Surveying Cis-Acting Sequences of Pre-mRNA by Adding Antisense 2'-O-Methyl Oligoribonucleotides to a Splicing Reaction

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We chemically synthesized antisense 12 mer 2'-O-methylribonucleotides and surveyed a scanning (signal-tracking) process as well as sequences within a β-globin transcript acting in the splicing reaction in vitro. The pre-mRNA transcript contained the sequences of the first exon, first intron, and a major part of the second exon of the human β-globin gene. We found that the antisense 2'-O-methylribonucleotides could anneal effectively to the target site in the pre-mRNA during the splicing reaction. A 2'-O-methylribonucleotide complementary to the donor (5') splice site completely inhibited authentic splicing and activated an upstream cryptic donor site. A 2'-O-methylribonucleotide complementary to the branch site inhibited normal branch formation and greatly reduced subsequent generation of the spliced product. Six other 2'-O-methylribonucleotides complementary to loci in the exons or the intronic region between the donor and branch sites had no significant effect on the splicing reaction. These observations suggest that an extensive scanning of the present pre-mRNA across the six regions tested is not essential for the splicing reaction. We propose that a short antisense 2'-O-methylribonucleotide provides a practical and convenient method to examine cis-acting sequences of RNA. The advantages of this method in comparison with site-directed mutagenesis or deletion are discussed.

The sequences around the donor (5') and acceptor (3') splice sites and those around the branch site of a pre-mRNA are known to be essential or at least important for splicing of the pre-mRNA (1-3). These sequence elements also serve as signals for selecting a correct splice site (4). However, they are not sufficient to account for the specificity of splice site selection, especially in the selection of a correct donor-acceptor pair (2-4). Some other determinant(s) not well defined probably serve to achieve correct splice site selection. Several potential determinants or mechanisms have been proposed such as scanning of pre-mRNA (5, 6), higher order structure of pre-mRNA (7-10), exon sequence or sequence context of a splice site (11, 12), and proximity of the donor and acceptor sites (11). Although there is evidence against a simple 5' to 3' or 3' to 5' scanning mechanism (7, 11, 13, 14), such a mechanism has not been conclusively ruled out (2-4). For example, processive movement of a splicing component from a 5' or 3' terminus of pre-mRNA might be required for splicing. Local scanning of pre-mRNA within an intron or exon may also be possible.

We therefore planned to examine a possible scanning process in pre-mRNA splicing by observing the effect of duplex formation in various, specific regions of a pre-mRNA. We postulated that a duplex region within a pre-mRNA may be an obstacle to a scanning process across that region. A convenient method to form a partial duplex is to add an antisense or complementary oligodeoxyribonucleotide (DNA) to a splicing reaction in vitro. However, the pre-mRNA was cleaved at the RNA-DNA hybrid region by RNase H activity contained in the HeLa cell nuclear extract (15; Itoh et al., unpublished results; see also Fig. 3). Although the RNase H does not cleave RNA duplex (16), it was necessary to use a much longer antisense RNA to achieve effective inhibition (40 nucleotides (nt) or longer; 17). Such a long RNA will not be suitable for pinpointing a region required for local scanning. Another factor is that RNA is generally unstable in the presence of a crude nuclear extract. We previously reported that 2'-O-methylribonucleotide (2'-O-Me RNA) formed a stable duplex with RNA (18) and it was resistant to RNase H activity (19). It has also been shown by other researchers that 2'-O-Me RNA is fully resistant to various RNase and DNase activities (20). Therefore, we have used antisense 2'-O-Me RNA instead of DNA or RNA to induce site-specific inhibition of pre-mRNA splicing. Examination of possible splicing inhibition with a partial duplex formation serves not only to facilitate observation of the scanning process in a DNA to RNA to induce site-specific inhibition of pre-mRNA splicing. Examination of possible splicing inhibition with a partial duplex formation serves not only to facilitate observation of the scanning process but also to make feasible a survey of unknown signals acting in the splicing reaction. To examine these signals systematically by site-directed mutagenesis would be a most difficult task.

We have used the transcript from a truncated human β-globin gene (21) as a substrate of in vitro splicing, since the sequence requirement for both the splice sites and
branch site have been well defined by naturally occurring and induced mutations (21-25). Thus we made use of antisense 2'-O-Me RNA specific to these sites to confirm the validity of the method. Most recently, successful application of antisense 2'-O-Me RNA to probing the structure and function of U2 (26) and U4/U6 (27) snRNPs has been reported. Their 2'-O-Me RNA contains 2'-O-Me inosine instead of 2'-O-Me guanosine. Guanosine is better for specific annealing than inosine, which can pair with various bases.

EXPERIMENTAL PROCEDURES

Synthesis of 2'-O-Me RNA and DNA—Each 12 mer 2'-O-Me RNA, R1 to R11 (Fig. 1), was synthesized on an automated DNA synthesizer (Applied BioSystems model 380A) using the phosphoramidite method and was purified by HPLC as described (18, 28). Twelve mer DNA (D1 to D11) with the sequences corresponding to R1 to R11, respectively, were also synthesized by a conventional use of the automated DNA synthesizer and purified by HPLC.

RNA Transcription and In Vitro Splicing—The human β-globin gene carried in plasmid pSP64-H β 6 for in vitro transcription was described previously (21). A pSP64-H β 6 derivative plasmid carrying an additional donor site sequence from SV40 small-t donor within the first intron has also been described (29). The plasmids were linearized by BamHI and used as the template for in vitro transcription, using SP6 RNA polymerase (Takara Shuzo) with a GpppG cap (Pharmacia) as a primer (29).

A HeLa cell nuclear extract was prepared as described (30), with minor modifications. The standard in vitro splicing reaction (25 μl) was carried out as described (21) except for the volume of the nuclear extract (10 μl), the deletion of RNasin (placental RNase inhibitor), and the addition of 20 mM HEPES-KOH (pH 7.3). This pH adjustment significantly increased the splicing activity (unpublished data). The radioactivity of the pre-mRNA added was equivalent to that of 20 fmol (0.8 nM) of the full size pre-mRNA.

Fig. 2. In vitro splicing of β-globin pre-mRNA in the presence of an antisense 2'-O-Me RNA. Standard splicing reactions (see “MATERIALS AND METHODS”) with 4 μM (5,000-fold molar excess over the pre-mRNA) of each antisense 2'-O-Me RNA (R1-R11) or without any oligonucleotide (−) were carried out and the products were analyzed as described in “MATERIALS AND METHODS.” The structures of the splicing products are schematically represented on the right. ● indicates a spliced product generated by activation of cryptic 2 donor site (see Fig. 4). Bands of about 250 nt observed in most lanes represent a cleavage product consisting of the second exon and a part of the intron (36). M, HpaII-digested pBR322 DNA marker, whose sizes (nt) are indicated on the left.

![Fig. 1](image-url)
transcript. The actual amount of the full size transcript added was 4 to 7 fmol estimated by the method reported previously (29). An indicated amount (0.6-200 pmol or 0.024-8 μM) of antisense 2'-O-Me RNA was added directly to the splicing reaction together with the other reagents, without any preincubation for annealing. After incubation at 30°C for 4 h, the RNA was extracted with water-saturated phenol and was precipitated with ethanol. The splicing products were analyzed on a denaturing 5% PAGE followed by autoradiography for 2-4 h with an intensifying screen at -80°C. Quantitation of the splicing products was performed as described (29).

RESULTS AND DISCUSSION

Antisense 2'-O-Me RNA Can Effectively Anneal to the Pre-mRNA in the Splicing Reaction—We synthesized ten kinds of 12 mer 2'-O-Me RNA which are complementary to ten specific loci of the truncated human β-globin transcript (R1-R10 in Fig. 1). R4 and R7 are complementary to the donor site and the branch site, respectively. We also synthesized 2'-O-Me RNA R11, which has no significant complementarity to the transcript, as a control.

Figure 2 shows the results of in vitro splicing in the presence of 4 μM (5,000-fold molar excess over the pre-mRNA) of the 2'-O-Me RNA. By adding the donor site specific 2'-O-Me RNA (R4), inactivation of the normal donor site was induced and the shorter spliced product was generated via activation of an upstream cryptic donor site. The branch site specific 2'-O-Me RNA (R7) reduced the spliced product greatly. R8, which is complementary to a region downstream of the branch site, significantly inhibited splicing at 10,000- and 20,000-fold excess although inhibition was not clear in the experiment shown in Fig. 2 (5,000-fold excess). To inhibit splicing, it was not necessary to preincubate a 2'-O-Me RNA with the pre-mRNA. Pre-annealing followed by removal of excess 2'-O-Me RNA before the splicing reaction failed to inhibit the splicing. A more detailed analysis of the effects of R4, R7, and R8 will be presented in the later sections. Control 2'-O-Me RNA (R11) had no effect on the splicing (Fig. 2, compare with the lane of no addition). Similar results were obtained in several other experiments with 5,000- or 10,000-fold excess 2'-O-Me RNA, using three different nuclear extracts. It thus appeared that each antisense 2'-O-Me RNA can anneal site-specifically to pre-mRNA in the splicing reaction containing a HeLa cell nuclear extract. To confirm this assumption, targeted RNase H cleavage was examined (31) in the same splicing reaction except for using 12 mer DNA D1 to D11 carrying the sequences corresponding to R1 to R11, respectively. If each DNA can effectively anneal to the specific region of pre-mRNA, the RNA must be cleaved into two specific fragments by an endogenous RNase H in the nuclear extract (15). As shown in Fig. 3, the pre-mRNA was indeed cleaved almost completely in the presence of each of D1 to D10 at the expected region, and both 5' and 3' RNA fragments were generated. We have already reported that 9 mer RNA (carrying the donor site sequence)-2'-O-Me RNA duplex has much higher thermal stability than the corresponding RNA-DNA duplex (18). These results taken together indicate that the antisense 2'-O-Me RNAs (R1-R10) effectively anneal to the target regions in the pre-mRNA under the present conditions.

Fig. 3. Site specific cleavage of the precursor under the splicing condition with an antisense DNA and endogenous RNase H activity. The pre-mRNA was incubated under the same splicing condition as for Fig. 1 except for an antisense DNA (D1-D11) instead of a 2'-O-Me RNA and the incubation time (20 min). A longer incubation obscured the cleavage products because of degradation. 5' and 3' fragments of the expected sizes are indicated. Non-specific fragments observed with D1, D5, D6, and D7 are presumably due to partial annealing of an antisense DNA to another site with a low stringency. Two faint bands found in the lane without antisense DNA (−) and control DNA (D11) are lariat intermediate and excised first exon, respectively, generated by a splicing reaction.
explanation for the non-specific inhibition by R2 is that by an unknown contaminant in R2 which had not been removed by repeated purification with HPLC.

2′-O-Me RNA Complementary to the Donor Site Can Activate a Cryptic Donor Site—To characterize the nature of splicing inhibition by donor site specific 2′-O-Me RNA (R4), the concentration of R4 added was varied extensively (Fig. 4). The authentic spliced product was decreased depending on the logarithm of the R4 concentration (0.024-8 μM). This correlation between splicing inhibition and R4 concentration can readily be explained if R4 hybridizes to the pre-mRNA according to the Rot curve as described (35) and if the hybridized pre-mRNA is not spliced. Thus, the result may support the basic assumption that a pre-mRNA with a hybrid structure in an essential region is not spliced.

Simultaneously with the decrease of the authentic spliced product, a shorter spliced product was increased (Fig. 4). This spliced product is presumed to be generated by activation of cryptic 2 (Cr. 2) donor site located 16 nt upstream of the authentic site (see Fig. 1A) because of its expected size and the appearance of related splicing intermediates as described (36). When both the authentic and cryptic 2 donor sites were annealed by R4 and R3, both spliced products clearly disappeared and cryptic 1 (Cr. 1) donor site located 38 nt upstream of the authentic site was activated, albeit with a low efficiency (Fig. 4A). It has been shown by other investigators in vivo (22) and in vitro (21, 37) that β-thalassemia mutations of authentic donor site of the human β-globin gene resulted in the activation of three cryptic donor sites and that the most efficient one was cryptic 2 site. We can induce similar effects by masking the authentic donor site with short antisense T-0-Me RNA, although only cryptic 2 product was prominent here (Fig. 4A). The authentic splice site could be completely inhibited by a 4 μM (5,000-fold excess) or higher concentration of R4 (Figs. 2 and 4A). The requirement of a great excess of 2′-O-Me RNA for inhibition is presumably due to the RNA unwinding activity present in the HeLa cell nuclear extract (8, 14, 38), competition with binding by splicing factors, and the small size of the 2′-O-Me RNA used.

To determine whether the cryptic donor site is activated even in the presence of an alternative donor site, we also examined antisense inhibition using the pre-mRNA carrying two active donor sites. We had already constructed SP6/human β-globin derivative plasmids including various donor site sequences within the first intron to study the mechanism of donor site selection (29). In the present work, we used one of these plasmids, which includes the SV40 small-t donor sequence within the intron as an alternative donor site. The transcript of this plasmid shows efficient utilization of the inserted, alternative donor site as well as that of the authentic donor site under the standard in vitro splicing condition (29; Fig. 5A, 2nd lane). When we added the 2′-O-Me RNA R4, the cryptic 2 spliced product appeared (Fig. 5), as in the case of the original pre-mRNA carrying only the authentic donor site (Fig. 4). Even though the alternative donor site is active, the inhibition of the authentic donor site still activates the silent cryptic 2 donor site just upstream of the authentic site. The spliced product from the alternative donor also increases upon inactivation of the authentic donor site, but to a lesser extent (Fig. 5). This observation may imply that the alternative choice of a donor site takes place locally, or more efficiently at a closer donor site. This idea might be related to the mechanism(s) by which cryptic donor sites remain silent when the authentic donor site is intact (39).
Fig. 5. Effect of the donor site specific 2'-O-Me RNA (R4) of various concentrations on the splicing of the β-globin transcript carrying an alternative donor site within the intron. The structure of the transcript is schematically shown at the bottom. The sequence inserted as the alternative donor site (Alt.) is that of SV40 small-t donor (29). Other abbreviations are as shown in the legend to Fig. 1. (A) An autoradiogram showing the splicing products. Alt. indicates splicing products generated from the inserted alternative donor site. See Fig. 4 for other descriptions. (B) Quantitative estimation of the spliced products shown in panel A. Concentration of R4 was plotted on a logarithmic scale.

Observation might be related to our previous observation that optimum splicing conditions for the authentic and the alternative donor sites (separated by 67 nt) differed (29).

2'-O-Me RNA Complementary to the Branch Site Induces Significant Reduction of the Spliced Product—The effects of branch site specific 2'-O-Me RNA (R7) of various concentrations were also examined (Fig. 6A). The spliced product was progressively decreased with increasing concentrations of R7 added (0.024-8 μM). The generation of the spliced product was almost completely inhibited by 8 μM R7.

Two new products X and Y are found in Fig. 6A. The mobility of X did not change after a debranching reaction in the nuclear extract (40; data not shown), suggesting that X is a linear molecule. The size of X was estimated to be approximately 430 nt, and a significant complementarity with R7 was found around position 425 of the precursor (GGCCUGGCUC). Therefore, although a duplex between RNA and 2'-O-Me RNA is not generally cleaved with the HeLa cell nuclear extract, it is possible that X was produced by cleavage of the precursor at this presumed duplex around 425 by some enzymatic activity. This possibility is supported by the concomitant appearance of products of approximately 255 nt (just above the 250 nt bands in other lanes) that can be produced by cleavage of the precursor at the presumed duplex with R7 around the branch site.

Similarly, the mobility of the product Y did not change after a debranching reaction, suggesting that Y is basically a linear molecule (100-110 nt). Y appears at the expense of the spliced product, and most significantly at R7 concentrations 0.24 and 0.8 μM which are lower than those at which X appears (2.4 and 8 μM). Therefore, Y seems to be related to the splicing intermediate carrying the exon 2 and a branched structure (exon lariat). A possibility consistent with these observations is that Y was produced by cleavage of the exon lariat within the duplex with R7 around the branch site, and by debranching of the branched structure.

In the case of the first intron of the human β-globin transcripts we used here, a point mutation of the branching adenosine or deletion of surrounding sequences induced activation of a cryptic branch site downstream of the authentic branch site (separated by 13 nt in the case of a point mutation), and the splicing efficiency was significantly reduced in vitro (24). Since there was little spliced product at 8 μM R7, splicing via the cryptic branch site was not significant. In addition, we did not detect a significant change in the splicing products when both R7 (2.4 μM) and R8 were added to inhibit both branch sites (data not shown). Thus, we presume that the branch site specific 2'-O-Me RNA suppresses the utilization of the cryptic branch site as well as that of the authentic branch site. Annealing of antisense 2'-O-Me RNA to the specific region of a transcript could be an obstacle for a trans-acting factor or a processive enzyme even if a point mutation or deletion is not. In fact, the antisense 2'-O-Me RNAs (1-10 μM) significantly blocked reverse transcription of the same β-globin tran-
Implications of the Results on Pre-mRNA Scanning and Advantages of Antisense 2'-O-Me RNA for Surveying Cis-Acting Sequences of RNA—

Suppression of the cryptic branch site can be explained by the duplex-induced block to some processive (scanning) event or to the binding of factors, such as U2 small nuclear ribonucleoprotein particle (snRNP), to the neighboring cryptic branch site (10 nt downstream from the end of duplex). Conversely, addition of R8 inhibited normal splicing (via the authentic branch site), albeit at higher concentrations (Fig. 6B). Requirement of higher concentrations of R8 for inhibition might be related to somewhat weaker annealing of R8, which was suggested by the presence of uncleaved precursor only in the case of D8 (Fig. 3). Possibly the R8 specific region, i.e. between branch site and the acceptor site, is rapidly protected by some trans-acting factor(s).

On the other hand, it is interesting that the antisense oligonucleotide annealed to the authentic donor site allowed utilization of upstream cryptic 2 donor site (13 nt upstream from the end of duplex). This difference may be correlated with the difference in the size of the RNA region to be occupied with the relevant trans-acting factor(s). The protection experiments using the human β-globin transcripts and specific antibodies against snRNPs identified an about 10 nt binding region around a donor site for U1 snRNP and an about 40 nt binding region around a branch site for U2 snRNP (41, 42). Our idea described above is consistent with this result.

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