A novel role for the 3' region of introns in pre-mRNA splicing of *Saccharomyces cerevisiae*

Brian C. Rymond, Dana D. Torrey, and Michael Rosbash

Department of Biology, Brandeis University, Waltham, Massachusetts 02254 USA

To investigate the importance of sequences between the yeast (*Saccharomyces cerevisiae*) branch point (TACTAAC box) and 3' splice site (AG), we generated a series of pre-mRNA substrates that differed in the length of RNA retained on the 3' side of the TACTAAC box. These pre-mRNAs were compared as substrates for the first step of in vitro splicing (5' cleavage and lariat formation) and in vitro spliceosome assembly (complex formation) in a whole-cell yeast extract. The results indicate that for rp51A pre-mRNA at least 29 nucleotides of RNA on the 3' side of the TACTAAC box are required for 5' cleavage and lariat formation, as smaller substrates fail to manifest any detectable cleavage or ligation events. Analysis of splicing complex assembly indicates that these smaller substrates undergo efficient yet incomplete complex formation; they are blocked at a late stage of spliceosome assembly, the complex I to complex II transition (Pikielny et al. 1986), a result which suggests that the failure to form lariats is due to a specific assembly defect. The lariat formation block (and assembly defect) can be relieved by the addition of ribohomopolymer "tails" to the 3' end of the shortened rp51A pre-mRNAs, and similar results were obtained with shortened actin pre-mRNAs. The results of this study indicate that this region of the pre-mRNA serves a specific function late in in vitro spliceosome assembly.

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The removal of intervening sequences from nuclear-encoded pol II transcripts (pre-mRNA splicing) is an aspect of gene expression common to all eukaryotic organisms. In combination with in vivo studies, in vitro approaches have led to and refined a two-step model for the splicing reaction (Domdey et al. 1984; Padgett et al. 1984; Rodrigues et al. 1984; Ruskin et al. 1984; Zeitlin and Efstratiadis 1984). The first step consists of cleavage of the pre-mRNA at the 5' splice site and the formation of a lariat structure in which the 5' terminal nucleotide of the intron (almost always a G residue) is attached by a 2'-5' phosphodiester bond to a residue (almost always A) near the 3' end of the intron. The second step of splicing consists of 3' splice site cleavage and exon ligation with release of the excised intron.

In the yeast *Saccharomyces cerevisiae*, as well as in metazoan organisms, in vitro splicing requires conserved pre-mRNA consensus sequences (Newman et al. 1985; Rymond and Rosbash 1985; Vijayraghavan et al. 1986) and extract-derived nucleic acid and protein factors (Lin et al. 1985; Cheng and Abelson 1986). Differences between *S. cerevisiae* (henceforth referred to as yeast) and metazoa are evident, however, in the nature and role of these consensus sequences and *trans*-acting

factors. For example, the yeast 5' splice site (GTAPyGT) and branch point position (TACTAAC) appear rigidly conserved (Langford and Gallwitz 1983; Pikielny et al. 1983; Langford et al. 1984; Jacquier et al. 1985; Newman et al. 1985; Parker and Guthrie 1985; Fouser and Friesen 1986; Jacquier and Rosbash 1986; Vijayraghavan et al. 1986), whereas metazoan 5' splice site and branch point sequences are more variable (Green 1986; Padgett et al. 1986). Recognition of the 3' splice site and the adjacent polypyrimidine stretch is critical for metazoan splicing (Frendewey and Keller 1985; Reed and Maniatis 1985; Ruskin and Green 1985a), whereas spliceosome assembly and branch point formation in yeast may take place efficiently without the participation of either a 3' splice site or a polypyrimidine sequence (Rymond and Rosbash 1985; Cellini et al. 1986; Fouser and Friesen 1987).

The abundant and highly conserved snRNAs that associate with the metazoan spliceosome (U1, U2, U4, U5, and U6), and that are required for splicing, do not appear to have precise counterparts in yeast. However, yeast spliceosomes do contain discrete snRNA species (Pikielny and Rosbash 1986; Pikielny et al. 1986). The largest of the yeast spliceosome snRNAs, snR20 (also re-

ferred to as LSR1), has extensive homology to mammalian U2 snRNA and, to a lesser extent, homology to mammalian U4, U5, and U6 (Ares 1986). While snR20 may represent a polyfunctional unit providing the function of several U series RNAs (Ares 1986), its size (1175 nucleotides) is much larger than any metazoan snRNA, and reinforces the notion that considerable differences exist between yeast and metazoan splicing.

We have previously demonstrated that, for yeast rp51A pre-mRNA, the first splicing step occurs efficiently in vitro without a 3' splice site and exon II (Rymond and Rosbash 1985). In this study we extend this analysis to determine more precisely the sequence requirements, 3' to the site of branch point formation (the TACTAAC box), for pre-mRNA splicing and for spliceosome assembly. Our data demonstrate a requirement of at least 29 nucleotides downstream of the rp51A TACTAAC box for lariat formation. Surprisingly, this result apparently reflects a simple length requirement, as the sequence of most of this 29-nucleotide stretch is inconsequential. Furthermore, the inhibition of lariat formation observed with shorter pre-mRNA correlates directly with a particular stage in spliceosome maturation, that of the complex I to complex II transition. The novel role played by this region of the yeast intron in the biogenesis of the yeast spliceosome is discussed.

Results and discussion

SP6 polymerase was used to transcribe the SP6/yeast (*S. cerevisiae*) fusion gene SPrp51AΔ2. The resulting pre-mRNA transcripts (Δ2 pre-mRNA) contain, in addition to a small SP6 leader sequence, intron and flanking exon sequences derived from the yeast rp51A gene (encoding ribosomal protein 51) (Fig. 1; Rymond and Rosbash 1985; Ruskin et al. 1986). Various truncated derivatives were prepared by incubating Δ2 pre-mRNA with RNase H and a series of single-stranded DNA oligonucleotides that hybridize to regions of RNA between the TACTAAC box and the 3' splice site (Fig. 1). Given the positions of hybridization and the sizes of the resulting trun-

cated products (see below), we estimate that these oligonucleotides (A, B, and C, Fig. 1) direct the deletion of exon II and approximately 45, 35, and 20 nucleotides, respectively, from the 3' end of the intron. Each of the pre-mRNAs was then assayed for its ability to serve as a substrate for in vitro splicing (Fig. 2).

Addition of the full-length pre-mRNA (Δ2 pre-mRNA) to the yeast extract generates the predicted pattern of intermediates [lariat intermediate (containing the intron and exon II) and exon I] and products (mRNA and excised intron). Pre-mRNA that has had exon II and ~20 nucleotides of intron removed (oligo C pre-mRNA) is also an efficient substrate, as the rate at which the intermediates of the splicing reaction (in this case a lariat form of the intron lacking exon II and a portion of the intron, plus exon I) appear is similar to that observed for Δ2 pre-mRNA. However, as oligo C pre-mRNA has no exon II, the intermediates of the normal reaction are the terminal products of this reaction. Indeed, the levels of oligo C exon I and lariat intermediate (as defined above for the truncated transcripts) are similar to the sum of the levels of intermediates and products observed with Δ2 pre-mRNA. More extensive deletions have a significant impact as the rate of lariat formation is greatly reduced with oligo B pre-mRNA (though clearly detectable upon longer exposures of this gel; see below) and virtually abolished with oligo A pre-mRNA.

While the average difference in size between the oligo B and oligo C transcripts is approximately 15 nucleotides, the latter is an efficient substrate for lariat formation whereas the former is inefficient. Therefore, it appears that a minimum length of intron sequence 3' to the TACTAAC box is required for the first step in pre-mRNA splicing.

Precise length requirement for splicing

To examine the relative efficiency of individual pre-mRNAs within a population to act as substrates for lariat formation, we compared the size distribution of the pre-mRNA substrates with the corresponding

Figure 1. Pre-mRNA splicing substrates. The hatched boxes represent exon I (77 nucleotides) on the left, exon II (35 nucleotides) on the right, and the open box the intron (137 nucleotides) of the Δ2 pre-mRNA. 5' SS and 3' SS are positioned to refer to the 5' and 3' splice site consensus sequences, respectively, and the asterisk indicates the position of branchpoint formation (the TACTAAC box). The expanded portion of this diagram represents the entire RNA sequence between the TACTTAC box and the 3' splice site. Under this sequence, in heavy lines, are indicated the positions of hybridization of oligonucleotides A, B, and C.

Figure 2. Splicing analysis of full-length and truncated pre-mRNAs. Uniformly labeled full-length ($\Delta 2$) and truncated (oligo C, B, and A) pre-mRNA were incubated, under standard splicing conditions, in a yeast whole-cell extract. At 1, 5, 10, and 20 min of incubation a portion of each reaction was removed, deproteinated, and the RNA analyzed on a denaturing 12% polyacrylamide gel. The intermediates (L, lariat intermediate; 5'E, exon I) and products (M, mRNA; I, intron) were identified based upon: (1) previous characterization (Rymond and Rosbash 1985; Ruskin et al. 1986); (2) their electrophoretic mobilities relative to the pre-mRNA (P) and single-stranded DNA standards [numbers at right refer to the sizes, in nucleotides, of these standards]; (3) their increases with time of incubation; and, in the case of the lariat molecules, (4) direct confirmation of their unique 2'-5' bond structure (see Fig. 3).

product lariats [linearized by cleaving the 2'-5' phosphodiester bond with HeLa cell debranching activity (Ruskin and Green 1985b)] (Fig. 3). For oligo C pre-mRNA, the heterogeneity of the debranched lariat population closely resembles the heterogeneity of the pre-mRNA population (compare lanes 6 and 1 and each with respect to the single-stranded size standards, lanes 7–11). There is, however, an underrepresentation of the smallest molecules in the lariat distribution, suggesting that the shortest members of the population (those with less than 35 nucleotides 3' to the TACTAAC box) are somewhat deficient substrates. Consistent with this observation is the fact that the pre-mRNA that persists through *in vitro* splicing is enriched for the shorter molecules (compare lanes 4 and 1).

A careful size determination of the oligo B pre-mRNA (lane 2) and the debranched lariat distribution (lower bands in lane 11) demonstrates that only the largest

oligo B pre-mRNA molecules (those with 29 or more nucleotides 3' of the TACTAAC box) are substrates for lariat formation. This result is consistent with the fact

Figure 3. Size distribution of pre-mRNAs and lariat intermediates. The sizes of the A, B, and C truncated pre-mRNAs and the linearized lariat intermediate derivatives were determined by comparison of these gel-purified species with a DNA sequence generated using the RP51A gene and a primer, RB1, which hybridizes to the exon II portion of this gene (Teem et al. 1984). [Lanes 1–3] Oligo C, B, and A pre-mRNA; [lane 4] oligo C pre-mRNA remaining unspliced after incubation for 20 min in the yeast whole-cell extract; [lane 5] oligo C circular lariat intermediate; [lane 6] linearized oligo C lariat intermediate; [lane 7–10] C, T, A, and G sequence reactions, respectively; [lane 11] linearized oligo B lariat intermediate (lower band); [lane 12] circular lariat B intermediate (lower band). In both lanes 11 and 12 the upper band is oligo B pre-mRNA which copurified with the lariat intermediate. The arrows on the left-hand side of this figure indicate the approximate positions of the pre-mRNA species (P) and the circular (L_c) and linearized (L_l) lariat intermediates.

that only a very small fraction of the added oligo B pre-mRNA undergoes 5' cleavage and lariat formation (Fig. 2). This small fraction of active substrates arises from the upper region of the oligo B pre-mRNA distribution, which constitutes the region of overlap with the oligo C pre-mRNA distribution (compare lanes 2 and 1, and lanes 11 and 6).

Length versus sequence

To determine whether a particular sequence in this region (for example, a polypyrimidine stretch) is necessary or preferred 3' of the TACTAAC box, we added to oligo A pre-mRNA (which retains 10–15 nucleotides 3' to the TACTAAC box) ~25 nucleotides of ribohomopolymer (which generates molecules of approximately the same length as the splicing-proficient oligo C pre-mRNA). Addition of ribohomopolymer tails (either A,G,C, or U) to oligo A pre-mRNA greatly facilitates lariat formation; indeed, the efficiency of lariat formation of the tailed oligo A pre-mRNA was comparable to that of oligo C pre-mRNA (Fig. 4A). To show that the simple length requirement indicated by this result does not represent a

gene-specific phenomenon peculiar to rp51A-derived pre-mRNAs, we analyzed transcripts derived from the yeast actin gene (Fig. 4B). Runoff transcripts prepared from the SP6/yeast actin fusion gene, LF49, cleaved at the naturally occurring *Clal* site within the actin intron, retain only the first two nucleotides 3' to the TACTAAC box (Ng and Abelson 1980). This RNA (Act/C) is an inactive substrate for lariat formation (lane 1) yet can be "activated" (as indicated by the release of the 116-nucleotide exon I and a slowly migrating lariat molecule) by the addition of ribohomopolymer tails (lane 2 and data not shown). Taken together with the rp51A experiments described above, this result makes it likely that the in vitro requirement for RNA downstream of the TACTAAC box reflects in large part a sequence-independent length requirement.

Analysis of splicing complexes

We have previously demonstrated that in vitro assembly of the yeast spliceosome progresses through three complexes (termed I, II, and III), which can be resolved by polyacrylamide gel electrophoresis (Pikielny et al. 1986). Based upon their kinetics of appearance, sensitivity to *cis*-acting mutations, the profile of associated snRNAs (Pikielny et al. 1986), and nuclease sensitivities (Rymond and Rosbash 1986), these complexes appear to define an assembly pathway: III $\rightarrow$ I $\rightarrow$ II. The characterization of this in vitro pathway indicates that the terminal complex, II, is most likely the mature spliceosome. To examine the effect of the truncations on spliceosome assembly, we compared complex formation of the oligo A and C pre-mRNAs with that of full-length pre-mRNA (Fig. 5).

One minute after addition of either $\Delta 2$ or oligo C pre-mRNA to the yeast cell-free extract considerable amounts of complexes III and I have formed (Fig. 5; $\Delta 2$, C). The appearance of complex II at 5 min correlates with the appearance of splicing products (compare Fig. 2 and 5) and is expected from previous studies of this kind (Pikielny et al. 1986). At 20 min, complexes III and I are much reduced in amount and complex II is the most prominent form of the spliceosome. Since the times of appearance, amounts, sizes, and relative proportions of the three complexes are indistinguishable for full-length and oligo C pre-mRNAs, we conclude that the 3' splice site region (including exon II, the 3' splice site junction and approximately 20 nucleotides of intron) does not associate with splicing specific factors that detectably influence these aspects of spliceosome assembly.

Addition of oligo A pre-mRNA to the yeast extract, however, results in a different assembly profile (Fig. 5, A). At 1 min, the spliceosome pattern closely resembles that shown above for the two longer substrates. As assembly progresses, however, complex II fails to appear. The time course suggests that complex I, rather than complex II, is the terminal assembly product with oligo A pre-mRNA. (The slowest migrating complex is of unknown origin and significance, but is also visible to a lesser extent with full-length pre-mRNA substrate.) The truncated actin-derived pre-mRNA also forms only two

Figure 4. Lariat formation of ribohomopolymer tailed pre-mRNAs. (A) Oligo C (lane 1), oligo A (lane 2), and oligo A pre-mRNA tailed (using RNA ligase) at its 3' end with ~25 nucleotides of poly A, C, G, or U (lanes 3, 4, 5, and 6, respectively) were incubated under standard splicing conditions for 15 min. The reactions were terminated with the addition of EDTA, deproteinated, and the RNAs analyzed on a denaturing 12% polyacrylamide gel. (L) Lariat intermediate; (P) pre-mRNA; (5'E) exon I. (B) Analysis of lariat formulation (as described above) using the actin-derived pre-mRNA Act/C before (lane 1) and after (lane 2) the addition of poly(A)₅₀ to its 3' end. The numbers on the right refer to the sizes, in nucleotides, of the single-stranded DNA standards in lane 3.

Figure 5. Native gel analysis of splicing complexes. Uniformly labeled full-length ($\Delta 2$), oligo C (C), and oligo A (A) pre-mRNAs were incubated under standard splicing conditions for 1, 5, 10, or 20 min. Act/C RNA was incubated for 20 min under standard splicing conditions. RNAs were analyzed for complex formation on a 3% polyacrylamide/0.5% agarose composite gel. I, II, and III refer to the previously described forms of the yeast spliceosome (Pikielny et al. 1986).

complexes, which approximately comigrate with complexes III and I of rp51A pre-mRNA (Fig. 5, Act/C). In contrast, full-length actin pre-mRNA (containing the entire intron and approximately 400 nucleotides of exon II) forms all three complexes (data not shown). As the products and intermediates of the splicing reaction are normally found in complex II (Pikielny et al. 1986), the oligo A and Act/C deletions may specifically inhibit lariat formation by inhibiting the complex I to complex II transition.

snRNAs

If specific yeast snRNAs interact with the 3' splice site, or the region of the intron required for the complex I to complex II transition, one might expect the truncated pre-mRNA molecules to be associated with only a subset of the snRNAs normally associated with full-length pre-mRNA. To examine this possibility, we exploited the biotin-avidin technology recently introduced by Grabowski and Sharp (1986) and compared the snRNA profile of spliceosomes assembled *in vitro* on full-length substrates with those assembled on truncated substrates (Fig. 6). Spliceosomal snRNAs are defined as RNA species that are reproducibly enriched with assembly-competent substrates (lane 4) [as compared with assembly-incompetent substrates (lanes 1-3)] and which can be immunoprecipitated using an anti-trimethyl cap antibody. By this criterion, spliceosomes from full-length pre-mRNA, oligo A pre-mRNA, and oligo C pre-mRNA contain the same snRNAs (lanes 4, 5, and 6). These include those snRNAs previously identified as associating with the yeast spliceosome, namely snR20 (LSR1), snR7L, snR7S, and snR14 (Pikielny and Rosbash 1986; Pikielny et al. 1986). Also detectable with this high-resolution technique are three additional species. Based on molecular weight analysis and a comparison with the recently published description of the yeast snRNAs (Riedel et al. 1986), one of these species corresponds to snR19. As none of these species asso-

ciates with mutant substrates lacking an intron (lane 1) and none (with the possible exception of snR19; data not shown) associates with mutant substrates lacking a proper 5' junction (lane 2) or a TACTAAC box (lane 3), we conclude that the association of all of these species with the substrate is splicing-complex specific. The experiment fails to reveal any snRNA component uniquely associated with the 3' junction.

Conclusion

Our results demonstrate that truncated pre-mRNAs, retaining >35 nucleotides on the 3' side of the TACTAAC box, undergo efficient complex formation and normal and efficient lariat formation. Truncations that approach more closely the TACTAAC box inhibit and ultimately eliminate splicing when <29 nucleotides remain 3' of the TACTAAC box. Surprisingly, the splicing-deficient pre-mRNAs are blocked at a precise and well-defined stage of spliceosome assembly, namely the complex I to complex II transition. This block, and the assembly time course shown in Figure 5, are consistent with the previously proposed assembly pathway, namely III $\rightarrow$ I $\rightarrow$ II (Pikielny et al. 1986). Addition of ribohomopolymers reactivates the inactive pre-mRNAs as assayed by lariat formation (Fig. 4) and by restoration of the complex I $\rightarrow$ complex II transition (data not shown), suggesting that the RNA 3' to the TACTAAC box (beyond perhaps the first few nucleotides) may have a relatively sequence-independent role in spliceosome assembly.

Previous results from this laboratory have shown that stable spliceosome complex formation requires both a 5' splice site junction and a TACTAAC box (Pikielny and Rosbash 1986; Pikielny et al. 1986; Rymond and Rosbash 1986). The efficient formation of complexes III and I with the oligo A pre-mRNA indicates that stable complex formation does not require extensive sequence information 3' of the TACTAAC box. Careful measurements of substrate length and the time course of oligo A

pre-mRNA assembly suggest that no more than 10–15 nucleotides 3' of the TACTAAC box are required for efficient complex formation (Fig. 3 and 5). These 15 nucleotides contain no significant pyrimidine runs (Fig. 1). Moreover, experiments with actin pre-mRNA, con-

Figure 6. snRNA analysis of splicing complexes. Biotinylated pre-mRNA was incubated under standard splicing conditions for 5 min, sufficient time to assemble significant quantities of complexes I, II, and III (see Fig. 5). Spliceosome assembly was terminated by the addition of EDTA and the pre-mRNA (and stably associated complexes), then bound to streptavidin agarose. After extensive washings the splicing complexes were disrupted with proteinase K. RNAs released from these complexes were 3' end-labeled, and then immunoprecipitated using an antibody directed against the trimethyl cap moiety of the snRNAs. The RNA in each sample was analyzed on a high-resolution, denaturing 7% polyacrylamide gel. The RNAs used to initiate spliceosome assembly were: (lane 1) a 1386-nucleotide SP6 control transcript containing no intron; (lane 2) 5'0, an rp51A derivative lacking a 5' splice site (Pikielny and Rosbash 1986); (lane 3) Δ3B, an rp51A derivative lacking a TACTAAC box (Pikielny and Rosbash 1986); (lane 4) full-length, SP6/rp51A pre-mRNA (Pikielny and Rosbash, 1986); (lane 5) oligo C truncated SP6/rp51A pre-mRNA; (lane 6) oligo A truncated SP6/rp51A pre-mRNA. The numbers on the left-hand side refer to the snRNAs associated with these transcripts according to the nomenclature of Guthrie and co-workers (Riedel et al. 1986). The two question marks [which lie between the 246 and 369 molecular weight markers (lane 7, with sizes indicated on the right-hand side)] refer to two immunoprecipitable RNAs which are enriched in spliceosomes but are of unknown origin. Based upon their apparent size they may correspond to snR17 (lower band) and snR18 (upper band). Yet, due to inherent ambiguities associated with identifying these snRNAs (see Riedel et al. 1986), it is uncertain whether these designations are correct.

taining only two nucleotides after the TACTAAC box, demonstrate that complexes III and I (but not complex II) also form with this substrate (Fig. 5). This result indicates that the block after band I observed with truncated rp51A-derived pre-mRNA is likely to be of general significance. Although these experiments do not exclude the possibility that sequence information 3' of the TACTAAC box affects the efficiency of complex formation (cf. Pikielny and Rosbash 1985; Cellini et al. 1986), they do suggest that stable complex formation requires only a properly positioned 5' splice site and TACTAAC box (see below) and relatively little RNA 3' to the TACTAAC box.

The fact that RNA 3' to the TACTAAC box is required for the complex I to complex II transition is somewhat difficult to interpret. Presumably this maturation event requires an interaction between the RNA downstream of the TACTAAC box and some other part [probably an extract-derived, *trans*-acting factor(s)] of the yeast spliceosome. However, since this interaction appears to be relatively sequence independent, it is likely that such a factor would initially recognize other conserved intron elements (or associated factors) and interact with this 3' region of the intron during a later stage of spliceosome maturation. One can imagine that a factor initiating complex assembly by associating, for example, with the TACTAAC box must dissociate or translocate to allow further complex maturation and lariat formation. The continuity of the RNA chain 3' to the TACTAAC box may be necessary for such a dissociation or translocation event, perhaps by providing a substrate for directed (5' to 3') movement or simply by providing a high local concentration of RNA. An alternative possibility is that the protein (or snRNP) interactions with this region are sequence-specific but are only modestly stronger than interactions with the homopolymers (Deckman and Draper 1985). A relatively high concentration of the binding factor in the extract might mask such a difference.

Previous *in vivo* studies have addressed the role of the 3' splice site region in yeast splicing (Cellini et al. 1986; Fouser and Friesen 1987). These studies indicate that the spacing between the TACTAAC box and the 3' splice site AG (normally 7–55 nucleotides) is rather flexible. Abnormally short or abnormally long spacing reduces the efficiency of the second step as exhibited by an increase in intracellular levels of lariat intermediate and a decrease in mRNA. (Indeed, this 3' region may have a clear sequence-dependent role in the second step.) In contrast, similar perturbations have little or no detectable effect on the first step of splicing *in vivo* and, by implication, on *in vivo* spliceosome formation. These conclusions are consistent with the results presented here, and, taken together, they raise the interesting possibility that for transcripts with very short distances between the TACTAAC box and the 3' splice site [for instance, *MATa1* (Miller 1984)], the 3' exon may play a sequence-independent role in complex formation.

These data reinforce the notion that significant differences exist between yeast and metazoan splicing; more-

over, they indicate that some of these differences are manifest at the level of splicing complex assembly. The 3' splice site AG and polypyrimidine stretch are required for mammalian splicing (Frendewey and Keller 1985; Reed and Maniatis 1985; Ruskin and Green 1985c; Aebi et al. 1986) and may play an obligate and early role in mammalian spliceosome assembly (Frendewey and Keller 1985; Ruskin and Green 1985a; Bindereif and Green 1986). Recent evidence implicates U5 snRNA and a specific protein which recognizes the polypyrimidine stretch as prominent candidates for *trans*-acting factors that recognize this region (Chabot et al. 1985; Gerke and Steitz 1986; Tazi et al. 1986). The experiments in this report also provide evidence for a role of the corresponding region of a yeast pre-mRNA intron in complex formation, but this role appears quite different as it is relatively sequence-independent and participates in a late stage of spliceosome assembly.

It is of some interest that, to date, all yeast *cis*-acting mutations that affect splicing also affect, quantitatively or qualitatively, *in vitro* spliceosome assembly (Brody and Abelson 1985; Pikielny and Rosbash 1986; Vijayraghavan et al. 1986). Similarly, many of the temperature-sensitive mutations, known to affect splicing *in vivo* (Fried et al. 1981; Rosbash et al. 1981; Larken and Woolford 1983) and *in vitro* (Lustig et al. 1986), also manifest a temperature-sensitive effect on *in vitro* spliceosome assembly (unpubl.). These observations suggest that much of the specificity and efficiency of splicing is determined at the level of spliceosome assembly. The results presented here, specifically the effects of the oligo A and actin truncations, are consistent with this generalization.

Methods and materials

Plasmids, DNA oligonucleotides

The various SP6/rp51A fusion constructs [pSPrp51A, pSPrp51A Δ 2, pSPrp51A(5'0), and pSPrp51A(Δ 3B)] have been described previously (Pikielny and Rosbash 1986; Ruskin et al. 1986). Promega SP6 "control template" DNA (derived from phage λ) was used to prepare the 1386-nucleotide, intronless RNA, pLF49, containing the SP6/yeast actin fusion gene (constructed by inserting a 0.7-kb *Xho*I–*Hae*III actin fragment into vector pSP65), was a generous gift of L. Fouser and J. Friesen.

Single-stranded DNA oligonucleotides A, B, and C [formerly referred to as RB23 (Rymond and Rosbash 1985)] were purchased from The DNA Synthesis Facility (University of Massachusetts Medical School, Worcester, Massachusetts). Each was phenol-extracted and then precipitated with ethanol prior to use in the RNase H cleavage reaction.

Preparation of pre-mRNA transcripts

Capped, uniformly labeled pre-mRNA transcripts were prepared from *Eco*RI-digested pSPrp51A, pSPrp51A(5'0), and pSPrp51A(Δ 3B), *Dde*I-digested pSPrp51A Δ 2, *Cla*I-digested pLF49, and Promega control template DNA using SP6 polymerase (Promega) and [32 P]UTP (NEN) according to previously established conditions (Ruskin et al. 1986). Full-length runoff transcripts were gel-purified prior to use as splicing substrates (see below). Truncated transcripts were derived from full-length

pre-mRNA using single-stranded oligonucleotides A, B, and C and the RNase H activity associated with the yeast whole-cell extract or, when used for homopolymer tailing, with purified *E. coli* RNase H as previously described (Rymond and Rosbash 1985). The 5' half of each RNase H-digested transcript was gel-purified prior to further manipulation.

Ribohomopolymer tailing

Random length, single-stranded RNA homopolymers were purchased from Sigma. Approximately 300 μ g of each was base-hydrolyzed (Bock 1973) at 100°C, in 0.1 M NH_4HCO_3 (pH 10), for a length of time sufficient to generate molecules with an average length of <100 nucleotides. The RNA populations were preparatively size-fractionated on a denaturing, 10% polyacrylamide gel and gel fractions containing molecules 20–30 or 40–60 nucleotides in length eluted overnight at 37°C in 0.1 M Tris-HCl (pH 7.5), 0.0125 M EDTA, 0.15 M NaCl, and 1% SDS. The size-selected homopolymers were extracted once with buffered phenol and then precipitated with ethanol. The 5' ends of approximately 2 μ g of homopolymer RNA were phosphorylated with T4 polynucleotide kinase (Pharmacia) for 30 min at 37°C in a 10- μ l reaction containing 0.05 M Tris-HCl (pH 7.6), 0.01 M MgCl_2 , 0.005 M DTT, 0.001 M ATP, and 10 units of kinase. The kinase reaction was terminated by the addition of 10 volumes of 0.005 M EDTA (pH 7.5), phenol-extracted, precipitated with ethanol, and then resuspended in H_2O to a final concentration of 1 mg/ml. The phosphorylated homopolymers were joined to the 3' ends of the truncated pre-mRNAs using RNA ligase (Pharmacia) in a reaction containing: 1 μ g of size-selected, phosphorylated homopolymer (~100 pmole), 100 ng of truncated pre-mRNA (~0.5–1.00 pmole), 10% DMSO, 0.05 M HEPES (pH 7.5), 0.0003 M DTT, 0.02 M MgCl_2 , 5 μ M ATP, and 5 units of RNA ligase for 12 hr at 0°C. The ligated ("tailed") pre-mRNA was separated from the unligated pre-mRNA on a denaturing polyacrylamide gel, eluted as described above, and then used as a splicing substrate.

In vitro splicing

Preparation of yeast whole-cell extracts and the *in vitro* splicing reactions were performed as described (Lin et al. 1985). Native gel analysis of splicing complexes was performed as previously reported (Pikielny et al. 1986).

snRNA analysis

Preparation of the biotinylated transcripts was performed essentially as described (Grabowski and Sharp 1986) with the nucleotide concentrations adjusted to: A, C, G, GpppG (1.0625 mM), U (0.05625 mM), biotin-11-U (BRL; 0.00625 mM). A 50- μ l splicing reaction was incubated for 5 min under standard conditions and then quenched by the addition of an equal volume of Q buffer (Pikielny and Rosbash 1986). Forty microliters (wet volume) of streptavidin agarose (BRL), equilibrated with NET-2 buffer [0.05 M Tris-HCl (pH 8.0), 0.5 M KCl, 0.05% NP-40, and 0.001 M DTT] was added and the tubes were gently shaken for 2 hr at 4°C. The avidin-bound, biotin-containing complexes were washed extensively [4 $\times$ (800 μ l of NET-2 buffer, 30 min, 4°C with intermittent, gentle agitation)], and then dissociated by the addition of 200 μ l of PK buffer [0.1 M Tris-HCl (pH 7.5), 0.0125 M EDTA, 0.15 M NaCl, 1% SDS, and 0.4 mg/ml proteinase K] for 1 hr at 50°C. The streptavidin agarose beads were pelleted by a brief (15 sec) spin in a Fisher microfuge, and the supernatant was removed, extracted with phenol, and precipitated (after the addition of 4 μ g of carrier DNA) with ethanol.

The complex-associated RNAs were 3' end-labeled using RNA ligase (Pharmacia), and the snRNA species among these were enriched by immunoprecipitation using an antibody preparation (a generous gift of R. Luhrmann) directed against their 5' terminal trimethyl cap moiety as previously described (Pikielny and Rosbash 1986).

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B C Rymond, D D Torrey and M Rosbash

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