfully (Table 1).

No relation was observed between exon skipping efficiency and exon length, or lengths of the upstream, downstream, or flanking introns (Fig. 2). In general, only the targeted exon was skipped, but occasionally unexpected results were observed. A striking example is that AONs targeting exon 8 always induce skipping of both exon 8 and 9 in human and dog transcripts (Aartsma-Rus et al. 2005; McClorey et al. 2006b; Wilton et al. 2007). The most likely explanation for this phenomenon is that the AONs somehow affect only the acceptor splice site but leave the donor splice site intact. Therefore, exon 8 can be joined to exon 9 and both exons are then spliced out together due to the AONs that disrupt the acceptor splice site of exon 8 (Fig. 3). This is consistent with the similar finding that an AON targeting the acceptor splice site of exon 17 resulted in the skipping of both this and the subsequent exon, whereas AONs targeting intraexonic sequences (abolishing donor splice site recognition) only cause single exon 17 skipping. However, AONs targeting acceptor splice sites can also result in skipping of only the targeted exon. Generally, upon the disruption of an acceptor splice site, the preceding donor splice site will be joined to the first accessible downstream acceptor splice site, leading to skipping of the targeted exon only. Thus, other factors such as the order of intron splicing must be involved as well. Transcription of the DMD gene takes ~16 h and the RNA has been shown to be cotranscriptionally spliced (Tennyson et al. 1995). However, it is unlikely that splicing occurs consecutively for all introns. In the case of exon 8, the preceding intron 7 is a hundred times longer than the following intron 8 (110 versus 1.1 kb), and splicing of the long intron 7 may take much more time so that in most transcripts exons 8 and 9 will already be joined before intron 7 is spliced out. This will enable the skipping of both exons 8 and 9 when AONs are used that do not disrupt the donor splice site of exon 8. We note that since it is not always possible to foresee whether AONs will induce

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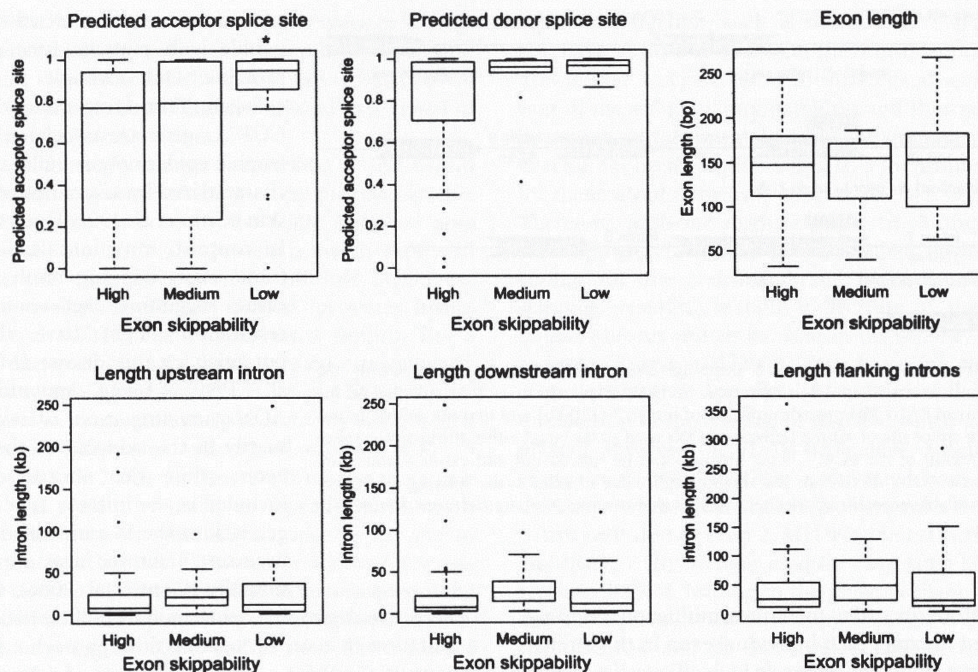

FIGURE 2. Box plots of the different groups of exons for the predicted donor and acceptor splice sites, exon length, and the lengths of the upstream, downstream, or flanking introns. Exon skippability is based on the report published by Wilton and colleagues (Wilton et al. 2007). The median value is indicated by a broad vertical line which is located within a box that contains all values between the 25th and 75th percentiles. The outer ranges are depicted by dotted lines and bordered by small horizontal lines. Outlying values are indicated by small circles. Splice site values were calculated with the Berkeley *Drosophila* Genome Project software for human splice site prediction. The predicted acceptor splice sites were significantly higher for poor skippable exons as calculated with the Kruskal–Wallis signed rank sum test (*P*-value 0.04, indicated with an asterisk). No significant differences were observed for the other parameters, although there is a trend for predicted donor splice sites to be somewhat lower for highly skippable exons (*P*-value 0.2).

skipping of a single or multiple exons, it is advisable to analyze not just the RNA directly flanking the targeted RNA, as this may lead to misinterpretation of results. For example, using primers in exons 7 and 9 would have led us to believe the exon 8 AONs were ineffective, because no skipping would have been observed. Another example is exon 23 skipping in the *mdx* mouse, where single exon 23 skipping is often accompanied by the out-of-frame skipping of exons 22 and 23 (Mann et al. 2001, 2002). In addition, using primers further away, the occasional skipping of longer stretches of exons has been reported (Dunckley et al. 1998; Fall et al. 2006). AONs targeting exon 54 induced equal levels of single exon 54 skipping and skipping of both exons 54 and 55, suggesting that intron 54 splicing occasionally precedes intron 53 splicing. These results imply that AONs can be a tool to study the splicing process per se in more detail. The timing and sequence of intron splicing is likely dependent on intron length, but nucleotide composition and secondary RNA structure may play a role as well. Knowing the sequence of intron splicing of a certain gene can explain the outcome of splicing mutations that result in complex splicing patterns (Schwarze et al. 1999).

The nonconsecutive splicing of the DMD gene explains why it is sometimes feasible to skip multiple consecutive exons targeting only the two outer exons (so-called multi-exon skipping). We first observed multiexon skipping after treating patient and control muscle cultures with AONs specific for exons 45 and 51, in a successful attempt to correct the reading frame of an exon 46–50 deletion (Aartsma-Rus et al. 2004a). In the control myotubes we observed low levels of single and double exon 45 and 51 skipping, but also exon 45–51 skipping. Notably, exon 45–51 skipping would be therapeutic for 13% of all DMD patients and would thus reduce the mutation specificity of the exon skipping approach. Furthermore, it would allow the generation of larger deletions, e.g., those known to be associated with a milder BMD phenotype. Unfortunately, despite many attempts it proved unfeasible to induce multiexon skipping of exons 17–48/51 and 48–59, which would be large deletions associated with extremely mild phenotypes (Aartsma-Rus et al. 2006b). In retrospect, this is not surprising given the long transcription time and the cotranscriptional splicing (Tennyson et al. 1995). Thus, exon 16 will be joined to exon 17 long before exon 48 is even transcribed (an estimated 4.5 h

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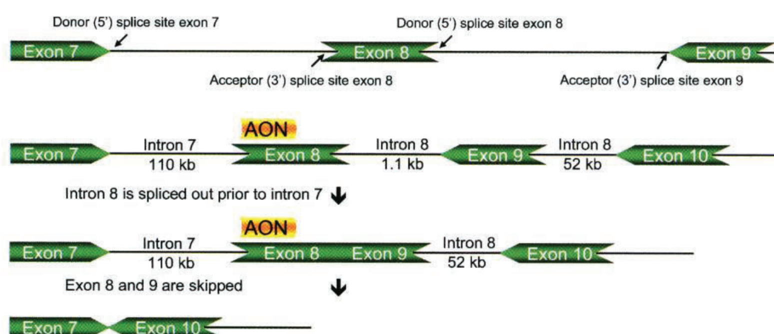

FIGURE 3. Antisense-mediated exon 8 skipping. Using AONs targeting exon 8 only the skipping of both exons 8 and 9 is observed. A likely explanation is that splicing of the downstream intron 8 (1.1 kb) precedes splicing of intron 7 (110 kb) and that the AONs do not affect the donor splice site of exon 8 (effective AONs used so far target either the acceptor splice site or the 5′ region of the exon). Thus, intron 8 can be spliced out and exons 8 and 9 are joined. As exon 8 AONs do disrupt the acceptor splice site of exon 8, the splicing machinery uses the first available acceptor splice site, which is that of exon 10 (because exons 8 and 9 are already joined).

later). Therefore, in order to induce multiexon skipping, one may need to target each individual exon in this stretch. This has been achieved at relatively high efficiencies in *mdx* mouse in vitro and in vivo for up to seven exons (Fall et al. 2006). However, targeting more exons was less efficient and typically led to many intermediate products where some, but not all, of the intended exons are skipped (S. Wilton, pers. comm.). Exon 42–55 and 45–60 skipping proved feasible but not consistently, and we often observed exon 45–55 skipping both in treated and nontreated samples (Aartsma-Rus et al. 2006b). Exon 45–55 skipping would potentially be therapeutic for ~40% of all DMD patients and an exon 45–55 deletion has been observed in asymptomatic individuals (Beroud et al. 2007). This multiexon skipping is probably more feasible than the very large skips, as the stretch involves less exons, while the flanking introns (44 and 55) are very long (248 kb and 120 kb, respectively). Thus, it is likely that for a significant number of transcripts exons 45–55 may already be joined, before introns 44 and 55 are spliced out, making exon 45–55 multiexon skipping an attractive target. Our preliminary results indicate that exon 45–55 multiexon skipping targeting the outer exons 45 and 55 is feasible, but levels are as yet too low to be beneficial (A. Aartsma-Rus, L. van Vliet, J.C.T. Deutekom, and G.-J.B. van Ommen, unpubl.).

EXON SKIPPING FOR OTHER APPLICATIONS

Reading frame restoration

The applicability of the exon skipping approach is not restricted to DMD (for an overview, see Table 1). A limited number of proteins share the feature of dystrophin that an in-frame, internal deletion is compatible with partially functionality of proteins. One example is type VII collagen. Truncating mutations in the *COL7A1* gene are associated with dystrophic epidermolysis bullosa, a disease characterized by severe blistering of the skin (Uitto et al. 1995; Fine et al. 2000). In contrast, mutations leading to in-frame exon skipping result in milder cases, suggesting that reading frame restoration might have therapeutic potential for this disease (McGrath et al. 1999). Using mutation-specific AONs targeting exon 70 resulted primarily in the skipping of the mutated exon, while the normal exon was included in the mRNA. Type VII collagen lacking the 16 amino acids encoded by exon 70 showed near normal functionality (Goto et al. 2006). Over 20% of recessive dystrophic epidermolysis bullosa patients carry a mutation in exon 70, making this approach a promising therapeutic tool for a significant subset of patients.

Isoform switching

Alternatively, AONs have been used to change levels of alternatively spliced genes. A striking example is the *Bcl-x* gene, which has two isoforms, Bcl-xS and Bcl-xL, that arise from two different 5′ splice sites in exon 2 (Mercatante et al. 2001, 2002). The Bcl-xS isoform is pro-apoptotic and sensitizes cells to chemotherapy. In contrast, Bcl-xL is anti-apoptotic and induces resistance to chemotherapeutic agents. Using AONs targeting the xL splice site, it was possible to shift the alternative splicing patterns toward the xS isoform. In vitro this shift by itself resulted in massive apoptosis in some cancer cell lines, and each cell line tested became sensitive to several chemotherapeutic agents (Mercatante et al. 2002). Unfortunately, after systemic delivery of AONs, one of the main target organs is the liver. Bcl-x AONs indeed induced liver apoptosis in treated mice after tail vein injection (Williams and Kole 2006), thus limiting the applicability of this approach.

A gene that is often inappropriately overexpressed in leukemia and solid tumors is the Wilms' tumor gene (*WT1*) (Scharnhorst et al. 2001). The gene product is thought to interfere with normal signaling, leading to maintenance of a malignant phenotype by increased proliferation and inhibition of differentiation and apoptosis. Most leukemic cells express high levels of *WT1* transcripts containing the alternatively spliced exon 5 (Renshaw et al. 2004). AON-mediated exon 5 skipping led to loss of cell viability and a decrease in cell survival in leukemic cell cultures (Renshaw et al. 2004). The expression of the *WT1*

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gene in adults is restricted to specific cell types in kidney, gonads, hematopoietic cells, the nervous system, and mesothelium (Reddy and Licht 1996). In vivo analysis of the AONs will have to determine potential adverse effects of exon 5 skipping in these tissues.

Another anti-cancer approach described is the use of AONs to induce isoform switching of the prostate-specific membrane antigen, encoded by the *folate hydrolase* gene (Williams and Kole 2006). This protein is mainly expressed in prostate cells and one isoform is 140-fold higher expressed in malignant versus normal prostate tissues (O'Keefe et al. 2004). The overexpressed isoform has a functional enzymatic domain that is located extracellularly and regulates folate uptake (Davis et al. 2005). Other prostate-specific membrane antigen isoforms exist, which arise from the alternative splicing of exons 6 and 18 (Williams and Kole 2006). In these isoforms the enzymatic domain is not present or inactive, respectively. A fourth isoform results from an alternative donor splice site in the first exon (Su et al. 1995). This isoform lacks the transmembrane domain of the antigen and as a consequence the enzyme activity is sequestered in the cytoplasm. Individual AONs targeting exon 1, 6, and 18 were able to induce isoform switching, which was accompanied by lower levels of the full-length isoform and decreased enzymatic activity (Williams and Kole 2006). AONs targeting the donor splice site of the full-length first exon are the most promising for therapeutic application, as the isoform without the membrane domain may be involved in a pro-apoptotic pathway (Williams and Kole 2006). In addition, side effects are expected to be low as in adults the expression of the full-length isoform is restricted to malignant cells.

Frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) results from tau deposits in the frontotemporal lobe of brain and neuronal cell death (Spillantini and Goedert 1998). Tau is normally involved in microtubule assembly and stability and has either three or four microtubule-binding domains (3R and 4R isoforms, respectively) (Goedert et al. 1989). The ratio between these isoforms is tightly regulated, as indicated by the finding that the majority of FTDP-17 patients carry mutations in the alternatively spliced exon 10 that lead to enhanced inclusion of this exon and increased expression of the 4R isoform (Varani et al. 1999). Using AONs targeting exon 10, levels of the 4R tau isoform were decreased in cultured rat cells that normally predominantly express the 4R isoform (Kalbfuss et al. 2001). This was accompanied by a changed morphology of the cytoskeleton of treated cells, suggesting that these AONs can be used to study the function of the different isoforms. It will be challenging to make this strategy therapeutically applicable, as the ratio between the 3R and 4R isoforms is tightly regulated and the target tissue (brain) is difficult to reach.

Isoform shifting is also useful for numerous inflammatory diseases. Receptors involved in immune stimulatory signal transduction, such as the IL-5 or TNFα receptors, also have an isoform that lacks the transmembrane domain. The soluble receptors bind to their ligand without induction of the inflammatory pathways and thus neutralize the bioactivity of the ligand. In many inflammatory diseases an effector has been identified, e.g., IL-5 for asthma and TNFα for rheumatoid arthritis (Panayi et al. 2001; O'Byrne 2006). Therefore, antisense-mediated isoform shifting has therapeutic potential for these diseases. AONs targeting exon(s) coding for the transmembrane induced efficient exon skipping, resulting in both a decrease of the membrane bound isoform and an increase in the soluble, neutralizing receptor (Karras et al. 2000, 2001; P. Sazani, pers. comm.).

An alternative approach to inhibit inflammation is modulation of the splicing of MyD88, which is an adaptor protein involved in IL-1β-dependent NFκB activation (Burns et al. 1998). $MyD88_L$ is translated from a full-length transcript, whereas the $MyD88_S$ isoform arises from the alternative exclusion of exon 2. $MyD88_L$ binds the IL-1 receptor and IRAK-1 (Interleukin receptor-associated kinase 1) and recruits IRAK-4, leading to the phosphorilation of IRAK-1 and subsequent NFκB activation. $MyD88_S$ is unable to recruit IRAK-4 and is, therefore, unable to induce NFκB activation (Burns et al. 2003). As $MyD88_S$ acts in a dominant-negative way, isoform switching from $MyD88_L$ to $MyD88_S$ may have therapeutic potential to treat inflammatory diseases associated with excessive IL-1 receptor signaling, such as atherosclerosis. AONs targeting exon 2 of the *MyD88* gene were indeed able to induce isoform switching, which was accompanied by diminishing pro-inflammatory signaling through the IL-1 receptor in vitro and in vivo (Vickers et al. 2006).

Gene knockdown studies

Recently, exon skipping has also been applied as an alternative way to achieve gene knockdown. This has advantages when compared to the standard RNase H gene knockdown as exemplified by AON-mediated exon skipping of Apolipoprotein B (*APOB*). There are two natural APOB isoforms. The full-length APOB100 protein is required for the assembly of VLDL, IDL, and LDL, is one of the ligands for the LDL receptor, and plays a central role in atherosclerosis (Soutar and Naoumova 2007). The other isoform, APOB48, is essential for chylomicron assembly and intestinal fat transport (Chester et al. 2000). APOB48 arises from intestine tissue-specific RNA editing of a CAA into a UAA termination codon in exon 26 (Chester et al. 2000). As a consequence, APOB48 lacks the LDL receptor binding domain. APOB100 knockdown is under investigation as a potential treatment for atherosclerosis. However, RNAi- and RNase H-induced degradation will result in knockdown of the detrimental APOB100 and the essential APOB48 isoform. Khoo and colleagues used the antisense-mediated exon skipping approach to target exon 27 of the

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APOB transcript (Khoo et al. 2007). As the RNA editing signal for *APOB48* is located in exon 26, exon 27 skipping will not affect this isoform. It will, however, disrupt the open reading frame of the *APOB100* transcript, leading to lower amounts of APOB100 and likely to lower LDL and cholesterol levels. This hypothesis is backed up by the finding that heterozygote individuals with truncating mutations in exon 27 have low LDL and cholesterol levels and are resistant to the development of atherosclerosis (Whitfield et al. 2004). Exon 27-specific AONs indeed resulted in skipping of the targeted exon leading to a truncated, nonfunctional APOB100 protein, while APOB48 levels were maintained (Khoo et al. 2007).

The exon skipping approach has finally been employed to achieve isoform specific knockdown in order to determine isoform functionality. The human titin gene consists of 363 exons, and over a hundred alternatively spliced transcripts have been described (Freiburg et al. 2000). The isoforms are categorized into long N2A and N2B isoforms and the short Novex isoforms. Titin is proposed to serve as a template for sarcomere assembly, but as yet little is known about the function of the different titin domains and isoforms. In zebrafish, there are two titin orthologs, *ttna* and *ttnb*, which are highly homologous and both can give rise to N2A and N2B isoforms, whereas only *ttna* can encode the Novex isoform (Seeley et al. 2007). Using AONs it was feasible to induce skipping in a homolog and isoform-specific way, allowing the dissection and analysis of the function of the different titin isoforms in zebrafish development (Seeley et al. 2007). This approach showed that different titin isoforms have distinct functions, e.g., the N2A domain is required for sarcomere assembly in the somites, while both the N2A and N2B domains are essential for sarcomere assembly in the heart.

AONs to induce exon inclusion

AONs have also been used to induce inclusion of exons that are skipped due to mutations that disrupt ESEs (Cartegni and Krainer 2003; Hua et al. 2007). The best-studied example is the survival of motor neuron 2 (*SMN2*) gene, which is a homolog of the *SMN1* gene that is mutated in patients with spinal muscular atrophy (SMA) (Munsat and Davies 1992). *SMN2* is a near perfect homolog of *SMN1* but cannot compensate for the lack of SMN1 protein due to a translationally silent mutation in exon 7 of *SMN2*, which disrupts an SF2/ASF-binding site and results in an exon 7 skipping exon in most transcripts (Cartegni and Krainer 2002). As the amount of full-length *SMN2* transcripts is inversely correlated with disease severity, enhancing exon 7 inclusion is a putative therapy for SMA (Jablonka et al. 2000). Exon 7-specific AONs with a tail containing an ESE motif, or AONs linked to a serine-arginine peptide domain to recruit SF2/ASF to the disrupted ESE, resulted in higher levels of full-length SMN2 (Cartegni and Krainer 2003; Skordis et al. 2003). The same result was obtained with AONs targeting exonic splicing silencer motifs (Hua et al. 2007). Exonic splicing silencers are the counterparts of ESEs and are involved in the induction of exon skipping in, e.g., alternatively spliced exons. SMN protein levels increased after treatment with AONs targeting exonic splicing silencers, implying that AONs do not interfere with mRNA translation. Thus, this approach has therapeutic potential for SMA and possible for other diseases caused by mutations that disrupt ESEs or induce exonic splicing silencers.

TOWARD CLINICAL APPLICATIONS

The current review shows that antisense-mediated exon skipping is a promising tool for many research and therapeutic applications. If AONs manage to reach the cytoplasm, they will be effectively transported to the nucleus through a so far undefined mechanism. However, the main obstacle toward clinical application of this approach is the actual AON delivery to the target tissues. Biodistribution studies have shown that after systemic delivery, the majority of the AONs end up in the liver and the kidney for each of the different AON chemistries (Sazani et al. 2002; Fluiter et al. 2003; C.L. de Winter, H.A. Heemskerk, S. de Kimpe, P. van Kuik, G. Platenburg, and J.C.T. Deutekom, in prep.). On one hand, this is good news when the target gene is mainly and/or highly expressed in the liver, as is the case for, e.g., *APOB*. On the other hand, when the target gene is expressed in another tissue and in liver and kidney as well, AONs may trigger unwanted side effects, such as the liver apoptosis observed after treatment with Bcl-x AONs (Williams and Kole 2006). To obtain high local AON levels, direct injection into a tumor may be an option for some cancers, but injecting each and every muscle in DMD patients is unfeasible as muscle makes up 30% of the body and some muscles such as the diaphragm and the heart are difficult to reach. However, sometimes diseased tissues may also be more accessible; e.g., dystrophic muscle fibers are more permeable than healthy muscle fibers, resulting in enhanced intramuscular AON levels after systemic treatment (C.L. de Winter, H.A. Heemskerk, S. de Kimpe, P. van Kuik, G. Platenburg, and J.C.T. Deutekom, in prep.).

AON chemistry

Currently, different AON backbone chemistries are available, each having different characteristics. The most commonly used AON chemistry for splicing modulation is 2′-*O*-methyl or 2′-*O*-methoxyethyl RNA with a phosphorothioate (PS) backbone (Kurreck 2003). The 2′-*O*- modification renders the AON RNase H resistant and increases affinity for target RNA. The phosphorothioate backbone enhances stability as it inhibits AON breakdown by endo- and exonucleases. This modification is relatively cheap and

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can be scaled up easily. Possible alternatives are morpholinos and locked nucleic acids (LNAs), which are both RNase H resistant. Morpholinos contain a six-membered morpholine moiety instead of the sugar ribose and phosphorodiamidate linkages (Summerton and Weller 1997). They have a nonionic backbone at physiological pH, making them notoriously hard to transfect in tissue culture experiments (Amantana and Iversen 2005). However, in vivo their nonionic nature results in higher tissue concentrations, due to the lack of nonspecific interactions with cellular components (Amantana and Iversen 2005). Morpholinos are nontoxic and very stable and have been shown efficient modulators of pre-mRNA splicing (Gebski et al. 2003). LNAs contain a methylene bridge that connects the 2′-*O* to the 4′-*C* of the ribose, forcing the nucleotide in the 3′ endoconformation (Obika et al. 1998). As a consequence LNAs are inflexible and have an extremely high affinity for RNA and DNA. In addition, they are nontoxic and nuclease resistant (Wahlestedt et al. 2000). LNAs have been reported to be extremely efficient modulators of pre-mRNA splicing (Aartsma-Rus et al. 2004b; Roberts et al. 2006). Ethylene-bridged nucleic acids (ENA) have an ethylene bridge instead of a methylene bridge and have comparable characteristics to LNAs (Morita et al. 2002, 2003).

To directly compare the effect of different AON analogs, the Kole group has developed an elegant read out system (Sazani et al. 2002). They generated a construct that contains a cryptic splice site in β-globin intron 2 linked to a green fluorescent protein gene. Without AONs the cryptic splice site will be used and GFP will not be produced, while effective AONs will redirect splicing and restore GFP synthesis. The amount of GFP reflects the efficiency of the AON. A mouse model stably expressing the GFP construct has been generated, allowing easy comparison of the biodistribution of different AON analogs (Sazani et al. 2002). Using this model, it was discovered that full-length LNAs generate an effect mainly in liver, colon, and small intestine after systemic delivery, thus providing a tool to manipulate splicing in these specific tissues (Roberts et al. 2006). Morpholinos have gained attention for DMD exon skipping since this chemistry is taken up at higher levels by the muscle (Sazani et al. 2002). Systemic delivery of morpholino in the *mdx* mouse was indeed more efficient than the 2′-*O*-methyl phosphorothioate counterpart (Alter et al. 2006; Fletcher et al. 2006; H.A. Heemskerk, G.-J.B. van Ommen, S. de Kimpe, P. van Kuik, J.C.T. van Deutekom, and A. Aartsma-Rus, in prep.). However, it is likely that the optimal chemistry partly depends on the target sequences as well. For example, LNAs were the most efficient chemistry to induce exon 46 skipping, but 2′-*O*-methyl PS AONs were optimal for exon 51 (A. Aartsma-Rus, unpubl.; Aartsma-Rus et al. 2004b). In addition, the morpholino targeting *mdx* mouse exon 23 is more efficient than the 2′-*O*-methyl counterpart, whereas for other exons morpholinos are equally efficient (McClorey et al. 2006a; H.A. Heemskerk, G.-J.B. van Ommen, S. de Kimpe, P. van Kuik, J.C.T. van Deutekom, and A. Aartsma-Rus, in prep.).

AON specificity

As the effect of the AONs is transient, due to AON clearance and breakdown of the targeted mRNA transcript and protein, patients will have to be treated repeatedly and chronically for genetic disorders like DMD. Therefore, one of the most important features of the AON has to be specificity, in order to avoid long-term side effects. During AON design BLAST analysis is performed for each AON to exclude annealing to other targets. However, this is based on the assumption that the AON only anneals to a completely homologous sequence. We compared the sequence specificity of LNAs, morpholinos, and 2′-*O*-methyl PS AONs (Aartsma-Rus et al. 2004b; H.A. Heemskerk, G.-J.B. van Ommen, S. de Kimpe, P. van Kuik, J.C.T. van Deutekom, and A. Aartsma-Rus, in prep.). As expected, LNAs showed very poor sequence specificity, and an AON containing two mismatches in a 14 mer was equally as efficient as the original LNA AON. The 2′-*O*-methyl PS AONs were much more sensitive to mismatches; a single mismatch either decreased exon skipping levels drastically or completely abolished AON efficacy. For morpholinos we obtained mixed results (H.A. Heemskerk, G.-J.B. van Ommen, S. de Kimpe, P. van Kuik, J.C.T. van Deutekom, and A. Aartsma-Rus, in prep.). In one case, two mismatches rendered the morpholino inefficient, for two other target sequences the same amount of mismatches reduced exon skipping levels or resulted in similar skipping levels as observed with the nonmismatched counterpart. In contrast, the mismatched 2′-*O*-methyl PS counterparts never induced a specific exon skipping.

AON delivery

As the majority of AON is cleared by the liver and kidneys, ways to decrease liver and kidney uptake and/or enhance tissue-specific uptake are under investigation. Certain cell-penetrating peptides enhance the uptake of morpholino AONs by muscle (Fletcher et al. 2007). This may also be achieved by linking muscle-homing molecules or peptides to AONs (Samoylova and Smith 1999; Ghosh and Barry 2005; Kolonin et al. 2006). Among the same line, one can envisage linking molecules or peptides specific for any given tissue to AONs to enhance tissue-specific uptake. Notably, alternative dystrophin isoforms are expressed in other tissues, such as the retina and the central nervous system. In DMD patients the muscle phenotype is most prominent, but deletions in the hot spot affect other isoforms as well. A complicating factor here is that the effect of the absence of the dystrophin isoforms in nonmuscle tissues is poorly understood. Nevertheless, AONs would

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restore the reading frame of these isoforms as well. Thus for DMD the AONs do not necessarily have to target only muscle tissue. Actually, the fact that exon skipping strategy targets the pre-mRNA transcribed from the endogenous gene allows for the systemic restoration of different defective isoforms in a patient with one single AON.

Alternatively, the antisense sequence can be delivered to cells using viral vectors carrying a gene from which the antisense sequence can be transcribed, such as the small nuclear ribonucleoproteins (snRNPs). U7 snRNP is normally involved in histone processing and hybridizes to the spacer element of histone pre-mRNA (Bond et al. 1991). Modified U7 snRNPs containing an antisense sequence against a β-globin cryptic splice site, several DMD exons and cyclophilin A, Tat or Rev (proteins involved in HIV multiplication) modulated splicing of targeted genes (Suter et al. 1999; Goyenvalle et al. 2004; Liu et al. 2004; Asparuhova et al. 2007). Alternatively, using bifunctional U7 snRNAs containing both an antisense and a splicing enhancer sequence inclusion of *SMN2* exon 7 could be established (Marquis et al. 2007). As the *U7* gene is small, it fits easily in adeno-associated virus (AAV) vectors, which is one of the few viral vectors that can efficiently infect muscle cells (Blankinship et al. 2004). Intramuscular and systemic treatment with AAV vectors containing U7 antisense constructs have shown promising results in the *mdx* mouse and golden retriever muscular dystrophy models (Goyenvalle et al. 2004; L. Garcia, pers. comm.). Exon skipping and dystrophin restoration were observed at high levels for at least 18 months and induced functional improvement. An advantage of this approach is that the antisense sequence is expressed for longer periods of time, thus eliminating the need for repeated injections. However, this is at the same time also a disadvantage. Using AONs, the treatment can be stopped in time, e.g., when better target sequences have been developed or when unexpected (long-term) adverse effects are observed. In addition, the use of a viral vector to deliver the antisense sequence converts the genetic exon skipping therapy into gene therapy. This will be accompanied by the typical gene therapy issues such as vector immunity and insertional mutagenesis. Finally, the manufacturing of AONs can be easily scaled up, whereas reproducibly and safely producing high titers of AAV vectors of excellent purity remain a major challenge. Unless these problems are solved, using AONs is probably a better option, especially since, so far, no toxicity has been reported after long-term systemic injections of AONs (Kurreck 2003; Takeshima et al. 2006).

BROADENING THE FIELD OF EXON SKIPPING APPLICATIONS

In addition to applications described in this review, antisense-mediated exon skipping can be implemented in numerous other therapeutic interventions or developmental studies. The number of applications where antisense-induced restoration of the open reading frame will be therapeutic, like for DMD and dystrophic epidermolysis bullosa, is probably limited. Generally, in-frame deletions of one or more exons will not result in partly functional proteins. However, numerous diseases, especially cancer, are associated with changes in the relative levels of alternative splicing (Srebrow and Kornblihtt 2006). Because the exon skipping effect is titratable, AONs can be employed to normalize levels of alternative splicing or to study the effect of disrupting normal splicing pattern. Finally, exon skipping is a useful tool for gene knockdown. In some areas this approach may be preferable over RNAi, which induces a catalytic process resulting in complete gene knockdown. Exon skipping on the other hand can be used to achieve variable, and more subtle and controlled, levels of knockdown, which is advantageous when (near) complete knockdown is detrimental for the cell, or when a certain amount of knockdown is required. Interestingly, this modulation mimics regulated unproductive splicing and translation, a naturally occurring mechanism, where alternative splice forms contain a premature stop codon and through nonsense-mediated RNA decay regulates protein expression levels (Lewis et al. 2003). In addition, AONs offer the opportunity for isoform or allele-specific knockdown. Ironically, antisense-mediated exon skipping is probably applicable to all areas said to benefit from RNase H-mediated knockdown. Thus, in this way AONs finally may be able to fulfill the promises made over a decade ago.

ACKNOWLEDGMENTS

We thank Johan den Dunnen, Judith van Deutekom, and Mark Einderhand for reading the manuscript and helpful comments. A.A.-R. is funded by a grant from ZonMw (The Netherlands).

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