Efficient in Vivo Manipulation of Alternative Pre-mRNA Splicing Events Using Antisense Morpholinos in Mice*

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Mammalian pre-mRNA alternative splicing mechanisms are typically studied using artificial minigenes in cultured cells, conditions that may not accurately reflect the physiological context of either the pre-mRNA or the splicing machinery. Here, we describe a strategy to investigate splicing of normal endogenous full-length pre-mRNAs under physiological conditions in live mice. This approach employs antisense vivo-morpholinos (vMOs) to mask cis-regulatory sequences or to disrupt splicing factor expression, allowing functional evaluation of splicing regulation in vivo. We applied this strategy to gain mechanistic insight into alternative splicing events involving exons 2 and 16 (E2 and E16) that control the structure and function of cytoskeletal protein 4.1R. In several mouse tissues, inclusion of E16 was substantially inhibited by interfering with a splicing enhancer mechanism using a target protector morpholino that blocked Fox2-dependent splicing enhancers in intron 16 or a splice-blocking morpholino that disrupted Fox2 expression directly. For E2, alternative 3' splice site choice is coordinated with upstream promoter use across a long 5'-intron such that E1A splices almost exclusively to the distal acceptor (E2dis). vMOs were used to test the in vivo relevance of a deep intron element previously proposed to determine use of E2dis via a two-step intrasPLICing model. Two independent vMOs designed against this intronic regulatory element inhibited intrasPLICing, robustly switching E1A splicing to the proximal acceptor (E2prox). This finding strongly supports the in vivo physiological relevance of intrasPLICing. vMOs represent a powerful tool for alternative splicing studies in vivo and may facilitate exploration of alternative splicing networks in vivo.

Alternative splicing enables individual genes to encode multiple proteins via the differential inclusion of exons during processing of pre-mRNA into translatable mRNA. As such, it is a major mechanism for regulating gene expression in metazoan organisms. An important subset of alternative splicing events in vertebrates is regulated in a developmental and differentiation stage-specific manner. Tissue-specific alternative splicing programs in conjunction with transcriptional programs give rise to cell type- and differentiation stage-specific proteomes that are necessary for normal function (1–7). Conversely, disruptions in these pathways underlie diseases such as myotonic dystrophy, paraneoplastic opsoclonus-myoclonus ataxia, and others (reviewed in Ref. 8). In addition to these systemic splicing diseases, many mutations interfere with splicing events in individual genes. It has been estimated that in some genes half of all disease-causing mutations act via disruption of splicing (9).

Mechanistic understanding of splicing regulation requires characterization of the cis-regulatory RNA sequences and trans-acting RNA-binding/splicing factor proteins that together mediate splicing decisions. These processes are typically analyzed in the context of minigenes transfected into cultured cells, conditions that facilitate systematic evaluation of splicing efficiency as a function of changes in sequence of candidate regulatory motifs or expression levels of candidate splicing regulatory proteins. Although much has been learned using this strategy, methods for in vivo evaluation of regulatory motifs or splicing factors are needed. Homologous recombination-based gene targeting strategies in mouse models are possible (e.g. Refs. 10 and 11), but this approach is expensive and cumbersome. A faster and more economical means of screening candidate regulatory elements would be invaluable.

Antisense oligonucleotides complementary to exon/intron boundary sequences can block access of the spliceosomal machinery and induce skipping of the associated exon. Alternatively, blocking negative regulatory elements can enhance exon inclusion (12–15). The splice-switching property of such oligonucleotides has been used to alter splicing for therapeutic purposes in cell line models of human disease (reviewed in Ref. 16). In a few cases, therapeutic switches in splicing have been achieved in the corresponding mouse models of β-thalassemia (17), muscular dystrophy (18–20), and spinal muscular atrophy (14, 21, 22).

Here, we report that antisense oligonucleotides in the form of vivo-morpholinos (vMOs)2 (23), represent a powerful and versatile tool for investigating normal alternative splicing regulation in a physiological context in live mice. Traditional morpholinos have been used with great success to study early zebrafish development. We used vMOs as a model system to manipulate two alternative splicing events in the complex gene encoding cytoskeletal protein 4.1R. One of these regu-

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‡ The abbreviations used are: vMO, vivo-morpholino; E, exon; MO, morpholino.
lates the protein’s affinity for spectrin and actin via inclusion/exclusion of alternative exon 16. The other regulates use of alternative 3′-splice sites in exon 2 that flank translation initiation site AUG1, thus controlling expression of an N-terminal headpiece that regulates affinity of 4.1R for its binding partners in the overlying membrane. Our results show that in vivo splicing events can be modulated substantially by vMOs directed against cis-regulatory elements at splice sites, branch points, and intron splicing enhancer elements. Splicing of target exons can also be altered by use of morpholinos that disrupt expression of splicing regulatory proteins such as Fox2. Importantly, vMOs provided unequivocal evidence for functionality of deep intron elements required for the nested intrasplicing events that activate the distal 3′-splice site in exon 2.

**EXPERIMENTAL PROCEDURES**

**Vivo-morpholino Experiments**—Vivo-morpholinos were purchased from Gene Tools, LLC (Philomath, OR) and maintained in sterile saline solution according to the manufacturer. vMO reagents are modified oligonucleotides synthesized with six-membered morpholino rings and nonionic phosphorodiamidates instead of five-membered ribose rings and anionic phosphates, but they retain the ability to base pair efficiently with complementary cellular RNA sequences. Vivo-morpholinos are further modified to contain a covalently linked delivery moiety composed of an arginine-rich octaguanidine dendrimer that facilitates uptake across cell membranes in the absence of exogenous transfection reagents. This latter feature allows vMOs, introduced via tail vein injection, to robustly alter splicing of transcripts in selected mouse tissues, including kidney, liver, and intestine. Other tissues (skeletal muscle, heart, lung, and spleen) are less efficiently targeted, and the technique does not work well in brain tissue. For each injection, 25 nmol/20-g mouse was delivered using a syringe with a 26-gauge needle. All mice were CD2F1 from Charles River Laboratories International, Inc. (Wilmington, MA), but other mouse strains presumably will respond equally well to morpholino treatment. Our Institutional Animal Care and Use Committee of the Lawrence Berkeley National Laboratory approved all protocols. Sequences of vivo-morpholinos were as follows: splice blocker for 4.1R exon (E) 16, agcttggaggctctccagctcg-3′; Fox-binding site blocker for 4.1R exon 16, tctctacctgattgtgctgagctc-3′; and Fox2 forward primer, 5′-tgcattgcttgctggaacg-3′. Primers used to assess splicing of Fox2 exon 5 were as follows: forward primer, 5′-ggagacaagagacagagagctc-3′; and reverse primer, 5′-gcctggcctattgctgct-3′.

**RESULTS**

**Antisense Vivo-morpholino Can Perturb Alternative Splicing Events in Vivo by Blocking Splice Sites, Splicing Enhancer Motifs, or Splicing Factor Expression**—We initially studied splicing of protein 4.1R alternative E16. The splicing efficiency of this exon is highly regulated in various mammalian cell types (24, 25) and has been shown to be activated in a stage-specific manner during erythroid differentiation. Mechanistic studies with minigenes in transfected cells have identified positive and negative regulatory motifs in E16 and in flanking introns, suggesting multifactorial regulation of alternative splicing (26–30). However, it has not previously been practical to test the relevance of these findings in a truly physiological setting.

Splice-blocking morpholinos (MOs) in zebrafish typically alter splicing patterns so as to induce exon skipping or activation of a nearby cryptic splice site. Recently, a modification of the MO technology has enabled induction of exon skipping in mice (20, 23). As a first step in exploring E16 splicing regulation in vivo, we asked whether a 5′-splice site–blocking vivo-morpholino could alter the partial E16 inclusion observed in normal kidney and liver in live mice (Fig. 1, upper left panel) to induce predominant E16 skipping (upper right panel). Semiquantitative RT-PCR indicated that endogenous E16 splicing efficiency was relatively lower in kidney and higher in liver (Fig. 1, lower panels, lanes 1 and 5). Treatment with control vMOs against an irrelevant gene (lanes 2 and 6) or against a different region of the 4.1R pre-mRNA (lanes 4 and 8) did not induce E16 skipping. However, splicing efficiency was significantly reduced in both kidney and liver by an E16-specific splice-blocking vMO that was complementary to the last five nucleotides of the exon and the first 20 nucleotides of the intron (lanes 3 and 7). In other experiments, we also found efficient and reliable alteration of splicing in intestine and partial effects in skeletal muscle and heart. However, tail vein injections do not efficiently target the brain or skin (23).

We next asked whether vivo-morpholinos can alter E16 splicing less directly by interfering with components of the splicing machinery that act at a distance to affect the decision to include or exclude this exon. Minigene studies showed previously that E16 is positively regulated by the Fox2 splicing...
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FIGURE 1. In vivo exon skipping induced by a splice-blocking vivo-morpholino. Upper panels, diagram showing the normal splicing pattern in which 4.1R E16 is partially included in mouse kidney and liver (left panel) and the predominant exon skipping that occurs in mice treated with splice-blocking vivo-morpholino against the 5'-splice site of E16 (right panel). E14 and E15 are alternatively spliced in other tissues. Lower panel, RT-PCR analysis of E16 splicing patterns in mouse tissues treated with the following reagents: normal saline (lanes 1 and 5), control vMO against human globin (lanes 2 and 6), vMO against the E16 5'-splice site (lanes 3 and 7), and control vMO against a different region of 4.1R mRNA (intra-E1B 5'-splice site) (lanes 4 and 8). E16 splicing was quantitated by densitometry and is reported as percent inclusion (incl). All results were reproducible in at least two independent mice for each morpholino. Lane 9 represents a negative PCR control.

factor by binding to three UGCAUG splicing enhancer motifs in the downstream intron (28, 29). In theory, antisense vivo-morpholinos should be able to antagonize this enhancer effect either by disrupting expression of the Fox2 splicing factor or by blocking access of the Fox2 protein to its binding sites (Fig. 2A). The strategy for disrupting Fox2 expression, using a splice-blocking vMO directed against E5, is diagrammed in Fig. 2B. E5 encodes part of the RNA recognition motif (RRM) required for RNA binding, and it is included in normal kidney and liver. Induction of skipping of this 93-nucleotide exon would yield a dominant-negative protein without splicing enhancer activity (31).

Experimental conditions that produced optimal E5 skipping are shown in Fig. 2C. Dose-dependent inhibition of E5 skipping was explored by tail vein injections of a nonspecific vivo-morpholino control (Fig. 2C, lanes 1) or the E5 splice-blocking vivo-morpholino at various doses (lanes 2–5). In both kidney and liver, E5 was predominantly included in control mice, whereas E5 skipping increased as a function of dose from 1.75 mg/kg up to 13.5 mg/kg (the latter being close to the manufacturer’s recommendation). Higher doses were not well tolerated by the mice because of toxicity of unknown etiology (data not shown). In parallel with the dose dependence study, a time course analysis determined that E5 skipping was induced efficiently 24 h after a single injection of 13.5 mg/ml (Fig. 2D, lanes 1 and 4); similar results were obtained using two daily injections with testing at 48 h (lanes 2 and 5). For comparison, the predominant inclusion of E5 in normal tissue is shown in lanes 3 and 6. For subsequent analysis of effects on Fox2-regulated splicing events, however, we utilized the double-injection protocol to maximize effects at the protein level.

To test the functional consequences of Fox2 splicing factor disruption, we assayed 4.1R E16 splicing efficiency in kidney and liver at 48 h after treatment with the highest dose of morpholino. RT-PCR analysis revealed substantial reduction in E16 splicing efficiency (Fig. 2E, lanes 3 and 7) relative to control tissues of mice injected with sterile saline (lanes 1 and 5) or an irrelevant vMO (lanes 2 and 6). Fig. 2E also shows that a second independent morpholino, designed to block two of the intronic Fox2-dependent enhancer elements, was able to reduce E16 inclusion substantially (lanes 4 and 8). In fact, blocking the Fox2-binding sites was almost as effective at inhibiting E16 inclusion as blocking the 5'-splice site directly (compare Figs. 1 and 2E). Together, these results provide in vivo evidence that Fox2 enhances splicing of E16 in the natural full-length pre-mRNA in a physiological context.

Antisense Vivo-morpholinos against Deep Intron Regulatory Elements Can Switch Downstream Splice Site Choice in Live Mice—An unusual coupling of alternative first exons with far downstream alternative 3'-splice site choice occurs during processing of the pre-mRNA for protein 4.1R (32–34). Whereas transcripts initiated at E1A ultimately splice exclusively to the second (distal) alternative 3'-splice site at E2 (Fig. 3A, splicing event 2), those initiated at E1C splice only to the first (proximal) E2 acceptor site (splicing event 3). On the basis of the splicing phenotype of short 4.1R minigenes, we proposed that E1A transcripts execute an ordered, two-step intrasplcing pathway to generate the mature mRNA (34). This mechanism involves successive processing of the intron at two pairs of nested splice sites such that the proximal E2 acceptor site is removed in splicing step 1, ensuring selection of the distal site in splicing step 2 (Fig. 3A, splicing events 1 and 2). However, this intrasplcing model was impossible to validate in the context of the natural gene, in part because of the large size of the intron separating E1A and E2 (~100 kb in humans).
Here, we applied the vivo-morpholino technology to rigorously test the intraexon requirement in a physiological context in the full-length natural gene. This intraexon, previously annotated as E1B, is ~6 kb downstream of E1A and 94 kb upstream of E2. Fig. 3A (upper right) depicts antisense vivo-morpholinos that were designed against the 5’ss of E1A splicing to the proximal E2 acceptor (upper band) in mouse kidney (Fig. 3B, left panel, lane 3). The induced splicing switch was also observed in liver, albeit somewhat less efficiently (Fig. 3B, right panel, lane 3). The branch point-blocking morpholino mediated a substantial switch in splicing in kidney and a modest switch in liver (lanes 4). Additional negative controls indicated that these switches in splicing were highly sequence-specific: the E16 5’ss-splice-blocking morpholino that inhibited E16 splicing (Fig. 1) did not alter E1A-E2 splicing (lanes 5), and morpholinos that altered E1A splicing in the 4.1R gene did not alter splicing E1A splicing in the paralogous 4.1B gene (data not shown).

According to the intrasplicing model, the morpholino-mediated switch in E1A splicing from the physiological site at E2dis to the inappropriate site at E2prox is caused by inhibition of intraexon function, which decreases the efficiency of nested splicing events 1 and 2 and activates cryptic splicing event 4 (Fig. 3A). This model predicts that a reduced amount

FIGURE 2. In vivo regulation of alternative E16 splicing by morpholinos that disrupt Fox2-mediated splicing enhancer activity. A, strategy for disrupting Fox2-enhanced E16 splicing. Fox2 binding at three intronic UGCAUG motifs comprising the intron splicing enhancer (ISE) promotes E16 inclusion (incl) in normal mouse kidney and liver (left) and E5 skipping in tissues from mice treated with the indicated splice-blocking vivo-morpholino (right). The lower panel indicates the predicted Fox2 protein generated from E5-skipped RNA, lacking a critical part of the RNA-binding motif. Fox2DN, dominant-negative Fox2. C, dose-dependent splicing changes induced in Fox2 RNA isolated from these tissues. Lanes 1, analysis in tissues treated with control morpholino at 13.5 mg/kg; lanes 2–5, Fox2 splicing analysis in tissues treated with splice-blocking vivo-morpholino at 1.75, 3.5, 7.0, and 13.5 mg/kg, respectively. D, time course of splicing changes induced in Fox2 transcripts. Lanes 1 and 4, Fox2 splicing analysis at 24 h after a single injection of vivo-morpholino at 13.5 mg/kg; lanes 2 and 5, analysis at 48 h after two injections of control morpholino. E, inhibition of 4.1R E16 splicing by morpholinos that disrupt Fox2 expression or block the Fox2-binding sites in intron 16. RT-PCR analysis shows E16 splicing behavior in mice treated with the following reagents: normal saline (lanes 1 and 5), control vMO (lanes 2 and 6), Fox2 splice-blocking vMO as in B (lanes 3 and 7), and intron-splicing enhancer-blocking vMO (lanes 4 and 8).
of splicing intermediate would be generated in morpholino-responsive tissues. To test this prediction, we assayed the intermediate product using a forward primer in the intron upstream of the intraexon and a reverse primer in E2 (modified from Ref. 34). As an internal control to normalize the intermediate product to total 4.1R mRNA, we amplified a downstream region of the mRNA between exons 10 and 12. Fig. 3D (lower panel) shows that the intermediate was more abundant, relative to the mRNA control, in kidney and liver treated with a nonspecific MO (lanes 1 and 3) than it was in tissues treated with the intraexon 5′-splice site-blocking MO (lanes 2 and 4).

Thus, two independent intraexon-directed morpholinos were able to switch splicing in E2 in a highly sequence-specific manner, operating from a distant position some 94 kb upstream of the regulated 3′-splice site. The virtually complete switching observed in kidney RNA, together with the reduced amount of RNA intermediate, supports an obligatory role for the intraexon in mediating E1A splicing to the distal acceptor in E2. This conclusion could not be fully justified by previous minigene studies.
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DISCUSSION

This study extends previous reports that antisense technologies can correct aberrant splicing in vivo by showing that vivo-morpholinos are also powerful and versatile tools with which to explore normal pre-mRNA splicing regulation in a natural physiological context. Antisense oligonucleotides with morpholino chemistry can base pair with complementary sequences in cellular pre-mRNA to block candidate cis-regulatory signals in endogenous full-length pre-mRNAs, allowing functional evaluation of these motifs during pre-mRNA processing in tissues of live mice. Among the motifs that could be blocked by morpholinos were splice sites, branch points, and intron splicing enhancers. The robust switching in splice site usage induced by masking these functional splicing motifs indicates that efficient delivery of vMO was achieved in kidney and liver by simple tail vein injection, consistent with earlier reports (20, 23). Importantly, several control experiments with gene- and exon-nonspecific MOs showed that splice-switching effects were sequence-specific. We propose that the vivo-morpholino technology can be used to target a wide variety of RNA functions, at least in tissues such as liver and kidney that are efficiently targeted by these antisense reagents (23).

Application of morpholino technology to the study of protein 4.1R pre-mRNA processing provided important mechanistic information regarding the regulation of two functionally important alternative splicing events. The first example concerned control of alternative E16 splicing. E16 encodes a high-affinity spectrin-actin-binding domain that is important for red cell membrane mechanical stability (36). Previous biochemical studies suggested that E16 inclusion is enhanced by Fox2 (29, 30), a sequence-specific RNA-binding protein hypothesized to be a major regulator of tissue-specific enhancer activity in a mammalian system (37). Here, the splice-switching capability of antisense morpholinos against intronexon 1B suggests that it indeed plays an obligatory role in E1A-E2 splicing across a very long intron in the natural full-length 4.1R transcript in mouse kidney. The lower splice-switching efficiency observed in liver is likely due to reduced vMO delivery, although the possibility of tissue-specific differences in regulatory protein concentration cannot be formally ruled out. We speculate that distal splicing regulatory elements may be fairly common and that morpholinos will be very useful for their functional validation in endogenous RNAs expressed either in cell lines or in animal models.

These experiments extend previous reports in which antisense oligonucleotide technologies were used for therapeutic applications to correct splicing aberrations in animal models of human disease, including Duchenne muscular dystrophy (18–20, 43), spinal muscular atrophy (21, 22), and β-thalassemia (17). Although most of these studies employed splice-blocking oligonucleotides to induce exon skipping, there is also precedence for improvement in exon inclusion by blocking a splicing inhibitory element in the adjacent intron (13). The ease and efficacy of vivo-morpholinos reported here promise to greatly expand the utility of this technology for in vivo validation of a wider array of RNA regulatory signals.

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