Reprogramming Alternative Pre-messenger RNA Splicing through the Use of Protein-binding Antisense Oligonucleotides*

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Alternative pre-messenger RNA splicing is a major contributor to proteomic diversity in higher eukaryotes and represents a key step in the control of protein function in a large variety of biological systems. As a means of artificially altering splice site choice, we have investigated the impact of positioning proteins in the vicinity of 5′ splice sites. We find that a recombinant GST-MS2 protein interferes with 5′ splice site use, most efficiently when it binds upstream of that site. To broaden the use of proteins as steric inhibitors of splicing, we have tested the activity of antisense oligonucleotides carrying binding sites for the heterogeneous nuclear ribonucleoprotein A1/A2 proteins. In a HeLa cell extract, tailed oligonucleotides complementary to exonic sequences elicit strong shifts in 5′ splice site selection. In four different human cell lines, an interfering oligonucleotide carrying A1/A2 binding sites also shifted the alternative splicing of the Bcl-x pre-mRNA more efficiently than oligonucleotides acting through duplex formation only. The use of protein-binding oligonucleotides that interfere with U1 small nuclear ribonucleoprotein binding therefore represents a novel and powerful approach to control splice site selection in cells.

The alternative splicing of pre-messenger RNA is now recognized as a most important generator of protein diversity in mammals. A recent estimate suggests that more than half of the human genes may be alternatively spliced (1). Given that some pre-mRNAs are spliced to yield dozens and potentially thousands of isoforms, the tight control of alternative splicing events is undoubtedly of the highest importance for many cellular and physiological functions. In addition, many human genetic diseases are caused by mutations that affect splice site utilization. Specific exonic or intronic sequence elements that conform to consensus sequences, an oligonucleotide complementary to any given splice site can potentially affect the use of many other splice sites, especially because an anti-splice site oligonucleotide with mismatches still displays weak activity (24). In addition, an anti-splice site oligonucleotide is less effective against a strong splice site possibly because it must compete with factors that normally bind to splicing signals (e.g. U1 snRNP)† (25). An alternative strategy to minimize these problems is to use oligonucleotides complementary to unique regions that are close to the target splice site. Indeed, an oligonucleotide that hybridizes to a region 20 nt upstream of the Bcl-xL 5′ splice site has been used with success (26), although the mechanism of inhibition has not been addressed. In the present report, we describe a novel strategy that combines the specificity of oligonucleotide-based approaches with the interfering capacity of proteins or complexes bound near splice sites. Antisense oligonucleotides carrying an extension bound by hnRNP A1/A2 proteins are active on different pre-mRNAs including β-globin, tau, dystrophin, and Bcl-x (reviewed in Ref. 23). Whereas notable successes have been reported with this approach, it is important to realize that because splice sites conform to consensus sequences, an oligonucleotide complementary to any given splice site can potentially affect the use of many other splice sites, especially because an anti-splice site oligonucleotide with mismatches still displays weak activity (24). In addition, an anti-splice site oligonucleotide is less effective against a strong splice site possibly because it must compete with factors that normally bind to splicing signals (e.g. U1 snRNP)† (25). An alternative strategy to minimize these problems is to use oligonucleotides complementary to unique regions that are close to the target splice site. Indeed, an oligonucleotide that hybridizes to a region 20 nt upstream of the Bcl-xl 5′ splice site has been used with success (26), although the mechanism of inhibition has not been addressed. In the present report, we describe a novel strategy that combines the specificity of oligonucleotide-based approaches with the interfering capacity of proteins or complexes bound near splice sites. Antisense oligonucleotides carrying an extension bound by hnRNP A1/A2 proteins are active on different model pre-mRNAs in vitro and in vivo. This approach could be useful in many systems where alternative or aberrant splicing events need to be manipulated.

MATERIALS AND METHODS

Cells and Oligonucleotide Transfection Procedures—HCT 116 cells were cultured in McCoy’s medium, HeLa S3 and EcrR 293 cells in

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1 The abbreviations used are: snRNP, small nuclear ribonucleoprotein; GST, glutathione S-transferase; RT, reverse transcription; nt, nucleotide(s); hnRNP, heterogeneous nuclear ribonucleoprotein; siRNA, small interfering RNA.
Dulbecco's modified Eagle's medium, PC-3 cells in Ham's medium/nutrient mixture F-12, and MCF-7 cells in α-modified Eagle's medium supplemented with 10% fetal bovine serum. All media were supplemented with 10% fetal bovine serum. Twenty-four hours prior to oligonucleotide treatment, cells were seeded in 6-well plates (35 mm) at a density of 6.5 × 10^4 cells/well for HeLa S3 and HCT 116 cells and 1 × 10^5 cells/well for PC-3 and MCF-7 cells. Eighteen hours prior to plasmid transfections, 5 × 10^4 EcR 293 cells were seeded in 60-mm plates.

Oligonucleotides and siRNAs used in splicing experiments were synthesized by ThermoFisher. For oligonucleotide treatments, cells were treated with Oligofectamine (Invitrogen) according to the manufacturer's instructions at the concentrations indicated. siRNAs against A1/A2 were transfected using the same procedure at a final concentration of 80 nM.

**Plasmids**—The pC5′−/− plasmid was described previously (25). Plasmids derived from pC5′−/− and containing MS2 binding sites were produced using meganuclease by overlap extension to obtain blunt restriction sites at positions −46, −37, −26, −17, +15, +23, and +31 relative to the 5′ splice site of exon 7B. MS2 binding sites were inserted in the sense and antisense orientations by inserting reannealed oligonucleotides bearing optimized and mutated MS2 binding sites. For the optimized MS2 binding sites, oligonucleotides MS2-I (TTTGCAGCCT-GACCATCAATACATAGGGTA) and MS2-IA (GTGACCTTGTG-TGTACAGGCGCGTACGGCTA) were inserted at positions −46, −37, −26, and −17 and oligonucleotides MS2-D (CTGACACATCAGG-TACGCAGGTATCTT) and MS2-DA (AATGCGAGCAGCG-TACCTGATGTACGG) were inserted at +15, +23, and +31. For the plasmid pC5′-M26SΔ, oligonucleotides MS2-Δ (TTTGCAGCCTG-GACCATCAATACATAGGGTA) and MS2-IA (GTGACCTTGTG-TGTACAGGCGCGTACGGCTA) were inserted at −26, pDUP5.1-MS2 and pDUP5.1-1SP were constructed from the stepwise assembly of various restriction fragments. pDUP4.1 and pDUP5.1 were described previously (29). The BamHI-SacI fragment of pDUP1.4 was inserted into the BamHI and SacI sites of pBluescript II KS+

**Reprogramming Alternative Pre-messenger RNA Splicing**—To allow DNA-liposome complexes to form, 1 mg/ml benzamidine) in the presence of 0.3 mg/ml lysozyme and 1% Triton X-100. Proteins were eluted from the columns with buffer B (200 mM piperazine-HCl, pH 9.8, 0.5 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). Nuclease-free water was used in the presence of 0.5 mg/ml lysine and 1% triton X-100. Purified proteins were dialyzed against buffer D (20 mM piperazine-HCl, pH 7.9, 150 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 20% glycerol). The concentration of the recombinant proteins was measured by Bradford (Bio-Rad) using serial dilutions of bovine serum albumin as a standard.

**Transcription and in Vitro Splicing Assays**—Plasmid substrates were produced from plasmids linearized with Scal and transcribed with T3 RNA polymerase (U. S. Biochemical Corp.) in the presence of cap analog and [α-32P]UTP (PerkinElmer Life Sciences). RNA was gel purified as described (32). HeLa nuclear extracts were prepared (33) and used in splicing reactions as described previously (34) for a final volume of 15 µl. creatine kinase was added to HeLa nuclear extracts at a final concentration of 1 unit/15 µl.

**Cell Culture**—HeLa extracts depleted of U1 snRNPs were produced by adding RNase H (Amersham Biosciences) and oligonucleotide TCAAGTTAAATGAT that is complementary to the 5′ end of U1 RNA (35). A mock-treated extract was obtained by incubation with RNase H in the absence of oligonucleotide. RNase H and 75 µmol of oligonucleotide complementary to the 5′ splice site of exon 7B (CTGATAC- CTCAG) were added to 12 µl of treated extract, 1 unit of creatine kinase, in the absence or presence of 150 µl of GST-MS2 or 10 µmol of oligonucleotides C5-M4 and C5-M41W after incubation for the specified times at 30 °C. Incubation was continued for 15 min at 30 °C.

**RNA Isolation and RT-PCR**—Total RNA was prepared with TRIzol (Invitrogen) and treated with DNase I following the manufacturer's protocols. Reverse transcription was performed with random hexamers and Superscript II RT (Invitrogen) and was carried out at 42 °C for 60 min and stopped at 95 °C for 5 min. These reactions were followed by PCR in the presence of α-32P]UTP (PerkinElmer Life Sciences) using the following procedure: 96 °C for 5 min; 35 cycles at 93 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min and a final extension at 72 °C for 10 min. Products related to the 5′ splice site were amplified using primers C5-M26SΔ (TCATTTCCGACTGAAGAGTGA) and Bclx-3 (ATGGCAGCAG-TAAA-GATAGCTGGTTATTG) and inserted in StuI sites of pCI-MS2-NLS. GST-StuI4 (GTTATAGGCCTATGTCCCCTATACC) and transferase of pGEX (Amersham Biosciences) was amplified by PCR in the presence of [α-32P]UTP (PerkinElmer Life Sciences) and Superscript II RT (Invitrogen) and was carried out at 42 °C for 60 min and stopped at 95 °C for 5 min. These reactions were followed by PCR in the presence of α-32P]UTP (PerkinElmer Life Sciences) using the following procedure: 96 °C for 5 min; 35 cycles at 93 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. Products related to the 5′ splice site were amplified using primers C5-M26SΔ (TCATTTCCGACTGAAGAGTGA) and Bclx-3 (ATGGCAGCAG-TAAA-GATAGCTGGTTATTG) and inserted in StuI sites of pCI-MS2-NLS.

**RESULTS**

The Binding of a GST-MS2 Protein Near a 5′ Splice Site Alters Splice Site Selection—As an alternative approach to current methods developed to alter splice site selection, we first tested the effect of targeting the binding of the protein in the vicinity of a 5′ splice site with the goal of interfering with its use through steric hindrance. Although one natural case of this type of splicing control exists (see Introduction), we wished to ascertain whether obstruction could be achieved by unrelated proteins, and if so, assess some of the parameters associated with such an effect. We used a pre-mRNA that contains two competing 5′ splice sites derived from the hnrNP A1 gene (C5′−/−; Fig. 1A), and tested the effect of targeting the binding of a recombinant form of the bacteriophage MS2 coat protein close to the proximal 5′ splice site. A high affinity MS2 binding site was inserted at various positions upstream or downstream of the proximal 5′ splice junction (−46, −37, −26, −17, +15, +23, and +31). The resulting pre-mRNAs were incubated in a HeLa extract in the presence or absence of the GST-MS2 protein. As seen in Fig. 1B, promoting the binding of GST-MS2 26 nt upstream of the proximal 5′ splice site decreased splicing at that site and stimulated the use of the distal 5′ splice site (compare lane 4 with lane 3). GST-MS2 did not affect 5′ splice site utilization when the MS2 binding site was substituted for its complementary sequence (Fig. 1B, compare lane 2 with lane 1), or when the MS2 binding site contained a point mutation that reduces binding by 3000-fold (36) (compare lane 6 with lane 5). A compilation of the effect on splicing with the MS2 binding site inserted at different positions is shown in Fig. 1C.
The effect on splice site selection was concentration-dependent (data not shown). The largest effect was observed when the MS2 binding site was placed 26 and 37 nt upstream of the 5′ splice junction. In contrast, the insertion of the MS2 binding site at similar distances downstream from the 5′ splice junction had little or no effect.

To determine whether the interference detected in vitro could also be obtained in vivo, we used the β-globin DUP5.1 reporter plasmid (29, 37). The internal exon 2 in DUP5.1 is preferentially skipped because of its small size, and its inclusion level did not change upon co-expression of GST-MS2 (Fig. 2B, lanes 1 and 2). Insertion of the MS2 binding site or a spacer element 26 nt upstream of the 5′ splice junction leads to almost complete inclusion of the central exon (lanes 3 and 5), most likely because of the increase in the size of the central exon. Co-transfection with the GST-MS2 expression plasmid promoted a decrease in the relative frequency of exon 2 inclusion only when DUP5.1 contained the MS2 binding site (compare lane 4 with lane 6). This result suggests that the binding of GST-MS2 upstream of a 5′ splice site can interfere with the use of this site in vivo.

A Protein-binding Oligonucleotide Can Switch 5′ Splice Site Selection in Vitro—Next, we asked whether positioning a protein near a 5′ splice site through the use of an hybridizing oligonucleotide could also affect 5′ splice site selection. For this purpose, we designed a series of antisense oligonucleotides complementary to nucleotides −4 to −23 relative to the proximal 5′ splice site on the C5′−/− pre-mRNA (Fig. 3A). The hybridizing portion of each oligonucleotide was made of 2′-O-methyl nucleotides. The C5-M4A1 oligonucleotide also contains at its 5′ end a 16-nt long non-hybridizing DNA extension carrying one high affinity binding site for the hnRNP A1/A2 proteins (TAGGGA). The C5-M4A1W oligonucleotide contains at the same position an RNA sequence for high affinity hnRNP A1 binding (38). A mutated version of this oligonucleotide harboring two GGG to CGC mutations was used as a control (C5-M4A1M). Oligonucleotides carrying a non-related 16-nt long tail (C5-M4CT) or lacking a tail (C5-M4) were also used as controls. All oligonucleotides were tested for A1 binding by using the UP1 protein, a shortened derivative of hnRNP A1/A2 proteins (TAGGGA). The C5-M4A1W oligonucleotide also contains a high affinity binding site for the MS2 bacteriophage coat protein at various positions upstream or downstream of the proximal 5′ splice site (−46, −37, −26, −17, +15, +23, and +31). We also tested a derivative containing the complementary sequence of the MS2 binding sites inserted at −26 (C5-M26AS), and a derivative containing a mutated version of the MS2 binding site (C5-M26SS). B, labeled pre-mRNAs were incubated in a HeLa nuclear extract for 2 h at 30 °C in the presence or absence of 100 pmol of GST-MS2 protein. The extracted RNA was fractionated on a 11% acrylamide denaturing gel. The position of the pre-mRNAs, splicing intermediates, and products is indicated. The distal 5′ exon and mRNA have run out of the gel because of their small sizes. C, histogram depicting the effect of positioning the GST-MS2 protein near the proximal donor site on 5′ splice site selection. For each position, the relative shifting ability was calculated by comparing the ratio of distal/proximal splicing products (lariats) in the presence of 100 pmol of GST-MS2 with the same ratio obtained in the absence of GST-MS2. In the absence of MS2 binding site, this value is 1.
A

DUP5.1

1
2
3

1
2
3

A

lanes 12

A

lanes 11

A

Combined a strong reduction in proximal 5’ shift was observed with the C5-M4A1W oligonucleotide that
tide carrying the A1 binding tail with the activity of an oligo-
terfering capability of the bound oligonucleotide.

The bottom panel, inclusion/exclusion ratio for the
central exon 2 is depicted in a histogram drawn on a logarithmic scale. The data were obtained from an experiment performed in triplic-
ate with the indicated samples (samples 1 to 6 are as in the top panel). The error bars represent standard deviations.

shift was observed with the C5-M4A1W oligonucleotide that combined a strong reduction in proximal 5’ splice site use with an important increase in distal 5’ splice site use (Fig. 1, C, lanes 11–13, and D). In this case, the amplitude of shift obtained was more than 25-fold at the highest concentration of the C5-M4A1W oligonucleotide. These results indicate that a 5’ tail carrying A1/A2 binding sites adds considerably to the interfering capability of the bound oligonucleotide.

We also compared the activity of the interfering oligonucleo-

tide carrying the A1 binding tail with the activity of an oligo-
nucleotide directly complementary to the proximal 5’ splice site (C5-5) (see Fig. 3A). Surprisingly, C5-M4A1W was more active than C5-5 at shifting splicing (Fig. 3E, compare lanes 2–6 with lanes 12–16). We also tested the effect of positioning A1 further upstream by using an oligonucleotide that hybridizes 26 to 45 nt upstream of the 5’ splice site of exon 7B (C5-M26A1; see Fig. 3A). Compared with an oligonucleotide that only forms a duplex with this sequence (C5-M26), the protein-bound C5-M26A1 oligonucleotide was slightly more effective than C5-M26 at repressing the proximal 5’ splice site and did not significantly stimulate distal 5’ splice site usage (Fig. 3F, compare lanes 2–4 with lanes 5–7). In contrast, the presence of the A1-binding tail on oligonucleotide C5-M4A1 promoted a more efficient shift toward the distal site when compared with the effect of the C5-M4 oligonucleotide (Fig. 3F, compare lanes 8–10 with lanes 11–13). Thus, more interference is obtained when the A1-binding tail is closer to the target splice site.

Modulation of Endogenous Bcl-x Alternative Splicing by an A1-binding Oligonucleotide in Vivo—The Bcl-x pre-mRNA was used on several occasions as a target for splice site modulation by duplex-forming oligonucleotides (e.g., Refs. 26 and 39–41). Two types of oligonucleotides have been tested: one oligonucleotide is partially complementary to the proximal 5’ splice site of Bcl-xL (positions +2 to −16 relative to the 5’ splice junction), whereas the other oligonucleotide is complementary to positions 16 to 35 nt upstream of the same 5’ splice site. Each oligonucleotide affects Bcl-x splicing such that the relative abundance of the mRNA isoforms shifts in favor of the production of Bcl-xS (41). To determine the modulating efficiency of protein-binding oligonucleotides in vivo, we used a series of 2’-O-methyl oligonucleotides (Fig. 4A). X-5 is complementary to the 5’ splice site of Bcl-xL (+7 to −13); X-M4 is complementary to the −4 to −23 region, upstream of the Bcl-xL splice site. The other two oligonucleotides contain the same complementary region and carry a 5’ tail with two high affinity binding sites for hnRNP A1 or a mutated version thereof (X-M4A1W and X-M4A1M, respectively). The A1 binding ability of these 2’-O-methyl oligonucleotides was confirmed by gel shift assays (Fig. 4B). The best U1P binder was X4-M4A1W (lanes 7–9), whereas no binding was detected using X-M4A1M, X-M4, and X-5. Transfection of the individual oligonucleotide was carried out in triplicate and at different concentrations in the prostate carcinoma cell line PC-3, the colon carcinoma cell line HCT 116, and the breast carcinoma cell line MCF-7 using control transfections with an unrelated oligonucleotide (C-RNA) or with no oligonucleotide (mock). Twenty-four hours post-transfection, RNA was extracted and analyzed by RT-PCR to monitor changes in the relative abundance of the endogenous Bcl-xL and Bcl-xS mRNAs. Compared with the control, we observed that the X-5 oligonucleotide had little activity at the concentrations tested in PC3, HCT 116, and MCF-7 cells (Fig. 4, C, lane 2; D, lane 3; and E, lane 3, respectively). The duplex-

forming X-M4 oligonucleotide displayed moderate activity in all cell lines (Fig. 4, C, lane 3; D, lane 4; and E, lane 4). The X-M4A1W oligonucleotide elicted the strongest shift toward the production of Bcl-xS in all three cell lines (Fig. 4, C, lane 4; D, lane 5; and E, lane 5). As expected, the X-M4A1M oligonucleotide was less efficient (Fig. 4, C, lane 5; D, lane 6; E, lane 6), thus supporting the conclusion that A1/A2 binding is important for the activity of the interfering oligonucleotide. The residual activity may reflect low affinity binding by A1/A2 or may in-
dicate that a 5’ tail can display intrinsic interfering activity in vivo. Although the X-M4A1W oligonucleotide was similarly active in PC-3 and HCT 116 cells, the amplitude of the switch was less important in MCF-7 cells. Given that the transfection efficiencies were similar for all cell lines (typically greater than 90%), cell-specific differences in the level of oligonucleotide intake or oligonucleotide stability may account for the noted differences in splicing switch. Alternatively or in addition, different levels of Bcl-x pre-mRNA and hnRNP A/B proteins may
contribute to cell line-specific differences.

To confirm the role of hnRNP A1/A2 proteins in the activity of the X-M4A1W oligonucleotide, we performed an RNA interference experiment using siRNAs against hnRNP A1/A2 to reduce the concentration of A1/A2 in HeLa S3 cells. siRNAs and interfering RNA oligonucleotides were co-transfected and total RNA was extracted 24 h later. Parallel transfections were continued for 96 h at which time proteins were extracted and analyzed by Western analysis. The level of A1/A2 proteins was reduced to represent less than 25% of the level observed in mock-treated cells (Fig. 5A). RT-PCR analysis of the endogenous Bcl-x expression levels indicated that the X-M4A1W oligonucleotide shifted splicing toward Bcl-xS production in HeLa S3 cells (Fig. 5, compare lane 5 with lane 1). The duplex-forming X-M4 oligonucleotide had little activity (lane 3). Notably, the activity of the X-M4A1W oligonucleotide was impaired when HeLa cells had been transfected simultaneously with siRNAs against A1/A2 (Fig. 5, lane 6), indicating that hnRNP A1/A2 proteins are important for the in vivo activity of the X-M4A1W oligonucleotide.

**Mechanism of Inhibition of GST-MS2 and Interfering Oligonucleotides**—We showed that positioning a protein in the vicinity of a 5' splice site, either directly or through the use of an antisense oligonucleotide, reduced splicing at this site and increased splicing at the other 5' splice site. This effect is presumed to be caused by an interference with splice site recognition or with spliceosome assembly. To confirm the mechanism of action, we performed an oligonucleotide-mediated RNase H cleavage assay (34). In this assay, a DNA oligonucleotide complementary to the targeted 5' splice site is added to a splicing mixture along with RNase H that degrades the RNA portion of the RNA/DNA duplex. Protection at time 0 is indicative of U1 snRNP binding, whereas a protection observed following incubation at 30 °C suggests that U1 snRNP-dependent splicing complexes have assembled onto the 5’ splice site. We performed a protection assay on the C5-M26S pre-mRNA using a DNA oligonucleotide complementary to the 5’ splice site of exon 7B. In the absence of GST-MS2 protein, protection was observed at time 0 and increased upon incubation at 30 °C (Fig. 6A, lanes 1–3). The bulk of this protection was U1 snRNP-dependent because protection strongly decreased when the assay was performed in an extract in which the 5’ end of U1 snRNA had been degraded (lanes 7–9). The addition of GST-MS2 protein decreased protection at time 0 and at later time points (Fig. 6A, compare lanes 4–6 with lanes 1–3), suggesting that GST-MS2 interfered with U1 snRNP binding and the assembly of U1 snRNP-dependent complexes. The effect of interfering oligonucleotides was also tested on the C5’ 5’–/– pre-mRNA (Fig. 6B). We noted that the duplex-forming C5-M4 oligonucleotide had little impact on the protection observed at time 0, but reduced the protection obtained following incubation at 30 °C (Fig. 6B, lanes 4–6), indicating that the oligonucleotide interferes predominantly with the assembly of U1 snRNP-dependent complexes. In contrast, the C5-M4A1W oligonucleotide almost completely eliminated early and late protections (Fig. 6B, lanes 7–9), consistent with the view that this oligonucleotide is more efficient at preventing the initial binding of U1 snRNP.

**DISCUSSION**

We have shown that the binding of a recombinant GST-MS2 protein in the vicinity of a 5' splice site decreases splicing at that site and improves the use of a competing splice site. Splicing interference by GST-MS2 is position-dependent because binding 17 to 46 nt upstream of the targeted 5' splice site affects splicing, whereas essentially no effect is observed when binding occurs 15 to 31 nt downstream of the splice junction. The requirement for unobstructed exonic space may be explained by invoking the results of RNase protection assays that have mapped regions on pre-mRNAs occupied by factors during spliceosome assembly (35, 42, 43). These studies indicated that as much as 19 nt upstream of a 5' splice site can be protected from nuclease digestion during spliceosome assembly. The binding of GST-MS2 in that region may therefore thwart the stable interaction of splicing factors or obstruct spliceosome assembly. The results of an RNase H protection assay showed that placing a GST-MS2 binding site 26 nt upstream of a 5' splice site reduces U1 snRNP binding and compromises the assembly of U1 snRNA-dependent splicing complexes. It is intriguing that the binding of GST-MS2 at position −17 was less inhibitory than the binding at more upstream positions (−26 and −37). The reason for this result is unclear but may be caused by differences in the binding affinity of GST-MS2 created by the local structure surrounding the MS2 binding site. The GST-MS2 protein can therefore recapitulate the activity of known factors that bind upstream of a 5' splice site to obstruct its use (4, 44). Because the spliceosome occupies a similar space downstream from the splice junction (42, 43), it is unclear why the binding of GST-MS2 at equivalent positions downstream from the 5' splice site has no effect on splice site selection. The asymmetric impact of protein binding near a 5' splice site may reflect intrinsic differences in the ability of the spliceosome to deal with structural impediments in exonic versus intronic flanking sequences.

To explore the applicability of protein-mediated steric interference as a tool to control splice site selection, we tested the activity of antisense oligonucleotides that contain protein-binding sites as part of a non-hybridizing 5' tail. We have shown that such oligonucleotides complementary to the exonic sequence directly upstream of the target 5' splice site can efficiently alter 5' splice site selection. Shifting 5' splice site use in vitro worked best when the oligonucleotide contained two binding sites for the hnRNP A1/A2 proteins. RNase H protection assays indicate that such an oligonucleotide decreased U1 snRNP binding to the 5' splice site even though the antisense portion is not complementary to sequences that pair with U1 snRNA. Thus, a protein-RNA complex emerging from the duplex portion 4 nt upstream of the 5' splice junction apparently constitutes a strong encumbrance to U1 snRNP binding. In contrast, a simple duplex-forming oligonucleotide complementary to the same position was less active and mostly prevented later steps of spliceosome assembly. Preventing early U1 snRNP binding can therefore be envisioned to favor splicing to the other 5' splice site because commitment complex formation can now occur more frequently at the competing site. Surprisingly, at similar concentrations, protein-binding antisense oligonucleotides were even more effective than an oligonucleotide directly complementary to the 5' splice site sequence. Apart from structural considerations, this result could be explained if the oligonucleotide complementary to the 5' splice site displays some affinity to other 5' splice sites found in endogenous pre-mRNAs, thereby reducing its effective concentration for the intended target.

In four different human cell lines, an antisense oligonucleotide carrying binding sites for A1/A2 was also the most active at shifting splicing of the endogenous Bcl-x pre-mRNA. This oligonucleotide was more active than a duplex-forming oligonucleotide against sequences upstream of the 5' splice site, or than an oligonucleotide directly complementary to the 5' splice site itself. Thus, oligonucleotides complementary to exonic sequences may improve their specificity of action, whereas maximal interference on splice site recognition is conferred by the hnRNP A1/A2-binding tail. In addition, if a fraction of the exonic oligonucleotide remains in the cytoplasm it may also
Fig. 3. *In vitro* splicing interference mediated by antisense oligonucleotides and protein-binding oligonucleotides. A, the position of the antisense oligonucleotides on the C5-5/− pre-mRNA is depicted. The hybridizing portion of each oligonucleotide is 2-O-methyl. Oligonucleotide C5-5 is complementary to the 5′ splice site of exon 7B, whereas the C5-M4 series are complementary to sequence directly upstream.
inhibit mRNA translation, a situation that should maximize the impact of the oligonucleotide on the production of the intended protein isoform.

Antisense oligonucleotides with a tail bound by hnRNP A1/A2 proteins bestowed strong splicing interference in vitro and in vivo. Our recommendation for maximal interference and stability in vitro and in vivo would be to use a 2′-O-methyl oligonucleotide containing two binding sites for hnRNP A1/A2. Because these proteins are abundant in growing cells (estimated A1 concentration of 0.2 mM in the nucleus of HeLa cells), providing an A1/A2-binding oligonucleotide at a concentration of 100 nM must have a negligible impact on normal A1/A2-mediated events. We have also tested the activity of a poly(U) tail that can be bound by many types of proteins including...

Fig. 4. Splicing interference mediated by the protein-binding antisense oligonucleotide in vivo. A, splicing map of the Bcl-x pre-mRNA depicting the alternative splicing events that lead to Bcl-xL and Bcl-xS mRNA production. The position and sequence of the 2′-O-methyl oligonucleotides used in vivo is indicated. B, native gel analysis of UP1 binding to oligonucleotides. The TS10 DNA oligonucleotide (60 nt) contains nine A1 binding sites. Each labeled oligonucleotide was incubated with increasing amounts of the shortened version of recombinant hnRNP A1 (GST-UP1) at concentrations of 1 and 2.5 μM. Complexes were fractionated in an 8% acrylamide gel. The position of the free oligonucleotide and the complexes is shown. In panels C, D, and E, PC-3, HCT 116, and MCF-7 cells were transfected with increasing amounts of oligonucleotides (25, 50, and 100 nM). C-RNA (AAUGUCUGCUACUGGAAG) was used as a negative control. Total RNA was extracted after 24 h and a RT-PCR assay in the presence of [32P]dCTP was performed to monitor the relative abundance of the Bcl-xS and Bcl-xL mRNA isoforms (the position of the primers used for amplification is shown in panel A). The ratios of these amplified products are depicted in each graph, and only the RT-PCR results on 4% acrylamide gels obtained at the 100 nM concentration are shown below each graph.

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Antisense oligonucleotides with a tail bound by hnRNP A1/A2 proteins bestowed strong splicing interference in vitro and in vivo. Our recommendation for maximal interference and stability in vitro and in vivo would be to use a 2′-O-methyl oligonucleotide containing two binding sites for hnRNP A1/A2. Because these proteins are abundant in growing cells (estimated A1 concentration of 0.2 mM in the nucleus of HeLa cells), providing an A1/A2-binding oligonucleotide at a concentration of 100 nM must have a negligible impact on normal A1/A2-mediated events. We have also tested the activity of a poly(U) tail that can be bound by many types of proteins including...
FIG. 5. Role of hnRNP A1/A2 in the activity of the protein-binding antisense oligonucleotides in HeLa cells. HeLa cells were mock treated or treated with 100 nM RNA oligonucleotides X-M4 and X-M4A1W. Another set of transfections was performed with the same oligonucleotides but was co-transfected with siRNAs molecules specific for human hnRNP A1 and hnRNP A2 (27). In panel A, a Western analysis was performed 96 h post-transfection with siRNAs against A1/A2. In panel B, total RNA was extracted after 24 h and a RT-PCR assay was carried out using Bcl-x-specific primers. A typical result is shown in the right panel. The ratios of the amplified products derived from Bcl-xL and Bcl-xS are depicted in the histogram.

FIG. 6. Monitoring U1 snRNP binding to the proximal 5′ splice site using an oligonucleotide-directed RNase H protection assay. A, the C5-M28S pre-mRNA used in Fig. 1 was incubated in a mock treated extract or an extract that had been depleted of U1 (U1A) by decapitation using a DNA oligonucleotide complementary to the 5′ end of U1 RNA and RNase H. Splicing mixtures were incubated for the indicated times (in min) in the absence or presence of GST-MS2 (150 pmol). A protection assay was then performed with a DNA oligonucleotide complementary to the 5′ splice site of exon 7B. B, the C5′−/− RNA was incubated for the indicated times (in min) with RNA oligonucleotides (C5-M4, C5-M4A1W, or no oligo) in a HeLa nuclear extract. Following incubation, an oligonucleotide complementary to the proximal 5′ splice site of exon 7B was added along with RNase H. Pre-mRNAs and cleavage products were fractionated on 5% denaturing acrylamide gels. The position of the fully protected pre-mRNA and molecules derived from the cleavage at the 5′ splice site of exon 7B are shown.
U2AF and PTB, as well as a tail containing a purine-rich sequence bound by SR proteins. None of these oligonucleotides elicited significant interference at concentrations that were active for the A1/A2-binding oligonucleotides (data not shown). However, we have observed that a 2′-O-methyl oligonucleotide with a tail containing a β-globin branchsite region, which is most likely bound by factors such as mBBP/SF1 or U2 snRNP, is at least as active as the A1/A2-binding oligonucleotide.2

Whether protein-binding antisense oligonucleotides will prove as effective to target 3′ splice sites remains to be tested. Positioning binding sites for GST-MS2 downstream from a 3′ splice junction had no impact on 3′ splice site use (data not shown). Steric interference at this position may not occur simply because the spliceosome does not establish strong interactions with sequences in the second exon (42). Targeting the hybridization in the intron near splicing signals should be interfering based on natural cases of interference caused by the binding of hnRNP A1 or ASF/SF2 near the branch site region (5, 11, 45).

One of the challenges emerging from the sequence of the human and mouse genomes is to assess the function of a large repertoire of proteins. Because the majority of pre-mRNAs are alternatively spliced to produce many different isoforms, approaches that will inhibit specific splicing events or that will shift selection toward specific isoforms will be very useful in deciphering protein function. Moreover, these approaches may find applications in the treatment of human genetic diseases caused by mutations that affect splice site utilization (46). Recently, bifunctional RNA oligonucleotides and peptide nucleic acid derivatives have been used to elicit splice site activation in the survival of motor neurons pre-mRNA (47, 48). The interfering method described here expands the choice of strategies that can be used to influence alternative splicing, and is unique in that it combines the obstructing capacity of proteins with the exquisite specificity of the antisense approach.

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