REPORT

Bimolecular exon ligation by the human spliceosome bypasses early 3' splice site AG recognition and requires NTP hydrolysis

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ABSTRACT

Here we report further characterization of an *in vitro* assay system for exon ligation by the human spliceosome in which the 3' splice site AG is supplied by a different RNA molecule than that containing the 5' splice and branch sites. By varying the time during splicing reactions when the 3' splice site AG is made available to the splicing machinery, we show that AG recognition need not occur until after lariat formation. Thus an early AG recognition event required for spliceosome formation and lariat formation on some mammalian introns is not required for exon ligation. Depletion/add-back studies and cold competitor challenge experiments reveal that commitment of a 3' splice site AG to exon ligation requires NTP hydrolysis. Because it both physically and kinetically uncouples exon ligation from spliceosome assembly and lariat formation, the bimolecular system will be a valuable tool for further mechanistic analysis of the second step of splicing.

Keywords: 3' splice site AG; exon ligation; RNA splicing

INTRODUCTION

The precise and timely removal of introns is critical for generation of eukaryotic mRNAs. Most nuclear introns are removed by the major spliceosome, a 60 S complex consisting of pre-mRNA, five small nuclear RNAs (snRNAs: U1, U2, U4/U6, and U5) associated into ribonucleoprotein particles (snRNPs), and a host of other protein factors (reviewed in Moore et al., 1993; Krämer, 1995). Within the spliceosome, intron excision occurs in two steps, both single transesterification reactions (Maschhoff & Padgett, 1993; Moore & Sharp, 1993): cleavage of the phosphodiester backbone at the 5' splice site GU coupled with lariat formation at the branch site A, followed by exon ligation at the 3' splice site AG. For each round of splicing, a spliceosome must be assembled from its component parts. This assembly is a highly ordered process wherein U1 snRNP and numerous proteins initially join the pre-mRNA to form a

stable commitment complex. This is subsequently chased into complex A by U2 snRNP addition. Complex B forms when the U4/U6/U5 tri-snRNP joins and, concomitant with lariat formation, additional proteins are added to form complex C wherein exon ligation occurs (Moore et al., 1993; Gozani et al., 1994; Krämer, 1995). Although the two chemical steps of splicing require no external energy input, all spliceosomal transitions subsequent to initial commitment complex formation require ATP hydrolysis.

Although both spliceosome assembly and lariat formation have been relatively well-studied, comparatively less is known about late steps in the pathway, such as 3' splice site AG definition, exon ligation, spliceosome disassembly, and snRNP recycling. One reason for this is that *in vitro* splicing reactions are rate limited by early steps, making the late steps difficult to study by biochemical means. Whereas assembly of active human spliceosomes *in vitro* often requires 10 min or longer, ligated exons can be detected almost immediately after lariat formation commences. Moreover, multiple recognition events involving all the splice site consensus sequences have been documented both before and after lariat formation (see Discussion). For example, mammalian introns with short polypyrimidine

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tracts (PPTs) between the branch and 3' splice sites require the 3' splice site AG for both spliceosome assembly (Frederick & Keller, 1985; Lang & Keller, 1990) and lariat formation (Aebi et al., 1986), even though the AG is not directly involved in the first chemical reaction. Such introns have been dubbed "AG-dependent" (Reed, 1989). Therefore effects specific to exon ligation caused by consensus sequence alterations are often obfuscated by accompanying effects on spliceosome assembly and lariat formation.

We recently reported a new assay for exon ligation in human splicing extracts where the 3' splice site AG is contributed by a different RNA molecule (3' substrate) than that containing the 5' splice site, branch site, and polypyrimidine tract (5' substrate). Because the 3' splice site AG is no longer attached to the sequence elements required for lariat formation, it is possible to address previously untestable questions with this system. For example, we showed that accurate 3' splice site selection is independent of either a covalently attached PPT or specific 3' exon sequences. Surprisingly, when appended to several different downstream sequences, the 5-mer GACAG was sufficient to specify a 3' splice site. When the 3' substrate contained more than one potential 3' splice site AG, the 5'-most AG was invariably chosen, indicating that 3' splice site selection in this system occurs with 5' → 3' directionality (Anderson & Moore, 1997).

In this paper we investigated the timing of 3' splice site AG selection for exon ligation relative to spliceosome assembly and lariat formation. We show here that the 3' substrate for bimolecular exon ligation has no detectable effect on spliceosome assembly or lariat formation. The 3' substrate need not be incorporated into spliceosomes until after lariat formation. Thus the early recognition event involving the 3' splice site AG in some introns is not required for subsequent chemistry. However, commitment of the 3' splice site AG to exon ligation does require NTP hydrolysis, indicating that at least one ATP-dependent step occurs coincident with or after 3' splice site AG recognition. Because exon ligation is fully uncoupled from lariat formation in this system, this assay will facilitate further mechanistic analysis of the second step of splicing.

RESULTS

In theory, the use of a 3' splice site AG for exon ligation could require its initial recognition at any of the following points in the splicing pathway (Fig. 1): (1) during the first stages of spliceosome assembly concomitant with 5' splice and branch site definition; (2) after spliceosome assembly but sometime before lariat formation; (3) after lariat formation but before the last ATP-dependent step required for exon ligation; or (4) after both lariat formation and all ATP-dependent steps. Because in the bimolecular exon

FIGURE 1. Possible stages in the splicing pathway where the 3' splice site AG could be committed to exon ligation.

ligation assay the 3' splice site AG is located on a different molecule than that containing the 5' splice and branch sites, the point during splicing when the 3' splice site is made available to the splicing apparatus can be varied experimentally. Thus this system is ideal for examining the time at which the exact site for exon ligation is chosen.

In all experiments described here, the 5' substrate was derived from an adenovirus major late intron variant (AdMLΔAG) in which the PPT had been lengthened to convert it to an "AG-independent" intron (Gozani et al., 1994)—that is, one not requiring the 3' splice site AG for early steps (Reed, 1989). Truncation of AdMLΔAG immediately downstream of the PPT (Anderson & Moore, 1997) yields an RNA that can support both spliceosome assembly (Fig. 2A) and lariat formation (Fig. 2B) in the absence of either a 3' splice site AG or downstream 3' exon. To initiate bimolecular exon ligation, three different 3' substrates were used as indicated in the text or figure legends. All began with the sequence 5'-GACAG, which defined the site of exon ligation. This was followed by a 55-nt exon derived from AdML sequences (AdML), the exact antisense of the AdML exon (AdMLas), or a

FIGURE 2. 3' splice site AG commitment occurs after spliceosome assembly. Native (**A**) and denaturing (**B**) polyacrylamide gels of labeled 5' substrate incubated under splicing conditions for the times indicated. Spliceosomal complexes A, B, and C are indicated to the left; RNA species (5' substrate, lariat, and free 5' exon) are denoted by symbols to the right. **C:** Denaturing polyacrylamide gel showing ligated exons from labeled AdMLas 3' substrate added to splicing reactions either simultaneously with unlabeled 5' substrate (lanes 1–9) or after the 5' substrate had been preincubated under splicing conditions for 30 min (lanes 10–18). The times indicated are from the point of 3' substrate addition. **D:** Graphical depiction of the data in **C**.

30-nt optimized cardiac troponin-T purine-rich enhancer (cTNT) (Ramchatesingh et al., 1995; Anderson & Moore, 1997). All three 3' substrates spliced with similar efficiencies, indicating that this assay is not highly dependent on any one 3' exon sequence (Anderson & Moore, 1997).

The 3' splice site AG need not be present during spliceosome assembly

To test whether use of a 3' splice site AG requires its incorporation into functional spliceosomes concurrent with the 5' splice and branch sites, the time of 3' substrate

addition relative to the 5' substrate was varied. Formation of functional spliceosomes or C complexes on the 5' substrate requires more than 10 min (Fig. 2A), with no first-step products (5' exon and lariat intron) detectable until later times (Fig. 2B). Therefore, if a 3' splice site AG can be used for exon ligation only if present during commitment complex formation, ligated exon products should not appear in any bimolecular reaction until at least 10 min after 3' substrate addition. On the other hand, if the 3' substrate is not required during spliceosome assembly, there should be no lag in ligated exon production if the 3' substrate is added at later times.

Labeled AdMLas 3' substrate was added to splicing reactions either simultaneously with unlabeled 5' substrate or after the 5' substrate had been preincubated at 30 °C for 30 min to accumulate functional C complexes. As expected, when both RNAs were added together, there was more than a 10-min lag in development of ligated exons (Fig. 2C, lanes 1–9). In contrast, in reactions where the 3' substrate was added after spliceosome formation, ligated exons were detectable within 1 min (Fig. 2C, lanes 10–18). Graphical representation of the data (Fig. 2D) reveals that once exon ligation is initiated, it evolves at similar linear rates, regardless of the time of 3' substrate addition. Identical results were obtained with the AdML and TNT 3' substrates (data not shown). Thus a 3' splice site AG can be incorporated into the splicing pathway after spliceosome assembly, and for efficient exon ligation there is no advantage for it to be present during commitment complex formation.

The 3' splice site AG can be recognized after lariat formation

A second possibility is that the 3' splice site AG must be recognized at some point after spliceosome assembly but prior to lariat formation (Fig. 1). To test this, we examined whether precleaved 5' exon from lariat formation on the 5' substrate alone could be chased into ligated exon product upon addition of a 3' substrate. Radiolabeled 5' substrate was preincubated alone under splicing conditions for either 30 or 60 min before addition of unlabeled 3' substrate. The data in Figure 3 represent composite results from three different experiments.

In the absence of 3' substrate, 5' exon accumulated for 60 min and then leveled off (Fig. 3A). However, if a 3' substrate was added after 30 or 60 min, the amount of free 5' exon decreased coincident with formation of ligated exon product (Fig. 3B,C). Therefore, precleaved 5' exon can be chased into ligated exon product by 3' substrate addition. Furthermore, in no case did addition of 3' substrate increase lariat formation on the 5' substrate (Fig. 3A). This is consistent with experiments in the previous section indicating that the 3' substrate does not participate in spliceosome assembly. Thus

the 3' splice site AG need not be defined until after lariat formation, and any recognition events involving the 3' splice site AG prior to the first step of splicing are dispensable. Additionally, the 3' substrate for bimolecular exon ligation can associate with complex C, as this is the functional complex present after lariat formation.

A requirement for NTP hydrolysis

Having established that the 3' splice site AG need not be present until after lariat formation, we next determined if ATP is required for its use. To test this, intermediate-containing spliceosomes were first accumulated on unlabeled 5' substrate for 30 min. ATP was then depleted by gel filtration over Sephadex G-50 spin columns that had been preequilibrated with nucleotide-free splicing buffer (Schwer & Guthrie, 1991). Thin layer chromatography of reactions spiked with [α - 32 P]ATP prior to the spin column verified that all free ATP, and therefore any molecules of similar or smaller size, was removed by this treatment (Fig. 4A).

When labeled 3' substrate was added to ATP-depleted spliceosomes, no spliced exon product was detected (Fig. 4B, lanes 4–5). In contrast, when ATP was also included, exon ligation was restored (Fig. 4B, lanes 6–7). Thus, bimolecular exon ligation requires ATP. Titration experiments revealed that optimal exon ligation required at least 1 mM ATP; spliced exon product could be observed with as little as 0.5 mM ATP, but increasing the ATP up to 5 mM had no benefit over 1 mM (data not shown).

To examine the nucleotide specificity of the requirement, other nucleotide triphosphates were tested (Fig. 4C,D). Interestingly, all were active to some degree with relative efficiencies at 1 mM being ATP = dATP $\gg$ CTP = dCTP > GTP = dGTP = UTP = dTTP = dUTP (Fig. 4D; data not shown). Therefore, although the specificity is rather broad, ATP and dATP are the preferred substrates.

To determine whether NTP hydrolysis is required, the nonhydrolyzable analogs AMP-C-PP and AMPP-C-P were also tested. Neither analog worked alone nor in combination. Moreover, 1 mM of either compound added to a reaction containing 1 mM ATP completely inhibited exon ligation (Fig. 4D). This indicates that these analogs can compete with ATP for binding to the species that confers the NTP requirement. Thus, both NTP binding and hydrolysis are required. Consistent with this, two slowly hydrolyzable analogs, ATP- α -S and ATP- γ -S, could rescue exon ligation to some degree, but not back to the levels obtained with ATP (Fig. 4C,D; data not shown).

ATP is required for 3' splice site commitment

Although the above experiments demonstrated that NTP hydrolysis is required for exon ligation in our bimolec-

FIGURE 3. Accumulated 5' exon can be chased into ligated exon product by 3' substrate addition. Graphical representation of data from three different experiments in which labeled 5' substrate was incubated under splicing conditions alone ($\square$) or with one of three different unlabeled 3' substrates (TNT, AdML, or AdMLas) added after either 30 ($\circ$) or 60 ($\triangle$) min. **A:** Relative lariat production. **B:** Relative 5' exon levels. **C:** Relative ligated exon product. Because the overall splicing efficiencies differed somewhat among the 3' substrates, the level of spliced exon product at each timepoint was normalized to the amount obtained at the longest time point for each 3' substrate. Error bars indicate standard deviations.

ular assay, they did not reveal whether it is needed for commitment of the 3' splice site AG to splicing or at a later step. Notably, the earliest steps of spliceosome assembly that commit the 5' splice and branch sites to splicing are ATP-independent (Séraphin & Rosbash, 1989). That is, preincubation of a labeled full-length splicing substrate under splicing conditions in the absence of ATP commits the RNA to the splicing pathway such that later addition of excess unlabeled substrate does not interfere (Legrain et al., 1988). Such commitment complexes, which contain U1 snRNP and numerous protein factors, have been observed in both yeast (Séraphin & Rosbash, 1989; Zhang & Rosbash, 1999) and mammals (Michaud & Reed, 1991; Bennett et al., 1992; Jamison et al., 1992). Although there are two documented stages of exon ligation in yeast, one requiring ATP hydrolysis followed by one independent of ATP (Horowitz & Abelson, 1993), it was not previously known at which stage AG commitment occurs.

We used a competition approach similar to that above to test whether 3' splice site AG commitment requires

NTP hydrolysis. Unlabeled 5' substrate was first incubated under splicing conditions to generate active spliceosomes containing splicing intermediates, and then NTPs were depleted as above. Labeled 3' substrate was then added with or without other components, and the incubations continued as indicated in Figure 5. Although ligated exon product was readily detectable when reactions contained both ATP and labeled 3' substrate (Fig. 5, lanes 1–4), simultaneous inclusion of a 100-fold excess of unlabeled 3' substrate completely abolished development of radiolabeled ligated exon product (Fig. 5, lanes 12–14). The same amount of a nonspecific RNA yielded no diminution in exon ligation (data not shown), indicating that competition by the unlabeled 3' substrate required a viable 3' splice site.

To test whether the labeled 3' substrate could be committed to exon ligation in the absence of ATP, spliceosome-containing reactions were first supplemented with labeled 3' substrate alone, incubated for 10 min, and then chased with ATP and excess cold competitor. Under these conditions, no labeled ligated

FIGURE 4. Bimolecular exon ligation requires ATP hydrolysis. **A:** Thin layer chromatography of splicing reactions before (1) and after (2) ATP depletion. Reactions were spiked with [α-³²P] ATP before separation over a G-50 spin column or (3) [α-³²P] ATP alone was spotted as a control. **B:** Denaturing polyacrylamide gel of bimolecular exon ligation reactions either depleted of ATP (lanes 4–7) or not (lanes 1–3) prior to 3' substrate addition. Depleted reactions in lanes 6 and 7 were supplemented with 2 mM ATP upon 3' substrate addition. Timepoints were taken at 0 (lane 1), 15 (lanes 2, 4, 6), or 30 (lanes 3, 5, 7) min after 3' substrate addition. All lanes represent identical exposures of a single gel. **C:** Abilities of various nucleotides (1 mM) to restore exon ligation. **D:** Histogram depicting the relative efficiencies of exon ligation restoration by addition of 1 mM indicated nucleotides to ATP depleted reactions. Error bars reflect standard deviations from three different experiments for NTPs and two experiments for the nonhydrolyzable ATP analogs.

exon product was observed (Fig. 5, lanes 9–11). Ten minutes was chosen as an appropriate time for the preincubation of labeled 3' substrate with spliceosomes, because under standard assay conditions, a significant amount of ligated exon product accumulated by that

time (Fig. 5, lane 1), indicating that at least a portion of the 3' substrate is committed to exon ligation at 10 min in the presence of ATP. As no ligated exon product was observed in Figure 5, lanes 9–11, commitment of a 3' splice site AG to exon ligation must require ATP.

FIGURE 5. ATP is required for 3' splice site AG commitment. Unlabeled 5' substrate (35 nM) was preincubated under splicing conditions for 30 min to accumulate intermediate-containing spliceosomes. After ATP depletion by gel filtration, labeled AdMLas 3' substrate (175 nM) was added alone (lanes 5–11), with 7.5 μ M unlabeled AdMLas 3' substrate (lanes 12–14), or with 2 mM ATP (lanes 1–4 and 15–18), and incubations continued at 30 °C for 10 min. Reactions were then supplemented with 2 mM ATP (lanes 12–14), unlabeled AdMLas 3' substrate (lanes 15–18) or both (lanes 9–11) and incubation at 30 °C continued for times indicated (min).

Finally, to test whether the ATP-dependent step was rate limiting for exon ligation under these conditions, the competition experiment was performed another way. Both labeled 3' substrate and ATP were added to intermediate-containing spliceosomes and incubated for 10 min prior to addition of unlabeled competitor (Fig. 5, lanes 15–18). If more labeled substrate had been committed to exon ligation during the 10 min in the presence of ATP than had yet undergone exon ligation, then when unlabeled competitor was added, some further accumulation of ligated exon product (as in Fig. 5, lanes 1–4) would have been expected. However, since no additional ligated exons accumulated in Figure 5,

lanes 15–18, the rate-limiting step is either 3' substrate binding or the step requiring ATP, with all subsequent steps including the exon ligation reaction being faster.

DISCUSSION

Experiments in this article demonstrate that we have developed a biochemical assay in which exon ligation is both physically and kinetically uncoupled from spliceosome assembly and lariat formation. This system will thus be valuable for further mechanistic analysis of the second step of splicing. In our laboratory, we have already employed this assay to examine the extent to

which sequences adjacent to the AG consensus contribute to 3' splice site recognition (Anderson & Moore, 1997), and to test various models of how the correct AG is chosen when relatively distant from the branch site (Chen et al., 2000). The bimolecular exon ligation assay was also recently used to probe the function of exonic enhancer sequences (Chew et al., 1999).

A number of interesting findings arose from the experiments reported here. First, although some mammalian introns require the 3' splice site AG for spliceosome assembly and lariat formation, this early AG recognition is not requisite for subsequent exon ligation. In fact, there is no detectable AG recognition prior to lariat formation in the bimolecular system. This is entirely consistent with recent findings regarding the nature of the early AG recognition event in full-length constructs. Green and coworkers (Wu et al., 1999) have established that U2AF³⁵, the small subunit of the U2AF heterodimer, is the species responsible for early AG recognition in mammalian introns. Previously, the large subunit (U2AF⁶⁵) was known to bind the PPT and promote U2 snRNP association with the branch site to form complex A (Ruskin et al., 1988; Valcarcel et al., 1996). When the PPT is long enough, as in "AG-independent" introns, the U2AF⁶⁵/PPT interaction alone is apparently sufficient to allow recruitment of U2 snRNP. However, when the PPT is short or weak, as in "AG-dependent" introns, additional binding energy must be supplied by interaction of U2AF³⁵ with an AG immediately adjacent to the PPT (Wu et al., 1999).

The U2AF³⁵/AG contact is clearly crucial for spliceosome assembly on AG-dependent introns, but several results indicate that this interaction does not specify the AG that is used as the site of exon ligation. First, although the AG required for spliceosome assembly in AG-dependent introns is generally also the one used as the 3' splice site, at least in one case different AGs in the same intron can serve the two functions (Zhuang & Weiner, 1990). Second, despite U2AF³⁵ being well conserved across evolution (Zhang et al., 1992; Rudner et al., 1996; Wentz-Hunter & Potashkin, 1996; Zorio & Blumenthal, 1999), there is no protein with obvious sequence similarity in *Saccharomyces cerevisiae*. Apparently yeast have no functional equivalent to U2AF³⁵, as neither mutation of the 3' splice site AG nor intron truncation downstream of the branch site inhibits lariat formation on yeast introns (Rymond & Rosbash, 1985). Third, consistent with our finding here that early AG recognition is not required for exon ligation, U2AF reportedly dissociates from mammalian splicing complexes after U2 snRNP addition but prior to lariat formation (Bennett et al., 1992). This fits well with data showing that the 3' splice site region is protected from RNase H digestion early in mammalian spliceosome assembly (presumably by U2AF), becomes accessible in more

mature splicing complexes (when U2AF leaves), and then becomes protected again after lariat formation and prior to exon ligation (Sawa & Shimura, 1991). Notably, although protection of the 3' splice site region against RNase H digestion after lariat formation is observed in yeast splicing reactions (Schwer & Guthrie, 1992), there is no early protection event. This is also consistent with there being no U2AF³⁵ equivalent in *S. cerevisiae*.

A number of factors have been implicated in recognizing the 3' splice site AG nearer the time of exon ligation. These include the splicing factors Prp16p, Prp8p, and Slu7p (Teigelkamp et al., 1995; Umen & Guthrie, 1995; Chiara et al., 1997), U5 snRNA (Newman & Norman, 1992), and the mammalian AG75/p70 protein (Chiara et al., 1997; Wu & Green, 1997). Non-Watson-Crick base pairing between the 5' and 3' terminal intronic nucleotides (Parker & Siliciano, 1993) and interactions with U6 snRNA (Lesser & Guthrie, 1993; Collins & Guthrie, 1999) may also contribute to AG recognition. Although the relative timing of many of these events is unknown, the action of Prp16p is required for UV crosslinking of Prp8p and Slu7p to the 3' splice site AG in *S. cerevisiae* (Umen & Guthrie, 1995).

Prp16p mediates the ATP-dependent structural change in *S. cerevisiae* spliceosomes that renders the 3' splice site AG inaccessible to RNase H cleavage after lariat formation (Schwer & Guthrie, 1992). The human Prp16p ortholog was recently identified (Zhou & Reed, 1998) and the 3' splice site protection that occurs after lariat formation in mammalian splicing reactions (see above; Sawa & Shimura, 1991) is also ATP dependent. Prp16p has both signature sequences for an RNA helicase (Schwer & Guthrie, 1991) and demonstrable RNA unwinding activity (Wang et al., 1998), but its exact target in the spliceosome has not yet been identified. Given that the only ATP-dependent step known between lariat formation and exon ligation is mediated by Prp16p, it seems likely that the NTP-hydrolysis requirement we found in this study reflects the activity of hPrp16p. The exact nucleotide specificity of the human ortholog has not been reported, but yeast Prp16p exhibits a variable NTP specificity similar to the one we observed (Schwer & Guthrie, 1992).

Our data indicate that, unlike the earliest stage of spliceosome assembly, which commits the 5' splice site and branch site to the splicing pathway independent of ATP, commitment of the 3' splice site AG at the time of exon ligation is ATP dependent. This suggests that whatever the ATPase, the last hydrolysis event must occur coincident with or after AG recognition; otherwise there would be no ATP requirement for bimolecular exon ligation. Two possible models consistent with our findings are: (1) ongoing ATP hydrolysis is required to maintain the spliceosome in a conformational state capable of binding the 3' splice site AG and, once bound, exon ligation occurs rapidly or (2) recognition of the 3'

splice site AG somehow triggers ATP hydrolysis. In the latter scenario, the 3' splice site AG might serve as the signal to initiate the structural transition that results in 3' splice site protection to RNase H digestion (Sawa & Shimura, 1991; Schwer & Guthrie, 1992). However, we favor the former possibility, because in no case have we been able to observe any stable association of a 3' substrate with the spliceosome prior to exon ligation (data not shown), even when a noncleavable phosphate analog is incorporated at the 3' splice site. Such modified substrates do specifically compete with active 3' substrates, indicating that they are able to bind the active site (data not shown).

Ongoing studies are focused on identifying the required ATPase and species that recognize the 3' splice site AG at the time of exon ligation, as well as determining the minimum set of required factors when exon ligation is uncoupled from lariat formation. The ability to individually vary the times of addition of these factors and the 3' splice site AG will facilitate our overall understanding of the mechanisms of 3' splice site definition and exon ligation.

MATERIALS AND METHODS

Splicing reactions

All splicing reactions (unless noted otherwise) were carried out in 10–25 μ L aliquots as previously described (Anderson & Moore, 1997), except that either KCl or potassium acetate was used to adjust the potassium concentration to 60–80 mM (which gave optimal splicing activity in these extracts; see Reichert & Moore, 2000). Concentrations of 5' and 3' substrates were typically 35 and 175 nM, respectively, unless otherwise noted. Splicing intermediates and products were separated on denaturing polyacrylamide gels that were quantitated using a MolecularDynamics PhosphorImagerTM and the accompanying ImageQuant software. For splicing complexes, reactions were stopped by adding heparin to a final concentration of 0.5 mg/mL, and 3–5 μ L of each reaction were loaded directly onto a 4% nondenaturing polyacrylamide gel. Complexes were visualized by autoradiography.

ATP depletions

ATP was depleted by buffer exchange using 1 mL bed volume G-50 Sephadex (Gibco) spin columns. Columns were preequilibrated by washing three times with 0.5 mL splicing buffer (Anderson & Moore, 1997) without nuclear extract, ATP, creatine phosphate, or tRNA. Splicing reactions (100 μ L per column) that had been preincubated with 5' substrate for 30 min were applied to washed columns and centrifuged in a clinical tabletop centrifuge for 1 min at 300 rpm. Depleted reactions were resupplemented with tRNA, but not creatine phosphate. To determine the extent to which reactions had been depleted of free nucleotides, splicing reactions were supplemented with trace amounts of [α -³²P] ATP prior to loading on the spin column. A fluorescent PEI-cellulose chromatography plate was spotted with 1 μ L of the spiked reaction

before and after ATP depletion, as well as with [α -³²P] ATP and unlabeled ATP, ADP, and AMP as controls. The plate was developed in 300 mM sodium citrate and visualized by autoradiography and UV shadowing.

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REFERENCES

- Aebi M, Hornig H, Padgett RA, Reiser J, Weissmann C. 1986. Sequence requirements for splicing of higher eukaryotic nuclear pre-mRNA. *Cell* 47:555–565.
- Anderson K, Moore MJ. 1997. Bimolecular exon ligation by the human spliceosome. *Science* 276:1712–1716.
- Bennett M, Michaud S, Kingston J, Reed R. 1992. Protein components specifically associated with prespliceosome and spliceosome complexes. *Genes & Dev* 6:1986–2000.
- Chen S, Anderson K, Moore MJ. 2000. Evidence for a linear search in bimolecular 3' splice site AG selection. *Proc Natl Acad Sci USA*. In press.
- Chew SL, Liu H-X, Mayeda A, Krainer AR. 1999. Evidence for the function of an exonic splicing enhancer after the first catalytic step of pre-mRNA splicing. *Proc Natl Acad Sci USA*. In press.
- Chiara MD, Palandjian L, Kramer RF, Reed R. 1997. Evidence that U5 snRNP recognizes the 3' splice site for catalytic step II in mammals. *EMBO J* 16:4746–4759.
- Collins CA, Guthrie C. 1999. Allele-specific genetic interactions between prp8 and RNA active site residues suggest a function for prp8 at the catalytic core of the spliceosome. *Genes & Dev* 13:1970–1982.
- Frendewey D, Keller W. 1985. Stepwise assembly of a pre-mRNA splicing complex requires U-snRNAs and specific intron sequences. *Cell* 42:355–367.
- Gozani O, Patton JG, Reed R. 1994. A novel set of spliceosome-associated proteins and the essential splicing factor PSF bind stably to pre-mRNA prior to catalytic step II of the splicing reaction. *EMBO J* 13:3356–3367.
- Horowitz DS, Abelson J. 1993. Stages in the second reaction of pre-mRNA splicing: The final step is ATP independent. *Genes & Dev* 7:320–329.
- Jamison SF, Crow A, Garcia-Blanco MA. 1992. The spliceosome assembly pathway in mammalian extracts. *Mol Cell Biol* 12:4279–4287.
- Krämer A. 1995. The biochemistry of pre-mRNA splicing. In: Lamond AI, ed. *Pre-mRNA processing*. Austin, Texas: RG Landes Company, pp 35–64.
- Lang KM, Keller W. 1990. Sequence requirements in different steps of the pre-mRNA splicing reaction: Analysis by the RNA modification-exclusion technique. *Mol Cell Biol* 10:4942–4947.
- Legrain P, Séraphin B, Rosbash M. 1988. Commitment of yeast pre-mRNA to the spliceosome pathway does not require U2 snRNP. *Mol Cell Biol* 8:3755–3760.
- Lesser CF, Guthrie C. 1993. Mutations in U6 snRNA that alter splice site specificity—implications for the active site. *Science* 262:1982–1988.
- Maschhoff KL, Padgett RA. 1993. The stereochemical course of the first step of pre-mRNA splicing. *Nucleic Acids Res* 21:5456–5462.
- Michaud S, Reed R. 1991. An ATP-independent complex commits pre-mRNA to the mammalian spliceosome assembly pathway. *Genes & Dev* 5:2534–2546.
- Moore MJ, Query CC, Sharp PA. 1993. Splicing of precursors to mRNA by the spliceosome. In: Gesteland R, Atkins J, eds. *The*

- RNA world. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 303–357.
- Moore MJ, Sharp PA. 1993. The stereochemistry of pre-mRNA splicing: Evidence for two active sites in the spliceosome. *Nature* 365:364–368.
- Newman A, Norman C. 1992. U5 snRNA interacts with exon sequences at 5' and 3' splice sites. *Cell* 68:743–754.
- Parker R, Siliciano PG. 1993. Evidence for an essential non-Watson–Crick interaction between the first and last nucleotides of a nuclear pre-mRNA intron. *Nature* 361:660–662.
- Ramchatesingh J, Zahler AM, Neugebauer KM, Roth MB, Cooper TA. 1995. A subset of SR proteins activates splicing of the cardiac troponin T alternative exon by direct interactions with an exonic enhancer. *Mol Cell Biol* 15:4898–4907.
- Reed R. 1989. The organization of 3' splice-site sequences in mammalian introns. *Genes & Dev* 3:2113–2123.
- Reichert V, Moore MJ. 2000. Better conditions for mammalian in vitro splicing reactions provided by acetate and glutamate as potassium counterions. *Nucleic Acids Res*. In press.
- Rudner DZ, Kanaar R, Breger KS, Rio DC. 1996. Mutations in the small subunit of the *Drosophila* U2AF splicing factor cause lethality and developmental defects. *Proc Natl Acad Sci USA* 93:10333–10337.
- Ruskin B, Zamore PD, Green MR. 1988. A factor, U2AF, is required for U2 snRNP binding and splicing complex assembly. *Cell* 52:207–219.
- Rymond BC, Rosbash M. 1985. Cleavage of 5' splice site and lariat formation are independent of 3' splice site in yeast mRNA splicing. *Nature* 317:735–737.
- Sawa H, Shimura Y. 1991. Alterations of RNase H sensitivity of the 3' splice site region during the in vitro splicing reaction. *Nucleic Acids Res* 19:3953–3958.
- Schwer B, Guthrie C. 1991. PRP16 is an RNA-dependent ATPase that interacts transiently with the spliceosome. *Nature* 349:494–499.
- Schwer B, Guthrie C. 1992. A conformational rearrangement in the spliceosome is dependent on PRP16 and ATP hydrolysis. *EMBO J* 11:5033–5039.
- Sraphin B, Rosbash M. 1989. Identification of functional U1 snRNP–pre-mRNA complexes committed to spliceosome assembly and splicing. *Cell* 59:349–358.
- Teigelkamp S, Newman AJ, Beggs JD. 1995. Extensive interactions of PRP8 protein with the 5' and 3' splice sites during splicing suggest a role in stabilization of exon alignment by U5 snRNA. *EMBO J* 14:101–111.
- Umen JG, Guthrie C. 1995. Prp16p, Slu7p, and Prp8p interact with the 3' splice in two distinct stages during the second catalytic step of pre-mRNA splicing. *RNA* 1:584–597.
- Valcarcel J, Gaur RK, Singh R, Green MR. 1996. Interaction of U2AF65 RS region with pre-mRNA branch point and promotion of base pairing with U2 snRNA [corrected] [erratum: *Science*, 1996, 274:21]. *Science* 273: 1706–1709.
- Wang Y, Wagner JDO, Guthrie C. 1998. The DEAH-box splicing factor Prp16 unwinds RNA duplexes in vitro. *Curr Biol* 8:441–451.
- Wentz-Hunter K, Potashkin J. 1996. The small subunit of the splicing factor U2AF is conserved in fission yeast. *Nucleic Acids Res* 24:1849–1854.
- Wu S, Green MR. 1997. Identification of a human protein that recognizes the 3' splice site during the second step of pre-mRNA splicing. *EMBO J* 16:4421–4432.
- Wu S, Rumfo CM, Nilsen TW, Green MR. 1999. Functional recognition of the 3' splice site AG dinucleotide by U2AF³⁵. *Nature*. In press.
- Zhang D, Rosbash M. 1999. Identification of eight proteins that cross-link to pre-mRNA in the yeast commitment complex. *Genes & Dev* 13:581–592.
- Zhang M, Zamore PD, Carmo-Fonseca M, Lamond AI, Green MR. 1992. Cloning and intracellular localization of the U2 small nuclear ribonucleoprotein auxiliary factor small subunit. *Proc Natl Acad Sci USA* 89:8769–8773.
- Zhou Z, Reed R. 1998. Human homologs of yeast prp16 and prp17 reveal conservation of the mechanism for catalytic step II of pre-mRNA splicing. *EMBO J* 17:2095–2106.
- Zhuang Y, Weiner AM. 1990. The conserved dinucleotide AG of the 3' splice site may be recognized twice during in vitro splicing of mammalian mRNA precursors. *Gene* 90:263–269.
- Zorio DA, Blumenthal T. 1999. U2AF35 is encoded by an essential gene clustered in an operon with RRM/cyclophilin in *Caenorhabditis elegans*. *RNA* 5:487–494.