Differential nuclease sensitivity identifies tight contacts between yeast pre-mRNA and spliceosomes

Brian C. Rymond and Michael Rosbash
Department of Biology, Brandeis University, Waltham, MA 02254, USA
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The oligonucleotide-directed RNase H sensitivity of a yeast (Saccharomyces cerevisiae) pre-mRNA was determined in an in vitro splicing reaction. While most of the pre-mRNA was sensitive to cleavage, the regions of the 5′ splice site and TACTAAC box were found to be highly resistant. The biochemical requirements for protection against nuclease attack parallel those of both spliceosome formation and splicing. Most of the uncleaved pre-mRNA remaining after RNase H challenge was found associated with two forms of the yeast spliceosome. Differences in the RNase H sensitivity of pre-mRNA found in the two spliceosomes forms indicate an increased association of splicing factors with the 5′ splice site during spliceosome assembly.

Key words: yeast/splicing/spliceosome/RNA processing

Introduction

The removal of intervening sequences from eukaryotic primary transcripts represents a fundamental aspect of gene expression. In yeast, as in the higher eukaryotes, this event (pre-mRNA splicing) can be represented by two cleavage–ligation reactions: (i) the first step, 5′ junction cleavage and lariat formation (joining via a 2′–5′ phosphodiester bond the 5′ terminal G of the intron with an A residue located near the 3′ end of the intron) followed by, (ii) 3′ junction cleavage and exon ligation (for reviews see Green, 1986; Padgett et al., 1986).

Mutational analyses of several yeast genes have led to the identification of three intron sequence elements, the 5′ splice site consensus sequence GTAPyGT, the internal conserved sequence or TACTAAC box, and the 3′ splice site (containing the invariant terminal AG dinucleotide). Deletions and point mutations within each of these sequences have been identified which specifically inhibit the first step of splicing, the second step or both (Langford and Gallwitz, 1983; Pikielny et al., 1983; Langford et al., 1984; Parker and Guthrie, 1985; Newman et al., 1985; Jacquier et al., 1985; Rymond and Rosbash, 1985; Fouser and Friesen, 1986; Cellini et al., 1986; Vijaraghavan et al., 1986).

While the pre-mRNA sequence requirements for splicing of yeast genes have been well established, much less is known about the mechanics of the splicing event. Splicing-specific ribonucleoprotein complexes, or spliceosomes, have been identified through the use of cell-free extracts which support the splicing of exogenously added transcripts (Brody and Abelson, 1985; Pikielny and Rosbash, 1986). Glycerol gradient separation of the yeast spliceosome indicates that the spliceosome is a large structure, sedimenting at approximately 40S (Brody and Abelson, 1985). We have recently demonstrated that the assembly of the spliceosome occurs in a stepwise fashion, progressing through at least three states, or complexes, which can be resolved on native polyacrylamide/agarose composite gels (Pikielny et al., 1986). These complexes, termed I, II and III based upon their increasing electrophoretic mobilities, appear to progress through the ordered pathway III→I→II. Although little is known about the biochemical components of the yeast spliceosome, we have demonstrated the association of at least four snRNAs (1170, 215, 185 and 160 nt in length) with spliceosome complexes (Pikielny and Rosbash, 1986; Pikielny et al., 1986). The genes for each of these yeast snRNAs have been identified and, in at least one case (the 1170 species), shown to be required for cell viability (Ares, 1986).

In the present study we address the question of where on the pre-mRNA splicing-specific factors associate and how this pattern changes during spliceosome assembly. Based on an assay developed by Ruskin and Green (1985a), we have employed an RNase H oligonucleotide-directed nuclease sensitivity assay to

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Fig. 1. (A) Schematic representation of the oligonucleotide-directed RNase H sensitivity assay. The assumption is made that during the in vitro splicing reaction specific contacts are made between the pre-mRNA and the spliceosome components. These interactions, if stable, will shield the pre-mRNA from cleavage by preventing the hybridization of a DNA oligonucleotide complementary to this region of the pre-mRNA and/or the enzymatic activity of RNase H. (B) The positions of hybridization of six single-stranded DNA oligonucleotides relative to the three yeast consensus sequences are indicated beneath the yeast RPS1A gene.
probe the accessibility of the pre-mRNA during the splicing reaction. Our results demonstrate that while most of the pre-mRNA is sensitive to digestion, the regions of the 5' splice site and TACTAAC box are highly resistant. Protection against nuclease digestion at either of these sequence elements requires ATP and is sensitive to mutations at either the 5' splice site or the TACTAAC box. The majority of this 'protected' RNA is found associated with spliceosomal complexes. Interestingly, differences in the protection pattern between complexes II and III indicate an increased association of a factor or factors, with the 5' splice site during assembly and correlate with the addition of the 185 and 215 nt snRNAs to the spliceosome.

Results
An experimental outline of the oligonucleotide-directed RNase H sensitivity assay is illustrated in Figure 1A. In brief, this procedure involves the addition of pre-mRNA to an in vitro splicing reaction under standard conditions of incubation. Then, after a given period of time, pre-mRNA is challenged with the addition of a complementary single-stranded DNA oligonucleotide and RNase H. Pre-mRNA which associates with the oligonucleotide and is accessible to RNase H is cleaved at the site of hybridization. Pre-mRNA that is not accessible to the oligonucleotide and/or RNase H is protected from cleavage. One can define, or estimate, the degree of protection by comparing the amount of pre-mRNA present after oligonucleotide and RNase H challenge with that present in a mock reaction to which no oligonucleotide has been added.

The six oligonucleotides used in our investigation are listed in Figure 1B. Each is 20 nt in length and capable of hybridizing to a unique, non-overlapping portion of RP51A RNA in the SP6/RP51A fusion gene pSpRP51A (Pikielny and Rosbash, 1986). Three oligonucleotides (#1, #3 and #5) are complementary to the three pre-mRNA consensus sequences (the 5' splice site, TACTAAC box and 3' splice site, respectively); two (#2 and #4) are complementary to internal regions of the intron between these consensus sequences; and one (#6) is complementary to exon II. In all of the experiments described in this study, a very active endogenous yeast RNase H activity was used to cleave the RNA/DNA hybrids; addition of purified *Escherichia coli* RNase H did not alter the pattern or extent of cleavage (data not shown).

The results of a typical experiment are presented in Figure 2. After a 5 min incubation under standard splicing conditions pre-mRNA, intermediates (lariat-intermediate and exon I), and products (mRNA and intron) were evident (lane A). The structure of all these RNAs has been described in detail elsewhere (Pikielny and Rosbash, 1986). Incubation for an additional 5 min resulted in a decrease in the level of pre-mRNA and a concomitant increase in the products of the reaction (compare lanes A and B), indicating that splicing continued over this interval. If, after the initial 5 min incubation, one of the oligonucleotides listed in Figure 1B was added for the subsequent 5 min of incubation, the RNase H digestion (cleavage) products were visible (lanes 1–6). The sizes and intensities of the cleavage products varied depending on the individual oligonucleotide and whether they resulted from digestion of the pre-mRNA, the intermediates, or the products of the splicing reaction. In addition, the 3' portion of a cleaved RNA was often under-represented due, at least in part, to the presence of a strong 5'-3' exonuclease in this extract (Rymond and Rosbash, 1985).

It is evident from this experiment that, after 5 minutes of prein-
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Fig. 3. ATP dependence of RNase protection. The oligonucleotide challenge of splicing reactions was performed as described in Figure 2 in the presence (+ATP) or absence (−ATP) of exogenously added ATP. Lane A represents a 10 min incubation in the absence of added oligonucleotide; lanes 1, 3 and 2 are numbered according to the oligonucleotide added to the reaction.

Fig. 4. 5’ splice site and TACTAAC box requirements for protection. Protection assays were performed as described in Figure 2 with pre-mRNA prepared from wild-type (WT), 5’ splice site point mutant (5’II) or TACTAAC box deletion mutant (Δ3B) gene constructions. Construction Δ3B lacks the site of hybridization with oligonucleotide 3 and was therefore not probed by this oligonucleotide. Lane A represents a 10 min incubation in the absence of added oligonucleotide; lanes 1, 3 and 2 are numbered according to the oligonucleotide added to the reaction.

Requirements of protection

If the nuclease insensitivities of the 5’ splice site and TACTAAC box regions of the pre-mRNA are due to splicing-specific interactions then the biochemical requirements of protection should resemble the requirements for in vitro splicing. Two parameters, the dependence upon ATP and the sensitivity to cis-acting mutations, were tested.

In the absence of ATP, cell-free extracts do not support splicing (Figure 3 and Lin et al., 1985). Under such conditions oligonucleotides 1, 2, and 3 direct the cleavage of the pre-mRNA with essentially uniform efficiency, indicating that in the absence of ATP the 5’ splice site and TACTAAC box sequences are no

branched RNA structure, as phenol-extracted lariat intermediate and intron, when added back to extract, were readily cleaved with oligonucleotide 3 and RNase H (data not shown).

The fact that incubation with the intron oligonucleotides 2 and 4, the 3’ splice site oligonucleotide 5, and the exon II oligonucleotide 6 resulted in the near complete digestion of the pre-mRNA by RNase H suggests that a large portion of the pre-mRNA may not be tightly associated with splicing factors. Also, it is unlikely that the entire intron is buried within the spliceosome. Rather, it appears that there are a limited number of tight contact points, including the 5’ splice site and TACTAAC box, detectable by this assay.
longer differentially protected (Figure 3). ATP itself does not inhibit the oligonucleotide-mediated cleavage since the simultaneous addition of the pre-mRNA and oligonucleotide 1 or 3 to the extract in the presence of ATP (i.e., no preincubation) resulted in efficient cleavage of the pre-mRNA (data not shown).

Two mutant derivatives of pSPPrp51A each containing an intron mutation, were used to test the pre-mRNA sequence requirements for protection (Figure 4). 5'II pre-mRNA contains a single base change (G to A) at position 5 of the 5' splice site consensus sequence. This change substantially reduces splicing efficiency in vivo (Jacquier et al., 1985) and virtually abolishes splicing in vitro (Figure 4). Protection of the 5' splice site was much reduced as a result of this change, as only 5–10% of the pre-mRNA remained intact after challenge with a 5' splice site oligonucleotide (identical to oligo 1 except for a complementary nucleotide change at the position of the mutation). Somewhat unexpectedly, protection at the TACTAAC box was simultaneously lost, indicating that protection at the TACTAAC box requires the sequence information from a proper 5' splice site. Similarly, the TACTAAC box was necessary for protection at the 5' splice site as mutant pre-mRNA Δ3B [which contains a 29 nt deletion encompassing the TACTAAC box (Pikielny et al., 1983)] was well-cleaved at the 5' junction.

Thus, protection against oligonucleotide-mediated RNase H cleavage shares with splicing a requirement for ATP and the presence of both the 5' splice site and TACTAAC box sequences. Taken together, the data suggest that the 'protected' regions result from splicing-specific interactions between the pre-mRNA and components of the splicing apparatus.

Protection of splicosomal complexes

Previous results for this laboratory have shown that the orderly assembly of pre-mRNA into splicing complexes can be visualized by polyacrylamide gel electrophoresis (Pikielny and Rosbash, 1986; Pikielny et al., 1986). Indeed, the requirements for in vitro splicosome assembly parallel those for in vitro splicing and for 5' junction and TACTAAC box protection. These studies indicated that pre-mRNA is assembled into a complex, called complex III, which is subsequently converted into a complex called complex I, and finally into the mature spliceosome (which contains the intermediates of the splicing reaction), complex II. (The complexes are named in order of increasing electrophoretic mobility.) It seemed likely that these previously identified spliceosome complexes contribute substantially to this protected RNA pool. Therefore we employed the splicing complex–RNA polyacrylamide gel assay to probe the accessibility of the RNA within the splicesome complexes to oligonucleotide-directed cleavage.

To this end, pre-mRNA was incubated in extract under splicing conditions, challenged with oligonucleotide 1, 2 or 3 (essentially as described above), and the RNP complexes resolved on polyacrylamide gels (Figure 5). Conditions were established such that the major splicesome complexes, III (the initial form) and complex II (the final form) were most prevalent (lanes A and B). Complex I is present at very low levels under these conditions and is not well resolved on this gel. The addition of oligonucleotides 1 or 3 to the splicing reaction had no detectable effect upon the amount of either complex II or III, but did result in a decrease in signal of the uncomplexed RNA at the bottom of the gel, suggesting that this RNA was well cleaved. Incubation with oligonucleotide 2 resulted in a substantial loss in signal of both complex II and III, consistent with the results presented above. As a substantial amount of complex formation had already occurred by 5 minutes of incubation (when the oligonucleotide was added), the data suggest that the pre-mRNA within complexes III and II is sensitive to oligonucleotide 2.

While the loss of signal with oligonucleotide 2 clearly demonstrates a destabilization of the complex and likely cleavage of the RNA, the lack of an effect with oligonucleotides 1 and 3 does not, by itself, demonstrate lack of RNA cleavage. It is conceivable that RNA cleavage occurred but the complex was not destabilized. To test this possibility, RNA was extracted from the individual complexes (and the uncomplexed region of the gel) and compared with unfractionated RNA from the same incubation (Figure 6A).

In the absence of added oligonucleotide, complex III contains mostly pre-mRNA, complex II pre-mRNA plus the intermediates and products of the reaction, and the uncomplexed RNA mostly pre-mRNA, as previously described (Pikielny et al., 1986). The cleavage products resulting from incubation with oligonucleotides 1 and 3 were clearly enriched in the uncomplexed RNA, confirming that this RNA is sensitive to digestion. Complex II contained no RNase H digestion products and therefore is indeed an oligonucleotide RNase H-resistant complex, at both the 5' splice site and the TACTAAC box regions of the intron. Complex III contained no oligonucleotide 3-directed digestion products and therefore is also oligonucleotide RNase H-resistant at the TACTAAC box. However, the 3' half of the oligonucleotide 1-directed cleavage products is clearly visible in complex III. The 5' half of this cleavage event is not in the complex as little or none of this RNA can be seen, even in long overexposures of this gel (Figure 6B).

These data, taken together, demonstrate that the splicesome
complexes, as defined in this polyacrylamide gel assay, harbor most of the RNase H-resistant RNA. Although the TACTAAC box is cleavage-resistant in both complexes, complex III is partially sensitive to cleavage at the 5' splice site. Furthermore, since complex III represents the initial visible form of the spliceosome and complex II the final form, the assembly pathway appears to progress through a state in which the 5' splice site is partially sensitive to one of apparently complete protection.

Branchpoint binding domain

In addition to destabilizing complexes II and III, oligonucleotide 2 resulted in the generation of a new complex of somewhat greater electrophoretic mobility than complex III. This new complex, III*, presumably is due to a cleavage-derived breakdown product of complex III and/or complex II. A single species of pSPrp51A RNA ~400 nt in length is present in this complex (Figure 7A). Primer extension analysis of this RNA, using an oligonucleotide complementary to exon II sequences, demonstrated that this RNA terminates over a series of ~18 nt, approximately 3–21 nt upstream of the TACTAAC box (Figure 7B). This result is consistent with the notion that the ~400 nt RNA is generated by 5'–3' exonuclease digestion from the site of oligonucleotide directed cleavage to a region just upstream of the TACTAAC box. Further digestion is presumably prevented by the interference of factors associated with the TACTAAC box. While other interpretations are possible we favor this notion and are supported by the fact that, like all of the assembly-mediated events in this and our previous reports, the generation of this 400 nt RNA is ATP dependent and sensitive to mutations at either the 5' splice site or TACTAAC box.
Discussion

Six oligonucleotides, complementary to unique segments of yeast (*Saccharomyces cerevisiae*) RP51A pre-mRNA, were used to probe the RNase H sensitivity of this transcript in an *in vitro* splicing reaction. Four of these, complementary to internal segments of the intron (oligonucleotides 2 and 4), the 3' splice site (oligonucleotide 5) and exon II (oligonucleotide 6), were able to direct the cleavage of the pre-mRNA (or pre-mRNA-derived molecules) to near completion. Oligonucleotides 2, 4 and 6 associate with non-essential regions of the yeast pre-mRNA which, therefore, would be unlikely to make tight, specific contacts with non-essential regions of the yeast pre-mRNA.

It was somewhat surprising that the 3' splice site, which clearly participates in the splicing reaction, was not protected against nuclease attack. Similar observations have been made in metazoan splicing extracts (Ruskin and Green, 1985a) where, unlike yeast, the 3' splice site participates in both the first and second steps of the splicing reaction (see below). If interactions occur between this region of the intron and the yeast spliceosome, they must be either transient or weak, associating such that RNase H cleavage is not inhibited. Recent biochemical fractionation of the yeast cell-free splicing extract has led to the identification of splicing factors unnecessary for lariat formation but required for exon ligation (Cheng and Abelson, 1986), characteristics which suggest an interaction with the 3' splice site. It remains to be determined, however, whether these factors act directly (by binding to the 3' splice site) rather than indirectly (e.g. by properly positioning exon I and the 3' splice site) to facilitate the second step of the splicing reaction.

Oligonucleotides 1 and 3 were complementary to the 5' splice site and TACTAAC box, respectively, and failed to direct the cleavage of significant quantities of pre-mRNA. Three lines of evidence indicate that this resistance to nuclease attack was due to the interaction with splicing-specific factors. First, the protected regions of RNA were limited to the two sequence elements required for spliceosome formation; oligonucleotides which hybridized to randomly selected regions of the intron, the 3' splice site, or exon II directed efficient cleavage. Second, spliceosome formation and RNase H protection have in common at least two requirements, a dependence upon ATP and the simultaneous participation of both the 5' splice site and the TACTAAC box. Third, and most convincingly, the majority of the protected pre-mRNA was found associated with spliceosome complexes.

Pre-mRNA from the two most prevalent complexes, III and II (which represent the initial and final forms, respectively, of the spliceosome as resolved by gel electrophoresis), were significantly more resistant to cleavage directed by oligonucleotides 1 and 3 than the unassembled pre-mRNA. Indeed, the overall level of protection was found to vary directly with the extent of complex formation (data not shown). Only pre-mRNA within complex III was cleaved to any measurable extent and then only with oligonucleotide 1. Furthermore, while the 3' half of this cleaved RNA was recovered from complex III, the 5' half was absent, presumably due to degradation or release. Based upon the predicted position of oligonucleotide 1 hybridization and the size distribution of the cleaved molecules (as assayed by direct RNA analysis without spliceosome analysis, ~50% of the cleaved RNA contained the entire exon I sequence. We conclude that the splicing factors which serve to hold exon I in complex II after 5' cleavage and lariat formation are not yet present or unable to function property in complex III, under conditions where the cleavage of exon I occurs prematurely and has been uncoupled from lariat formation.

The TACTAAC box of pre-mRNA in complex III, and the TACTAAC box of the pre-mRNA, lariat intermediate and excised intron of complex II were well protected against RNase H cleavage. This suggests that in yeast as in metazoan transcripts (Ruskin and Green, 1985a), splicing factors associate with this region at a very early stage of spliceosome assembly and remain bound (or are replaced by other factors) through the final step of the splicing reaction. This interpretation is supported by the fact that disruption of the spliceosome by RNase H cleavage between the 5' splice site and the TACTAAC box generates a degradation product, III*, containing an ~400 nt RNA whose 5' boundary maps just upstream of the TACTAAC box. The identification of this novel species of RNA explains the selective persistence of an ~400 nt RNA after oligonucleotide 2 treatment relative to treatment by oligonucleotides 4, 5 and 6. We believe that this RNA is distinct from the ~397 nt mRNA, as this RNA species can be produced by RNase H cleavage under conditions where little or no mRNA is present (Figure 6). Exonuclease digestion of metazoan pre-mRNA also terminates upstream of the branchpoint, adjacent to where U2 snRNPs are presumed to associate (Krainer et al., 1984; Ruskin and Green, 1985b; Black et al., 1985). We believe that the 5' ends of the 400 nt RNA define the 5' border of an interaction between extract factors and the TACTAAC box.

Comparison to metazoan protection experiments

The splicing of both yeast and metazoan transcripts appears to occur by fundamentally similar mechanisms (for reviews see Green, 1986; Padgett et al., 1986). The consensus sequences at the 5' splice site, branchpoint and 3' splice site are related between these two groups of organisms. Yet differences are apparent. The consensus sequences at the 5' splice site and branchpoint are much more stringent in yeast, representing a small subset of the allowable metazoan sequences (Teem et al., 1984). In addition, the metazoan 3' splice site, in particular the polypyrimidine stretch upstream of the 3' terminal AG, is essential for directing the position and efficiency of branchpoint formation (Ruskin and Green, 1985b; Reed and Maniatis, 1985; Fredewey and Keller, 1985). In yeast, intron sequences upstream of the 3' PYAG are not well conserved and may be deleted without greatly affecting the rate of lariat formation (Rymond and Rosbash, 1985; Cellini et al., 1986; and L.A. Fouser and J.D. Friesen, personal communication).

RNase H analysis of the human β-globin gene has likewise established ATP-dependent, splicing-specific interactions between the 5' splice site and branchpoint sequences, on the one hand, and the biochemical components of the extract, on the other (e.g. Ruskin and Green, 1985a). A strong interaction at the 5' splice site requires a 3' junction and branchpoint, a result not unlike those presented here for yeast splicing. In contrast to our results, however, a strong interaction at the branchpoint preceded protection at the 5' splice site and was independent of the 5' splice site sequences (Ruskin and Green, 1985a).

These and other more recent observations offer an attractive and relatively simple picture of pre-mRNA/snRNP interactions which can explain these metazoan protection studies. U2 snRNP develops a strong interaction with the branchpoint region, independent of the 5' junction. Subsequently or simultaneously, the 5' junction becomes nuclease resistant [through a strong association with U1 snRNP (Mount et al., 1983)], in a manner
which is dependent on the U2 snRNP-branchpoint interaction.

The results reported here and in our previous communication on yeast splicing suggest some interesting parallels with this metazoan scheme. The only snRNA identified in complex III is LSR1, the large yeast RNA with extensive U2 homology (Ares, 1986). Thus, protection of the TACTAAC box can be accomplished by this yeast U2 RNA in a manner precisely analogous to the metazoan scenario described above. Perhaps one of the additional snRNAs present in complex II (Pikielny et al., 1986) accounts for the subsequent protection of the 5' splice site in a U2-dependent manner, once again not unlike the metazoan description. What is surprising, however, is the fact that TACTAAC box protection (these studies) and complex III formation (Pikielny et al., 1986) are dependent on a proper 5' splice site, unlike branchpoint protection in mammalian extracts (Ruskin and Green, 1985a). While this difference might be due to a quantitative difference between systems, we feel this to be an unlikely explanation. Rather, and based on the fact that the RNase H assay is identical in this study to that of Ruskin and Green, we suggest that this difference is qualitative in nature and that a strong branchpoint (TACTAAC box):U2 interaction in yeast, in contrast to metazoan branchpoint:U2 interaction, requires recognition of the 5' junction, either by LSR1 itself or by some other as yet unidentified protein or RNA.

Materials and methods

Plasmids, RNAs and reverse transcription

The RPS1A containing plasmid pSPrp51A and its derivatives 5'II (Jacquier et al., 1985) and δ3B (Pikielny et al., 1983; Pikielny and Rosbash, 1986) were constructed by inserting the intron-containing HindIII–PvuII fragment of the wild-type or mutant DNA into plasmid pSP6A as previously described (Pikielny and Rosbash, 1986). In vitro transcriptions of these fusion genes, cleaved with EcoRI, were performed using SP6 polymerase (Promega) and [β32P]UTP (NEN) according to the manufacturer's specifications. This RNA was size-fractionated on a denaturing 5% polyacrylamide gel, to exclude incomplete transcripts, prior to in vitro splicing. The specific activity of the resulting 797 nt pre-mRNA transcript was ~4 × 10^6 c.p.m. per ng of RNA.

Reverse transcriptase (Life Sciences) and oligonucleotide RB1 (Teem and Rosbash, 1983) were used to prepare run-off transcripts from complex III RNA (Teem and Rosbash, 1983). The transcript sizes were determined by fractionation on a denaturing 8% polyacrylamide gel using a DNA sequence generated from the same primer and a single-stranded DNA template as molecular weight markers.

Splicing reactions, RNase H digestions, native gels

Yeast cell-free splicing reactions were prepared and splicing assays carried out as described previously (Lin et al., 1985). RNase H challenge of the pre-mRNA was performed by adding, 5 min after splicing was initiated, 1 nmol of complementary single-stranded DNA oligonucleotide (purchased from The DNA Synthesis Facility, University of Massachusetts Medical School, Worcester, MA) to each 10 μl splicing reaction. The oligonucleotide treated splicing reactions were incubated at 25°C for 5 min then terminated by adding an equal volume of 0.4 M KCl, 0.002 M MgOAc, 0.02 M EDTA, 0.05 M Hepes, pH 7.5. Portions of each sample were deproteinized for RNA analysis and the remainder prepared for spliceosome analysis on 3% polyacrylamide/0.5% agarose gels as previously described (Pikielny and Rosbash, 1986; Pikielny et al., 1986).

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