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Achieving targeted and quantifiable alteration of mRNA splicing with Morpholino oligos

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Abstract

This work represents the first guide for using steric-block antisense oligos as tools for effective and targeted modification of RNA splicing. Comparison of several steric-block oligo types shows the properties of Morpholinos provide significant advantages over other potential splice-blocking oligos. The procedures and complications of designing effective splice-blocking Morpholino oligos are described. The design process requires complete pre-mRNA sequence for defining suitable targets, which usually generate specific predictable messengers. To validate the targeting procedure, the level and nature of transcript alteration is characterized by RT-PCR analysis of splice modification in a β-globin splice model system. An oligo-walking study reveals that while U1 and U2 small nuclear RiboNucleoProtein (snRNP) binding sites are the most effective targets for blocking splicing, inclusion of these sites is not required to achieve effective splice modifications. The most effective targeting strategy employs simultaneously blocking snRNP binding sites and splice-junctions. The work presented here continues to be the basis for most of the successful Morpholino oligos designed for the worldwide research community to block RNA splicing.

Keywords: Morpholino; Splicing; RNA processing; Splice-blocking; Antisense; Transcript

Background

Morpholino oligos allow experimental manipulation of pre-mRNA splicing. Such manipulation is powerful given that about 74% of all transcripts from multi-exon genes undergo alternative splicing [1].

A powerful technique for studying functions of individual transcripts is to use steric-blocking antisense oligos to block RNA processing events, splicing events in particular, and thereby force the expression of altered transcripts. Morpholino oligos have been shown to be the most effective steric-block oligo type for splice modification, completely and specifically blocking splicing events [2–5]. In splice-blocking, Morpholinos can be used as tools to characterize functions of alternatively spliced transcripts or generate "loss of function" (knockdowns) by means of exon deletion. While this paper focuses on the use of Morpholino oligos to directly alter splicing events by blocking snRNP binding sites and splice-junctions, Morpholino oligos have also been successfully used to block other sequences involved in control of RNA processing, including miRNA [6], intronic splicing silencers (ISSs) [7], exonic splicing enhancers (ESEs) [8], and ribozyme catalytic sites [9]. These studies and the work described herein demonstrate that Morpholinos are effective for controlled modification of nuclear processing thereby forcing expression of altered messengers.

PNA (peptide nucleic acid) and 2′-*O*-methyl RNA steric-block oligos have been used to block splicing, however they lack the specificity and efficacy of Morpholino oligos [4]. Morpholinos have been shown to alter splicing in zebrafish development by targeting a normal gene and generating an exon deletion to produce a phenotype equivalent to a loss-of-function mutation [2]. Utilizing the targeting

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rules in this guide, Morpholinos have since been used to block splicing in a variety of other organisms. The etiologies of many human genetic diseases involve splicing errors [10–12]; Morpholinos were recently shown to correct Hutchinson–Gilford progeria syndrome (HGPS) by modifying splicing of mutant Laminin A transcript in human cells [13] and to correct splicing in muscular dystrophy in myocytes [3]. While Morpholinos have been very successful in altering splicing, a complete and reliable procedure for targeting splice-blockers and predicting the products of splice targeting with Morpholino oligos has not previously been published.

I describe the simple targeting procedures in a 3-exon example pre-mRNA assaying the level and nature of transcript alteration with RT-PCR analysis. I will show that our methods typically generate expected results when targeting primarily intronic sequence that includes snRNP binding sequences. The ramification of poor targeting and characterization of unexpected outcomes from splice-blocking experiments is also discussed.

Results and discussion

The test system and general guidelines

Our 3-exon test system is a human β-globin intron fusion with firefly luciferase stably integrated in HeLa cells [14]. Blocking a dominant splice mutant with a Morpholino oligo edits out a stop codon and brings luciferase in-frame, up-regulating luciferase expression. In this test system, splice-modification can be characterized by luciferase activity or RT-PCR analysis (Fig. 1A) and it provides all the components necessary to define optimal splice targeting.

The 3-exon test system looks no different than a multi-exon gene you might be studying. The splicing of a typical multi-exon gene is defined by snRNP binding sites and consensus splice-junctions. Targeting primarily intron sequence on either side of an exon and covering the splice-junction usually results in deletion of the targeted exon. There are qualifications to achieve deletions and they will be discussed shortly; however the effectiveness of a targeted exon deletion can be characterized with careful choice of RT-PCR primers derived from neighboring exon sequence from each side of the targeted exon (Fig. 1B).

Primer sites should be selected so that RT-PCR end products are over 100 and fewer than 1500 bases with a 10% or greater mass change anticipated in RT-PCR products with splice-blocking. These primers should anneal within exons present in the end products and not directly affected by the Morpholino oligos chosen for the experiment.

A very important aspect of successful splice-blocking is using Morpholino oligos that satisfy the same general rules required for blocking translation. Splice-blocking Morpholinos should bind specifically to target sequence while at the same time contain little extra- or intramolecular complementarity, have good solubility, and not bind too tightly (favoring off-target effects), or too loosely (causing low efficacy). Fortunately, Morpholino oligos are extremely specific, requiring at least 12 bases of contiguous pairing to block translation at typical concentration. Additionally, self-complementarity is usually not a problem as Morpholinos containing self-complementary moieties with fewer than 16 contiguous extra- or intramolecular hydrogen bonds (forming dimers or stem-loops, respectively) retain significant activity. Selecting sequences with G content of 36% or less favors aqueous solubility, while selecting CG content between 40% and 60% gives ideal binding affinity for 37 °C systems. In a previous length vs. efficacy study comparing Morpholino oligos to PNAs and 2′-*O*-methyl RNA in our test system, Morpholinos longer than 23-mer lengths surpassed the activity of any comparable and optimal PNA or 2′-*O*-methyl RNA [4]. For this reason the typical Morpholino oligo designed for splice-blocking is 25-mer in length.

Targeting snRNP binding sites and splice-junctions

The familiar models for RNA processing includes the formation of a spliceosome that includes five small ribonu-

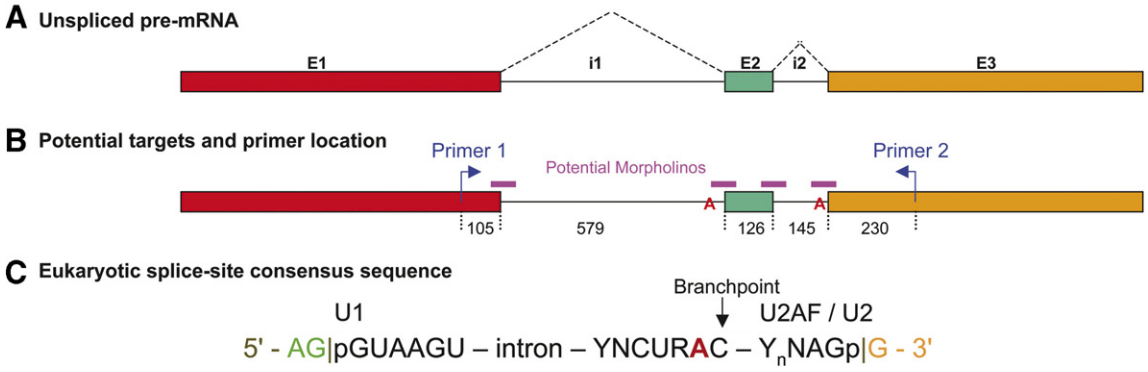

Fig. 1. The β-globin test system represents a typical 3-exon pre-mRNA transcript (A). The potential targets for Morpholino oligos include snRNP binding sites and splice-junctions (B). The consensus sequence for splicing is defined primarily by intronic sequence with more complexity in the 3′-end of an intron (C).

cleoprotein particles and the nucleophilic attack of an intronic adenine base against a phosphate on the 5′-end of an intron, forming a lariat structure. It has been well established that the first steps in generating a complete prototypical spliceosome are the binding of a U1 snRNP to the 5′-end of an intron (splice donor, exon–intron boundary) and the U2AF and U2 snRNP to the polypyrimidine tract in the 3′-end of an intron (splice acceptor, intron–exon boundary) [15]. This interaction is thought to occur via hydrogen bonding of snRNA to highly conserved sequences at intron ends, with substantially more complexity conserved in the U2AF and U2 snRNP binding sites (Fig. 1C) [16].

This 3′-end of the intron is comprised of the branchpoint adenine residue that closes the intermediate lariat structure characteristic of eukaryotic splicing and the poly-pyrimidine tract found just 3′ of the branchpoint near the acceptor junction. While the prototypical spliceosome for introns with consensus GU-AG ends initially binds pre-mRNA with U1 and U2 snRNPs, a secondary spliceosome has been characterized that replaces U1 and U2 snRNPs with U11 and U12 snRNPs and is involved in splicing the majority of AU-AC intron ends as well as a small subset of introns with GU-AG ends [16,17]. As you will soon see, these snRNP binding sites and the splice-junctions serve as the primary targets for Morpholino oligos used to block splice site recognition.

The model for predicting the outcome of altering splicing by blocking snRNP binding and splice-junctions predicts different outcomes for blocking internal exons than for blocking flanking exons. For internal exons, targeting an exon–intron or intron–exon boundary usually results in the deletion of the included exon in the final messenger (Fig. 2A), while targeting the first exon–intron boundary in a pre-mRNA sequence or the last intron–exon boundary in pre-mRNA usually results in a final messenger with the first intron or last intron inserted, respectively (Fig. 2B).

Targeting intron ends and splice-junctions yield best results

The basic targeting guidelines were validated with Morpholino oligos designed against each of the exon–intron boundaries (e1i1 and e2i2), intron–exon boundaries (i1e1 and i2e2) (Fig. 3A), sequences at the intronic branchpoint (i1BP and i2BP) and a collection of intronic sequences (i1-1, i1-2, i1-3, and i1-4) (Fig. 4) in our 3-exon test system.

The oligos were scrape-delivered into HeLa cells at 10 μM Morpholino in the medium. While the limited delivery efficiency of scrape loading caused incomplete modification of splicing, all targeted Morpholino oligos generated measurable amounts of predictable products (Fig. 3A). In addition, oligos designed to bind outside of the model target sites can yield significant splice-blocking more than 50 bases away from the splice-junction (Fig. 4).

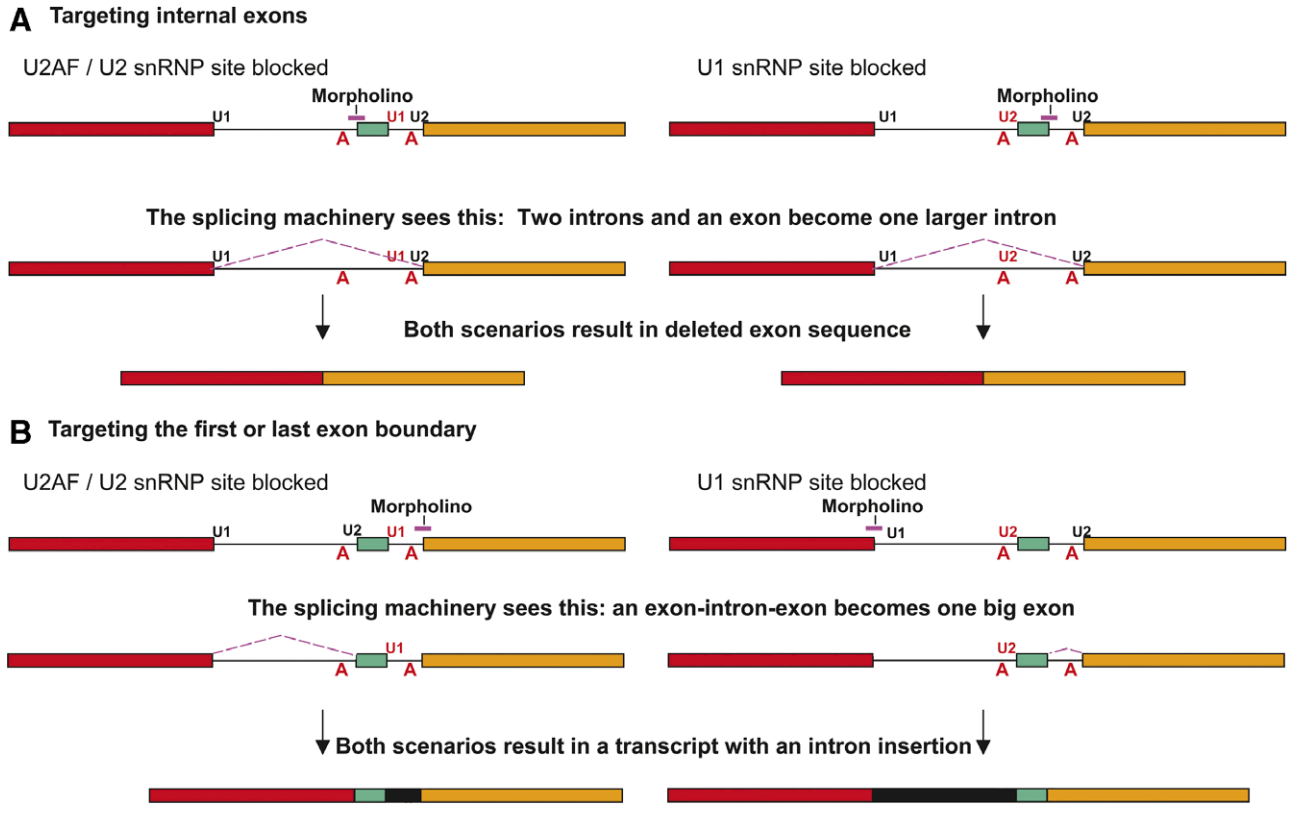

Fig. 2. The typical splice-blocking scenario involves deletion of an internal exon by masking a U2AF/U2 snRNP binding site or a U1 snRNP binding site with a Morpholino resulting in deletion of the proximal exon (A). Targeting junctions at the first or last exons of a transcript can create intron insertions (B).

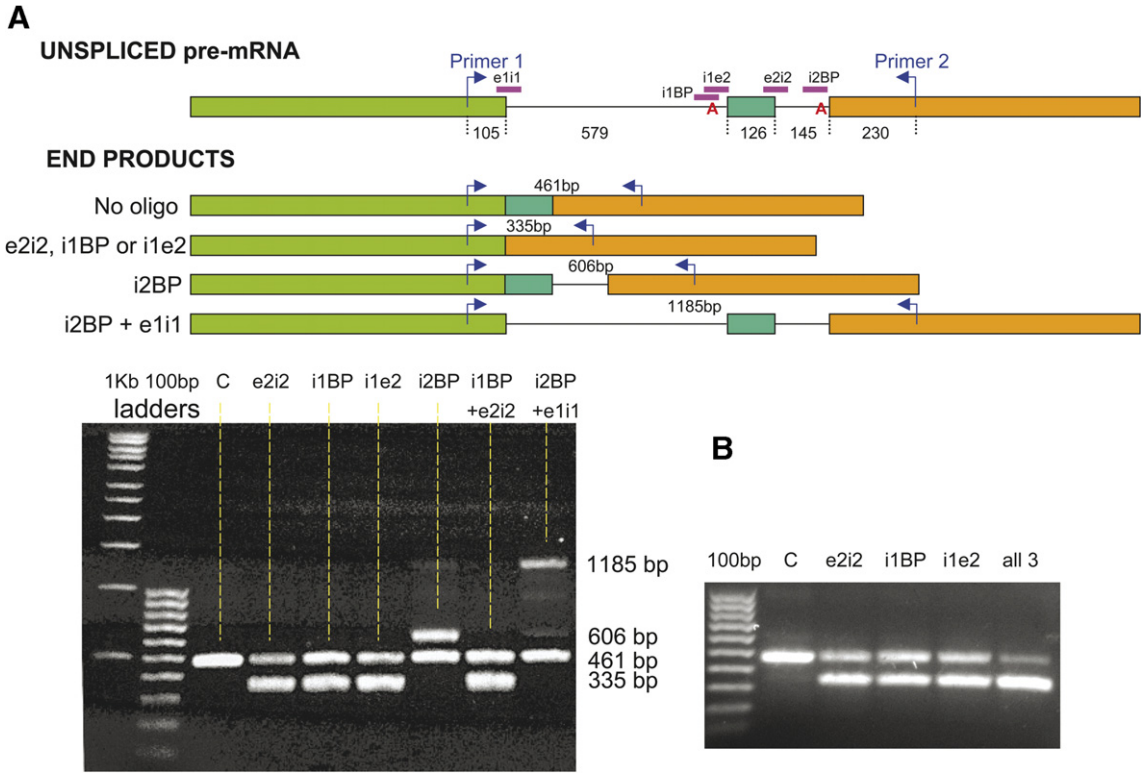

Fig. 3. Quantitative analysis and RT-PCR analysis of splice-blocking with Morpholino oligos in the β-globin test system. Four splice-junctions and the branchpoint of intron1 were targeted with a total of five Morpholino oligos scrape delivered at 10 μM. The end products are represented schematically and by RT-PCR analysis (A). The last two lanes show results from delivering a combination of two oligos. A combination of three oligos targeting both sides of exon2 yielded a 90% exon2 deletion as compared to approximately 50% for each oligo delivered individually (B).

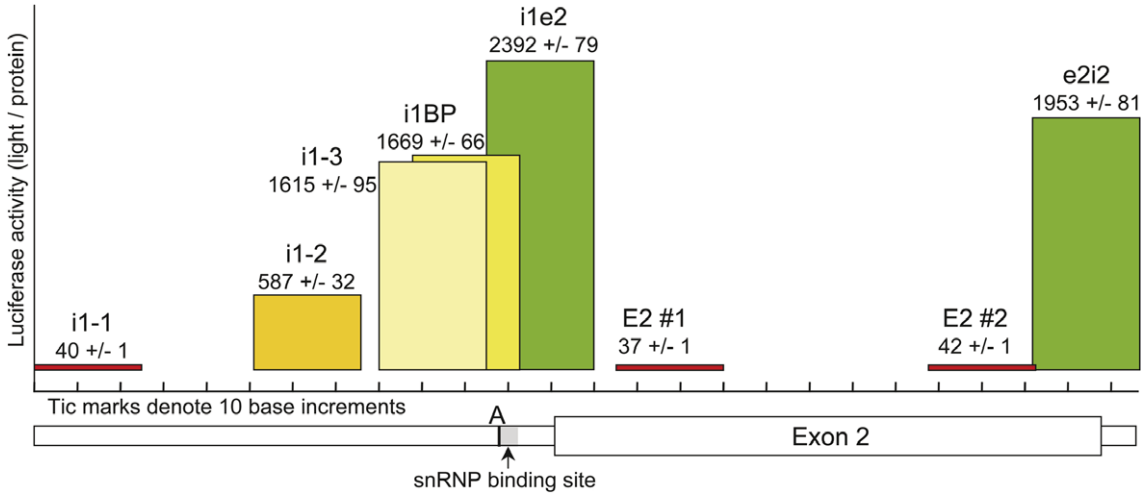

Fig. 4. A quantitative analysis of intron targeting revealed the greatest splice-blocking effect was achieved when targeting the branchpoint and splice-junction simultaneously. Two oligos, E2 #1, and E2 #2, targeted 13 bases from either splice-junction for exon2 had no impact on splicing.

The e1i1 and i2BP oligos, which target the first and last exons in this test system, resulted in RT-PCR products containing unspliced introns. The e1i1 oligo alone also generated an intron1 insertion (data not shown). While it is unclear whether the intron-retaining mRNAs are nuclear or cytosolic, clearly the Morpholinos are capable of interfering with intron removal. Interestingly, simultaneous introduction of both e1i1 and i2BP oligos resulted in a subset of completely unspliced RT-PCR products (Fig. 3A, last lane). Targeting the splice-junctions blocked splicing events. The oligos e1i1 and e2i2, which target the short U1 snRNP consensus donor sites and span their splice-junctions, also blocked splicing. For splice acceptors, targeting both the branchpoint and the

splice-junction blocked splicing more effectively than targeting the junction or branchpoint alone (Fig. 4). In the case of the i1e2 boundary for the β-globin test system, the branchpoint adenine residue is 14 bases from the splice-junction. A single Morpholino oligo targeting both the splice-junction and the entire U2 snRNP binding sequence (i1e2) yielded the greatest splice-blocking activity out of 6 oligos targeting exon2 deletion. However, an oligo that included the branchpoint but not the splice-junction (i1BP), or an oligo targeting sequence just 5′ to the branchpoint and splice-junction (i1-3), remained effective with 60% of the activity of i1e2. An oligo targeting 33 bases 5′ to the branchpoint (i1-2) continued to yield exon2 deletion at 20% the level of i1e2. An oligo targeting 83 bases 5′ to the branchpoint had no detectable activity. These data suggest that targeting the snRNP binding sites with Morpholino oligos may be the most effective means of blocking splicing, but targeting those sites directly is not required for splice-blocking activity. In this example, targeting the splice-junction, branchpoint, or sequence further upstream all caused the desired splice modifications albeit to a lesser extent for oligos targeted away from the splice-junction. Interestingly, targeting entirely within exon2 and only 13 bases away from either end of exon2 sequence had no impact on splicing (Fig. 4).

Targeted intron insertions are possible

In the β-globin test system, targeting the first exon–intron boundary (e1i1) or last intron–exon boundary (i2e3) yielded intron insertions. Previous experiments by others have shown that the first steps in spliceosome assembly include U1 snRNP binding to the 5′-end of the intron and displacement of branchpoint binding protein (BPP) and U2AF helper proteins by the U2 snRNP near the 3′-end of an intron [15,18–20]. Our data is consistent with the current splicing model: snRNPs, bound to target sequence via hydrogen bonding between the target mRNA sequences and complementary snRNA within snRNPs, mark potential splice sites and these sites are evaluated to process the final transcript that leaves the spliceosome. In the case of the e1i1 Morpholino, the oligo is blocking the U1 snRNP binding site of intron1 but leaving the U2 binding site available. The i2e3 Morpholino oligo is likely blocking the U2AF and U2 snRNP from binding the polypyrimidine tract of intron2 but leaving the U1 binding site available. The observation that intron1, exon2 and intron2 are included in the final mRNA transcript when the e1i1 and i2e3 oligos are simultaneously delivered suggests that the absence of favorable secondary splicing patterns can result in final mRNA transcripts with an intron insertion. In the case of targeting the first and last exons this is likely given that there are not options for additional splice elements outside of the existing pre-mRNA transcript.

Which is the better target: splice donor or splice acceptor?

There were no surprises in this relatively simple 3-exon test system. The majority of reported successful Morpholino splice-blocking experiments targeted exon–intron boundaries. This is partly due to early concerns that intron–exon boundary targeting could yield intron insertions if the 5′-end of the intron did not include a strong donor consensus sequence. It was further speculated that blocking the donor site would not likely impede the more robust acceptor consensus sequence of the 3′-end of an intron from forcing a deletion by finding an alternative 5′-donor, whether it be a cryptic site or preferably the donor site from the upstream intron. There is not yet enough data to definitively favor either donor site or acceptor site targeting, but both have been effective (see: pubs.gene-tools.com).

In our test system the i1e2 and e2i2 oligos were equally effective at blocking splicing, signifying that either intron–exon or exon–intron targets can be chosen to force deletion of the same internal exon. However, cryptic splice sites have been activated by targeting consensus splice sites with Morpholino oligos [2,5]. The cryptic splice sites characterized in these papers are splice donors, where near-matches with the consensus sequence (4 bp) are short enough to occur randomly. However, splice acceptor sites are unlikely to form by random mutation as they are more complex and must be associated with a polypyrimidine tract to be active, thus decreasing the probability of a mutation in a near-consensus splice site activating a cryptic splice site. In general, this means that targeting any exon–intron boundary with a Morpholino oligo can potentially activate a cryptic splice donor site and generate unpredictable transcripts. Targeting an intron–exon boundary is unlikely to reveal a cryptic splice acceptor site, which suggests that intron–exon targeting may be more desirable if avoiding cryptic splice sites is important. However, Morpholinos including stretches of the acceptor polypyrimidine tract may be less specific than donor-site Morpholinos since this tract is constrained to two bases, thereby increasing the possibility of off-target effects due to lost specificity.

Achieving greater splice alteration with multiple oligos

The data described here indicate that targeting the splice-junctions on either side of an internal exon or the intronic snRNP binding site (branchpoint) just 5′ of the exon each yield deletion of the same targeted exon. Does simultaneous targeting of all three of these sites increase the level of transcript deleted for exon2 in this test system? Using a novel and non-toxic delivery reagent called Endo-Porter, the intron1 branchpoint oligo (i1BP) and the splice-junction oligos (i1e2 and e2i2) were delivered individually at 5 μM each and compared to a cocktail of all three oligos delivered together at 5 μM each (Fig. 3B). The individual oligos each generated the same exon2 deletion at roughly 50% conversion, whereas the triple cocktail resulted in a

90% conversion. Therefore, blocking multiple sites expected to generate the same outcome can alter splicing more effectively. The branchpoint adenine residue of an intron is usually found 15–30 bases from the intron–exon boundary. This leads to another advantage of targeting intron–exon boundaries to generate exon deletions: sometimes a single Morpholino oligo can span sequence from the branchpoint residue to the splice-junction, targeting two critical sites involved in splicing.

The pitfalls and critical observations

The results from blocking the splice-junctions in the 3-exon globin-luciferase fusion led to a simple model where targeting internal exon–intron or intron–exon boundaries results in exon deletion, and targeting the first exon–intron or last intron–exon boundary results in intron insertion. I applied the internal exon rules of this model for targeting an exon–intron boundary in Cathepsin B and an intron–exon boundary in P53 and achieved exon deletions consistent with results achieved in the 3-exon test system (data available upon request). Proper targeting requires both consideration of expected outcomes and selection of a target sequence optimal for a Morpholino oligo. Critical limitations include selecting primers and targeting an exon sized to optimize RT-PCR analysis.

One should also be aware that even after the most careful targeting and oligo design, a splicing experiment can yield unexpected outcomes, such as activation of a cryptic splice site. These unexpected outcomes are difficult to guard against, but it is important to know that they can occur and to be able to identify them and find alternatives. For example, cryptic splice sites are often present and are not used unless a more-favorable splice site (snRNP binding site) is mutated or blocked. Highly conserved but different genes (such as pseudoalleles in tetraploid *Xenopus laevis*) may have similar splice sites, but a splice-blocking oligo may reveal differences that make one splicing event more favorable than another. Activation of a cryptic splice site illustrates how a lesser snRNP binding site that may not normally be utilized can end up dictating splicing.

And finally, there are ESEs, ISSs, and possibly other unidentified factors which may interfere with Morpholino targeting. However, the target mRNA sequences for these elements are also prime candidates for targeting with Morpholinos to block their activity [7,8].

Conclusion

We have focused on the use of Morpholino oligos as tools to alter splicing events and have shown that with appropriate sequence analysis and by targeting internal exons, the likely outcome is an exon deletion. The targeting methods described in this paper have been used to design oligos for hundreds of successful splice-blocking experiments in tissue culture and in developmental model systems including zebrafish and frog embryos. A large percentage of splice-blocking results have yielded complete exon deletions allowing the researcher to focus on specific phenomenon associated with complete loss of an exon, whether loss of function or expression of secondary transcripts.

Experimental procedures

Materials. HeLa cells stably transfected with pLUC/705 [14], a plasmid comprising the firefly luciferase gene interrupted with human β-globin intron2 containing a favored splice site mutation (IVS2-705) and referred to as the positive test system, were from Dr. Ryszard Kole. Morpholino oligos were synthesized by GENE TOOLS, LLC (http://www.gene-tools.com). The sequences of the Morpholino oligos targeting the β-globin test system were derived from pLUC/705 sequences as follows: e1i1, 5′-ATAGACTCACCTGAAGGGATCGTAA; i1-1, 5′-CCCTGATTTGGTCAATATGTGTACA; i1-2, 5′ AAGTATATTAAAAGAAGAAAGCATT; i1-3, 5′-GGGAAAGTATTAGAAATAAGATAAAC; i1BP, 5′-GAGATTAGGGAAAGTATTAGAAATA; i1e2, 5′-ATTGCCCTGAAAGAAAGAGATTAGG; e2i2, 5′-CCTCTTACCTCAGTTACAATTTATA; i2BP, 5′-AGGAAGATAAGAGGTATGAACATGA. The single-stranded DNA primers used for RT-PCR analysis of the β-globin test system were purchased from Operon and had the following sequences: e1-1263, CCATCACGGTTTTGGAATG; e3R-2448, 5′-CCCTCGGGTGTAATCAGAAT.

Reagents. Tissue culture cells were grown in D-MEM/F12 (Invitrogen catalog 11330-032) supplemented with 10% fetal bovine serum (Invitrogen catalog 16000-044). Cells were trypsinized with 0.25% trypsin–EDTA (Sigma catalog T-4049). Reduced serum media for transfection was purchased from Invitrogen (catalog 31985-070). Dulbecco's phosphate-buffered saline (PBS) was purchased from Invitrogen (catalog 14040-133). The firefly luciferase assay system (catalog E1500) and passive cell lysis buffer (catalog E1941) were purchased from Promega. The protein assay reagent was purchased from Bio-Rad (catalog 500-0006). The Access RT-PCR kits were purchased from Promega (catalog A1250). Perfect RNA, Eukaryotic Mini prep kits were purchased from Eppendorf AG (catalog 0032 006.108).

Oligo delivery. Morpholino oligos were delivered by scrape-delivery [21], EPEI delivery [22], or using Endo-Porter delivery reagent [23].

RT-PCR analysis and luciferase assays. RT-PCR analysis was carried out as described elsewhere [24]. Firefly luciferase assays were performed as a modification to the protocol supplied by the manufacturer. In a clean test tube, 10 μl of cell lysate was mixed with 40 μl of Promega Luciferase Assay Reagent (catalog E1483) at ambient temperature and vortexed for 5 s. Light emission was immediately measured for 15 s in a Model TD-20e luminometer (Turner Designs, Inc.).

Following assessment of light emission, protein in the lysate was quantitated by adding 10 μl of cell lysate to 790 μl sterile water, and 200 μl protein assay reagent, vortexing for 1 min, and then reading the optical absorbance in a spectrophotometer at 595 nm. Luciferase activity was calculated by dividing the measured light units by optical absorbance measured in the protein assay.

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