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The ATP requirement for U2 snRNP addition is linked to the pre-mRNA region 5′ to the branch site

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ABSTRACT

Association of U2 snRNP with the pre-mRNA branch region is the first ATP-dependent step in spliceosome assembly. The basis of this energy dependence is not known. Previously, we identified minimal intron-derived substrates that form complexes with U2 independent of ATP. Here, we identify the intron region linked to the ATP dependence of this step by comparing these substrates to longer RNAs that recapitulate the ATP requirement. This region needed to impose ATP dependence lies immediately 5′ to the branch site. Sequences ranging from 6 to 14 nt yield a near linear inhibitory effect on efficiency of complex formation with U2 snRNP, with 18 nt yielding near maximal ATP dependence. This region is not protected prior to U2 addition, and RNase H targeting of the region within nuclear extract converts an ATP-dependent substrate into an ATP-independent one. Within this region, there is no sequence specificity linked with the ATP requirement, as neither a specific sequence is needed, nor even nucleobases. These data and the results of other modifications suggest models in which the 18-nt region is a target for interactions with U2 snRNP in an ATP-bound or -activated conformation.

Keywords: anchoring site; ATP; intron; pre-mRNA; spliceosome; U2 snRNP

INTRODUCTION

The removal of introns from nascent RNA transcripts (pre-mRNA) is catalyzed by the spliceosome, a dynamic 50-60S complex. U1, U2, U5, and U4/6 small nuclear ribonucleoprotein (snRNP) particles are involved in an ordered pathway of spliceosome assembly (reviewed in Moore et al., 1993; Nilsen, 1998; Burge et al., 1999), during which numerous rearrangements of snRNAs, pre-mRNA, and proteins occur (reviewed in Staley & Guthrie, 1998). Most of these transitions are likely coupled to ATP hydrolysis by helicase-like proteins (reviewed in Schwer, 2001). However, the exact events that result in such rearrangements are not well understood.

The spliceosome assembles de novo on each pre-mRNA molecule, and distinct intermediates in the assembly pathway can be observed in vitro. The first binding events form the commitment or early complex (CC or E) and are ATP independent. In this complex, the 5′ splice site is recognized by U1 snRNP and mammalian proteins SF1 and U2AF^{65}, or yeast proteins BBP and MUD2, bind the branch region and pyrimidine tract, respectively. In the mammalian system, recent evidence suggests that U2 snRNP is loosely associated at this time, but not yet stably engaged (Das et al., 2000), although such an association has not been detected in yeast (Liao et al., 1992). In both mammals and yeast, the first ATP-dependent step is the stable binding of U2 snRNP to the pre-mRNA branch site (Cheng & Abelson, 1987; Konarska & Sharp, 1987; Krämer, 1988; Pruzan et al., 1990; Michaud & Reed, 1991; Liao et al., 1992), in part, through U2 snRNA•branch region base pairing (Parker et al., 1987; Wu & Manley, 1989; Zhuang & Weiner, 1989). This forms complex A in the mammalian system. Subsequently, a larger complex, B, is formed by association of U4/U5/U6 tri-snRNP. Complex C follows B after significant rearrangements and is the active spliceosome, containing U2/5/6 snRNPs and splicing intermediates (reviewed in Moore et al., 1993).

In the mammalian system, both the mechanistic basis for the ATP requirement during U2 snRNP addition and the factor(s) utilizing the ATP remain unclear. U2 snRNP is a 17S complex composed of U2 snRNA and approximately 20 proteins that form ordered subdomains

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(Krämer et al., 1999; reviewed in Will & Lührmann, 1997). A major U2 protein component, SF3, composed of two heteromeric complexes SF3a and SF3b, has been implicated in stabilization of the U2 particle via contact to the RNA 5′ to the branch region (also called the anchoring region; Gozani et al., 1996). One SF3b protein, SF3b-155, additionally contacts the RNA on the 3′ side of the branch region (Gozani et al., 1998), and another SF3b-associated protein, U2-p14, contacts RNA within the branch region (Will et al., 2001). In both yeast and mammals, SF3 can dissociate from U2 snRNP in vitro, indicating that a remodeling of the particle may be important for its function (Krämer, 1996; Pauling et al., 2000). Other factors, including U2AF^{35} and SR proteins, also contribute to stable U2 snRNP binding. Yeast U2 addition also requires the presence of two DExH/D ATPases (Prp5p and Sub2p), but their targets are not yet known (Ruby et al., 1993; Kistler & Guthrie, 2001; Libri et al., 2001; Zhang & Green, 2001).

Previously, we had recognized that a small RNA containing only a branch sequence and pyrimidine tract (BS-PPT RNA; Fig. 1A) was an efficient substrate for U2 addition (Query et al., 1997). Formation of these A-like or "Amin" complexes on BS-PPT RNA required similar elements and factors as the full-length substrate. Both the branch site and pyrimidine tract were necessary and required in the correct orientation; U2 snRNA was required for, and present in, the complexes formed on BS-PPT RNA; protein factors such as U2AF^{65} and other SR proteins were required; and proteins that crosslinked from the branch site adenosine were the same as on full-length substrates. However, complex formation on the minimal RNA did not require ATP. This difference between the short BS-PPT RNA and the full pre-mRNA suggests that some additional feature of the pre-mRNA imposes the requirement of ATP in adding U2 snRNP.

In this study, we have used additions to the minimal ATP-independent substrate to determine the pre-mRNA region responsible for the ATP requirement. Only a short sequence 5′ to the branch site is needed to impose the ATP requirement, with 18 nt yielding near-maximal ATP dependence. Within this region, neither specific sequences nor even nucleobases are needed to confer ATP dependence. These data and the results of other modifications suggest models in which the 18-nt region is a target for interactions that can only occur with an ATP-bound conformation of U2 snRNP.

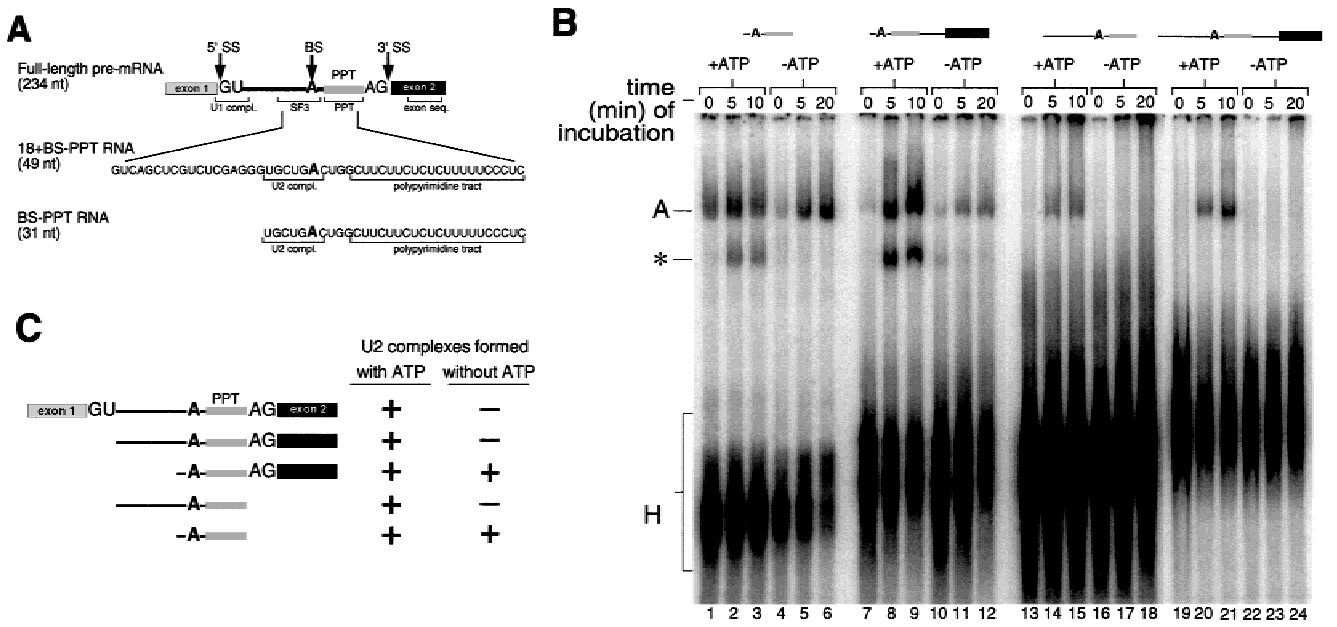

FIGURE 1. Effects of ATP on U2 complex formation are related to sequences flanking the BS-PPT. **A:** Schematic comparison of RNAs that form complex A (full-length pre-mRNA, upper), the minimal ATP-dependent complex A (18+BS-PPT RNA, middle), and the ATP-independent Amin complex (BS-PPT RNA, lower). The region coupled to ATP dependence of U2 addition is indicated underneath. Regions that promote formation of complex A on pre-mRNA are bracketed. SS: splice site; BS: branch site adenosine; PPT: polypyrimidine tract; U1 compl.: region complementary to U1 snRNA; SF3: binding site for SF3a and SF3b components; U2 compl.: region complementary to U2 snRNA; exon seq.: exon sequences that typically include enhancer elements, SR protein binding sites, and/or downstream 5′ splice sites. **B:** Formation of U2-containing complexes on RNAs with or without 50-nt sequences either 5′ or 3′ to the BS-PPT. BS-PPT RNA (lanes 1–6), 50+BS-PPT RNA (lanes 13–18), BS-PPT+50 RNA (lanes 7–12), or 50+BS-PPT+50 RNA (lanes 19–24) were incubated in HeLa nuclear extract at 30 °C for the times indicated, adjusted to 0.5 mg of heparin per mL, and separated on a native 4% polyacrylamide gel. A: U2 snRNP complexes; H: nonspecific complexes; *: a faster-migrating complex observed on ATP-independent substrates in the presence of ATP. **C:** Summary of sequences and ATP requirements from **B**.

RESULTS

U2 snRNP binding and ATP-dependent steps can be separated

Previously, we had demonstrated that a small RNA consisting only of a consensus branch site and pyrimidine tract (BS-PPT RNA) efficiently formed complexes with U2 snRNP. One unanticipated property of this complex was its formation independent of ATP. Because this is not observed with full-length pre-mRNA, it suggests, in principle, that ATP dependence is linked in some way to regions of the pre-mRNA not present in the minimal BS-PPT RNA.

To test this, we examined the ability of pre-mRNAs truncated on either side of the branch region to form complexes with U2 snRNP independent of ATP (summarized in Fig. 1C). RNAs containing intron sequence 5′ to the branch site did not form such ATP-independent complexes with U2 but did form complexes in the presence of ATP, regardless of the presence or absence of additional sequences 3′ to the PPT (Fig. 1B, cf. lanes 16–18 to lanes 13–15 and 22–24 to 19–21). In contrast, RNAs lacking any sequence 5′ to the branch region did form complexes with U2 in the absence of ATP, regardless of the presence or absence of sequence 3′ to the PPT (Fig. 1B, lanes 4–6 and 10–12). RNAs without sequence 5′ to the branch region also formed low levels of a faster migrating complex in the presence of ATP, which we have not characterized (Fig. 1B, ∗). Full-length pre-mRNA only formed complexes in the presence of ATP, and mutation of either branch region or pyrimidine tract abolished formation of complexes, as expected (data not shown). It should also be noted that the ATP-dependent complexes formed on substrates with sequence 5′ to the branch region but not 3′ to the PPT were unstable at later time points (data not shown). This ATP-dependent dissociation has previously been described for the minimal BS-PPT substrate (Query et al., 1997). This destabilization is distinct from the ATP-dependent complex formation on substrates with sequence 5′ to the branch region. We conclude that the intron region 5′ to the branch site is linked to the ATP requirement for U2 snRNP association.

A short region 5′ to the branch site is sufficient to impose the ATP requirement for U2 binding

To localize what region 5′ to the branch site was responsible for the ATP requirement, we first tested formation of U2-containing complexes on RNAs that included either 44, 28, or 14 nt 5′ to the branch region in comparison to no sequences added (Fig. 2A). In contrast to the efficient formation of complexes on BS-

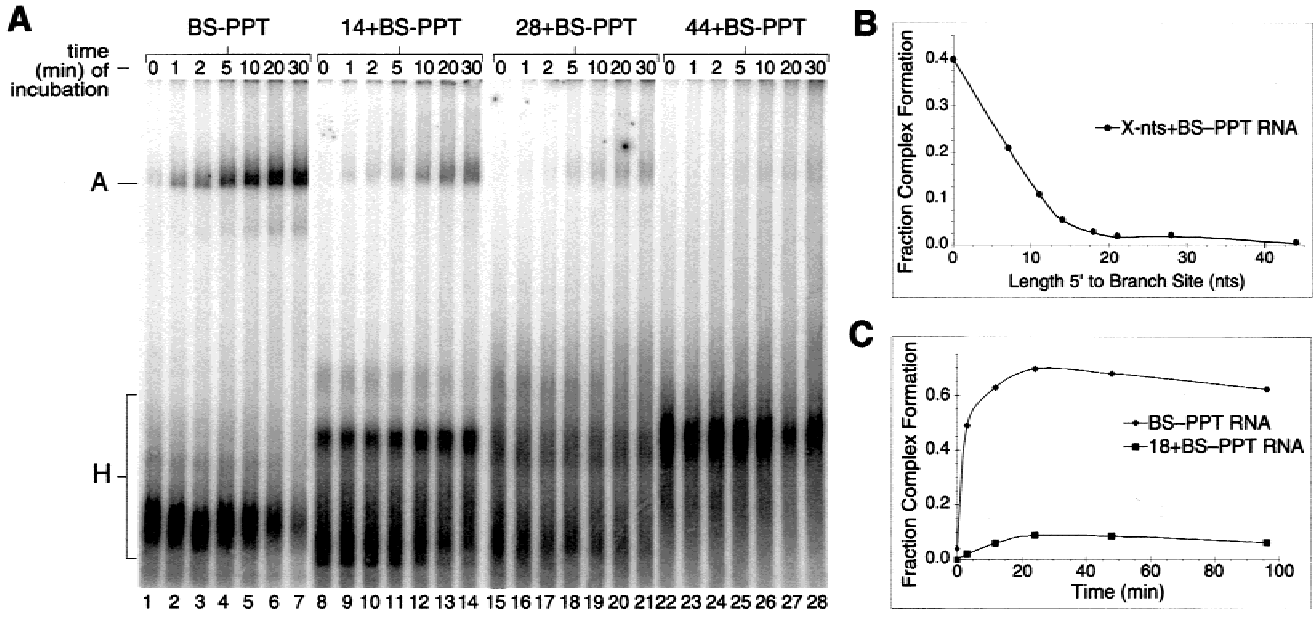

FIGURE 2. Analysis of length of sequence 5′ to the BS-PPT required to confer ATP dependence. **A:** BS-PPT RNA (lanes 1–7), 14+BS-PPT RNA (lanes 8–14), 28+BS-PPT RNA (lanes 15–22), or 44+BS-PPT RNA (lanes 22–28) were incubated in HeLa nuclear extract depleted of ATP at 30°C for the times indicated, adjusted to 0.5 mg of heparin per mL, and analyzed as described for Figure 1B. **B:** Graphical analysis of formation of U2 complexes on RNAs containing 0, 6, 11, 14, 18, 22, 28, or 44 nt 5′ to the branch region. All RNAs were incubated in nuclear extract at 30°C across time courses and analyzed as in **A**. Relative complex formation was determined as the fraction of maximal complex A formation (corresponding in each case to 30 min of incubation) relative to the total input RNA. X: number of nucleotides added 5′ to the branch sequence. **C:** Kinetics of formation of complexes on BS-PPT RNA (—●—) versus 18+BS-PPT RNA (—■—).

PPT RNA (40% of input RNA after 30 min of incubation), complexes were nearly undetectable on an RNA containing 44 additional nt (0.6%), greatly reduced on an RNA containing 28 additional nt (1%), and intermediate on an RNA containing 14 additional nt (5%). These data suggest that the region responsible for ATP dependence lies immediately 5′ to the branch region.

To more precisely define the length of RNA required for this effect, we next tested RNAs containing 6, 11, 14, 18, or 22 nt 5′ to the branch region (Fig. 2B). Even for the 6-nt 5′ extension, there was a loss of approximately half the complexes, from 40 to 21%, and longer extensions formed progressively fewer complexes. The relationship between length of sequence added and decrease in complexes formed without ATP was close to linear up to 14 nt, and nearly maximum dependence on ATP was obtained with 18 nt.

The difference in amounts of complex formed with differing length RNAs might reflect either a difference in the absolute amount of complexes formed (i.e., equilibrium) or a difference in kinetics. To distinguish these, longer time courses were compared for RNAs either with or without 18 additional nt (Fig. 2C). The difference in formation of complexes in the absence of ATP did not represent kinetic differences of the different length RNAs, but rather an absolute effect on the amount of U2 complex formed. Thus, we conclude that a short region immediately upstream of the branch site is responsible for the coupling of U2 snRNP addition to the ATP requirement. We chose a length of 18 nt for further characterization.

The coupling of ATP and U2 addition is sequence independent

The strict dependence of the ATP requirement on the region upstream of the branch site suggests that this region is a target for the ATP-dependent activity, either directly or indirectly. A number of possible scenarios by which this could happen can be envisioned. One possibility is that some factor binds in a sequence-specific fashion and must be removed to allow access to U2 snRNP. Although there is little sequence bias in this region in natural introns (Burge et al., 1999) and no apparent sequence requirement was observed previously for complex A formed on full-length pre-mRNAs (Gozani et al., 1996), such a scenario is known to occur in an adenovirus pre-mRNA, where binding of a protein factor to this exact region regulates U2 snRNP access and thus splicing of the intron (Kanopka et al., 1996, 1998). To test for this possibility with the sequence used here, the 18-nt region was added in *trans* to reactions depleted of ATP to potentially compete out binding of such a factor. This did not result in ATP independence of U2 binding to RNAs containing this region (e.g., 18+BS-PPT RNA). Nor did addition of this sequence in *trans* with BS-PPT RNA result in ATP dependence (data not shown). In contrast, addition in *trans* of PPT RNA, which binds to protein factor U2AF^{65}, did compete out formation of U2 complexes on the same RNAs, as expected, either with or without ATP (not shown; see Fig. 2B in Query et al., 1997). Both above results demonstrate that the 18-nt region is required in *cis* with BS-PPT RNA to confer ATP dependence, and are consistent with there being no sequence specificity within the 18-nt region to confer ATP-dependence.

To test directly for a sequence-specific effect of this region when present in *cis* with the BS-PPT RNA, the original sequence was replaced with its reverse complement, or with a different, unrelated sequence (Fig. 3A). Without ATP, U2 complex formation on these new substrates was similarly inefficient as on the original substrate (Fig. 3B, cf. lanes 7–9 and 10–12 to lanes 4–6), although both new substrates did form U2 complexes in the presence of ATP (Fig. 3B, lanes 15–16). Thus, the ATP requirement is not limited to a specific sequence. Although we have not exhaustively altered this sequence, this conclusion is confirmed by results below demonstrating that nucleobases are not required for the conveyance of ATP dependence.

A second possible explanation for the ATP requirement could be related to inhibitory secondary structure

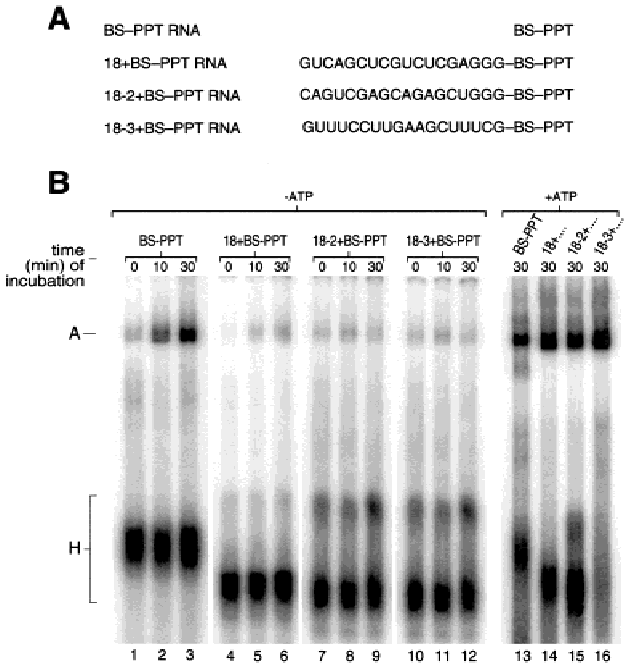

FIGURE 3. The coupling of ATP and U2 addition is sequence independent. **A:** Comparison of sequences altered 5′ to the branch region and analyzed in **B**. 18+BS-PPT RNA contains the original sequence 5′ to the branch region, as given in Figure 1A. 18-2+BS-PPT RNA contains the reverse complement of the first 15 nt of 18+BS-PPT RNA. 18-3+BS-PPT RNA contains a different sequence within the first 18 nt (derived from sequences used 5′ to the branch region in Gozani et al., 1996). **B:** RNAs from **A** were incubated in nuclear extract at 30 °C for the times indicated, either in the absence of ATP (lanes 1–12) or in the presence of ATP (lanes 13–16), and analyzed as in Figure 1B.

in the sequence 5′ to the branch site. In this case, ATP would be required to remove this structure, enabling U2 snRNP to engage the RNA. Although we cannot rule out the existence of any structure involving this region, the above results using different sequences argue against a specific structure (Fig. 3). More importantly, an abasic sugar–phosphate backbone, as mentioned above, still imposed ATP dependence of complex formation (see Fig. 6), arguing strongly that features other than secondary structure impose the ATP requirement.

A third scenario, but related to the first, is that the 18-nt region is bound in a sequence-independent fashion prior to U2 complex formation and thereby inaccessible to U2 snRNP. To address this possibility, we asked whether the 18-nt region could be cleaved by RNase H within the extracts. After incubation of 18+BS-PPT RNA in HeLa nuclear extract depleted of ATP, the availability of the 18-nt region was tested by addition of an antisense DNA oligonucleotide and RNase H during a second incubation. Analysis of a portion of this reaction by denaturing gel demonstrated that the 18-nt region was accessible to the DNA oligonucleotide and RNase H, as evidenced by cleavage to a size similar to BS-PPT RNA (Fig. 4B, cf. lanes 1 and 5–6). Mock RNase H treatment (either without RNase H or without DNA oligonucleotide) did not result in efficient cleavage (Fig. 4B, lanes 5 and 8). RNase H treatment using a different DNA oligonucleotide (of irrelevant sequence; Fig. 4B, lane 7) resulted in some increased degradation, but not efficient and specific cleavage. Cleavage reactions were also examined on a native gel to test for ability of these cleaved RNA substrates to form complexes with U2 snRNP. Indeed, after cleavage of the 18-nt region, the RNA did efficiently form complexes with U2, in contrast to the similarly incubated control reactions (Fig. 4C, lanes 5–8). Thus, the 18-nt region is accessible to hybridization and degradation in nuclear extracts; and,

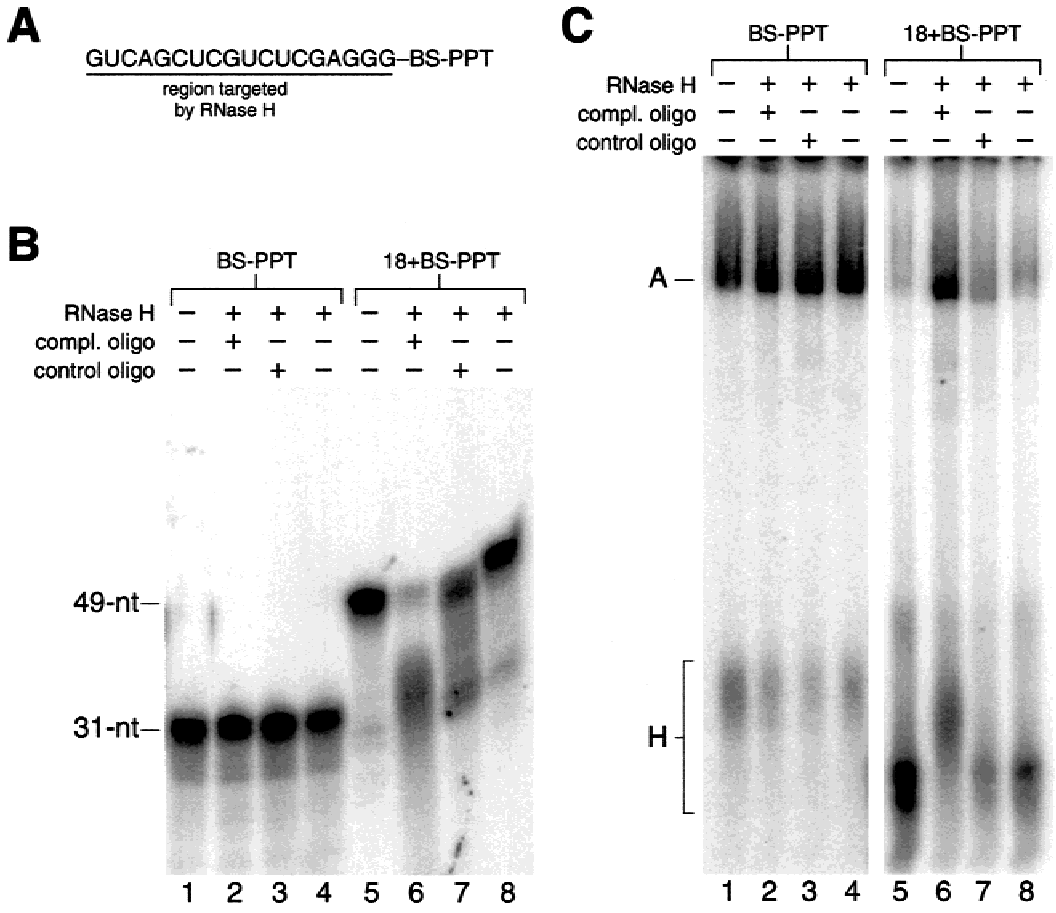

FIGURE 4. The 18-nt region is accessible prior to ATP-dependent complex formation. **A:** Schematic representation of the region cleaved by a complementary oligonucleotide and RNase H. **B:** Labeled BS-PPT or 18+BS-PPT RNAs were incubated in HeLa nuclear extracts at 30°C for 30 min (lanes 1 and 5). Aliquots of these reactions were further RNase H treated with either an oligonucleotide complementary to the 5′ region (lanes 2 and 6), or an irrelevant sequence (lanes 3 and 7), or no oligonucleotide as a controls (lanes 4 and 8) and incubated for 30 min at 30°C. RNA was extracted and degradation of labeled RNAs analyzed on a 20% 8 M-urea polyacrylamide gel. RNAs were visualized using a Molecular Dynamics PhosphorImager. **C:** Samples of the reactions in **B** were separated on a native 4% polyacrylamide gel, and quantified as in Figure 2B.

removal of this sequence converts an otherwise ATP-dependent substrate into an ATP-independent substrate for U2 addition.

Nucleotide requirements for U2 addition

To assess the NTP requirement of the U2 addition reaction, RNAs containing the 18-nt region upstream of the branch site were incubated in nuclear extracts depleted of ATP, and supplemented with each of the four rNTPs or rITP, with dNTPs, or with nonhydrolyzable ATP analogs (summarized in Fig. 5). Among rNTPs, there was a clear preference for ATP over CTP or GTP (approximately twofold), and stronger preference over UTP or ITP (approximately four- to fivefold). The same pattern of usage was observed for the dNTPs, except that generally all dNTPs stimulated somewhat more U2 complexes than rNTPs. Nonhydrolyzable analogs, AMP-cPP, AMP-PcP, or AMP-PnP, could not replace ATP, although these analogs most likely did not bind, as they did not compete against ATP (data not shown). ATPγS, which is hydrolyzed slowly compared to ATP, could replace ATP with equivalent efficiency. This is essentially the same result as obtained in yeast (Liao et al., 1992) and previously in mammalian systems for complex A formation (Pruzan et al., 1990; Tazi et al., 1992). Thus, ATP binding, rather than hydrolysis, is the feature required to allow U2 binding. Alternatively, hydrolysis is not rate limiting under the conditions used.

A sugar–phosphate backbone is sufficient to impose the ATP requirement for U2 snRNP binding

Because no sequence dependence within the 18-nt region was detected, we next asked whether other RNA elements made specific contributions to the ATP requirement. To examine the role of the nucleobases, we replaced the RNA with abasic residues (a deoxyribose phosphate backbone without nucleobases) or DNA as a control for the 2′ position of the abasic residues (Fig. 6A). In the absence of ATP, formation of U2-containing complexes on RNAs with 18 abasic residues was similarly inefficient as on the all-RNA substrate (Fig. 6C, cf. lanes 15–21 to 8–14) or the substrate containing 18 DNA residues (Fig. 6C, lanes 22–28), in comparison to BS-PPT RNA (Fig. 6C, lanes 1–7). In contrast, in the presence of ATP, these substrates did form complexes with U2 snRNPs, confirming that they are viable substrates for U2 addition (Fig. 6D). We conclude from these data that nucleobases are not required within the 18-nt region to confer ATP dependence. Thus, ATP is not used to disrupt interactions involving the bases, nor are the bases needed for the ATP to act. Further, this result demonstrates that a sugar–phosphate backbone alone is sufficient to couple the ATP requirement with U2 snRNP binding.

We next examined the role of the sugar–phosphate backbone. The 18-nt region was replaced with 18 spacers composed of three carbons interspersed with a phosphate (C3), the same spacing between phosphates as in natural sugar–phosphate backbones, and the ability to form complexes with U2 snRNP was tested (Fig. 6A). Similar to the all-RNA, or the DNA- or abasic-containing substrates, in the absence of ATP, formation of U2 complexes on substrates containing C3 residues was inefficient, although consistently improved (approximately threefold) over the all-RNA substrate. This indicates that the lack of ribose in the backbone does not alleviate the ATP requirement (Fig. 6C, lanes 29–35). However, in contrast to all-RNA, or the DNA- or abasic-containing substrates, complexes did not form efficiently in the presence of ATP on the C3-containing substrate (Fig. 6D). This suggests that a sugar–phosphate backbone is required to couple the ATP requirement with U2 snRNP binding.

The above results might have been due to the abasic or spacer regions nonspecifically interfering with U2 binding. However, several control experiments suggested that the abasic and C3 spacer RNAs neither blocked access to the branch region nor generally affected the ability of U2 to bind to an RNA. First, the RNAs containing abasic or spacer residues could hybridize to an antisense oligonucleotide with similar efficiency to BS-PPT RNA (or 18+BS-PPT), indicated both by native gel shifts and by their abilities to undergo template-directed RNA ligation with comparable efficiencies (data not shown). Second, addition of the abasic or spacer sequences in *trans* did not inhibit binding of U2 snRNP to 18+BS-PPT RNA or to BS-PPT RNA (with and without ATP, respectively). This indicates that the modified regions did not generally disrupt the ability of U2 snRNP to interact with branch sites.

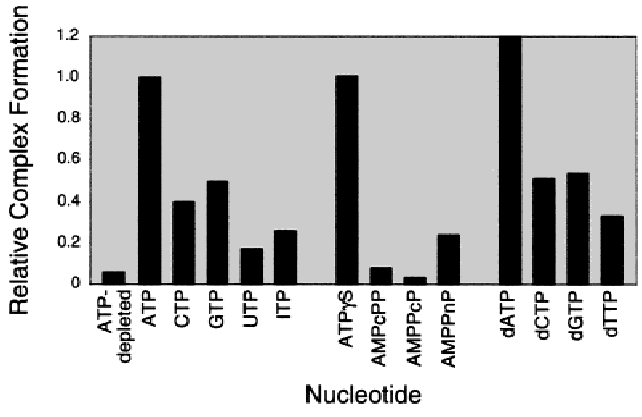

FIGURE 5. NTP requirement for U2 addition. Graphical representation of formation of U2 complexes on 18+BS-PPT RNA in the presence of various nucleoside triphosphates or analogs. HeLa nuclear extract was depleted of ATP, then incubated with labeled RNA at 30 °C for 30 min in the presence of the NTP indicated. ATPγS: adenosine 5′-*O*-(3-thiotriphosphate); AMPcPP: adenylyl-(α,β-methylene)-diphosphonate; AMP-PcP: adenylyl-(β,γ-methylene)-diphosphonate; AMP-PnP: adenylyl-imidodiphosphate.

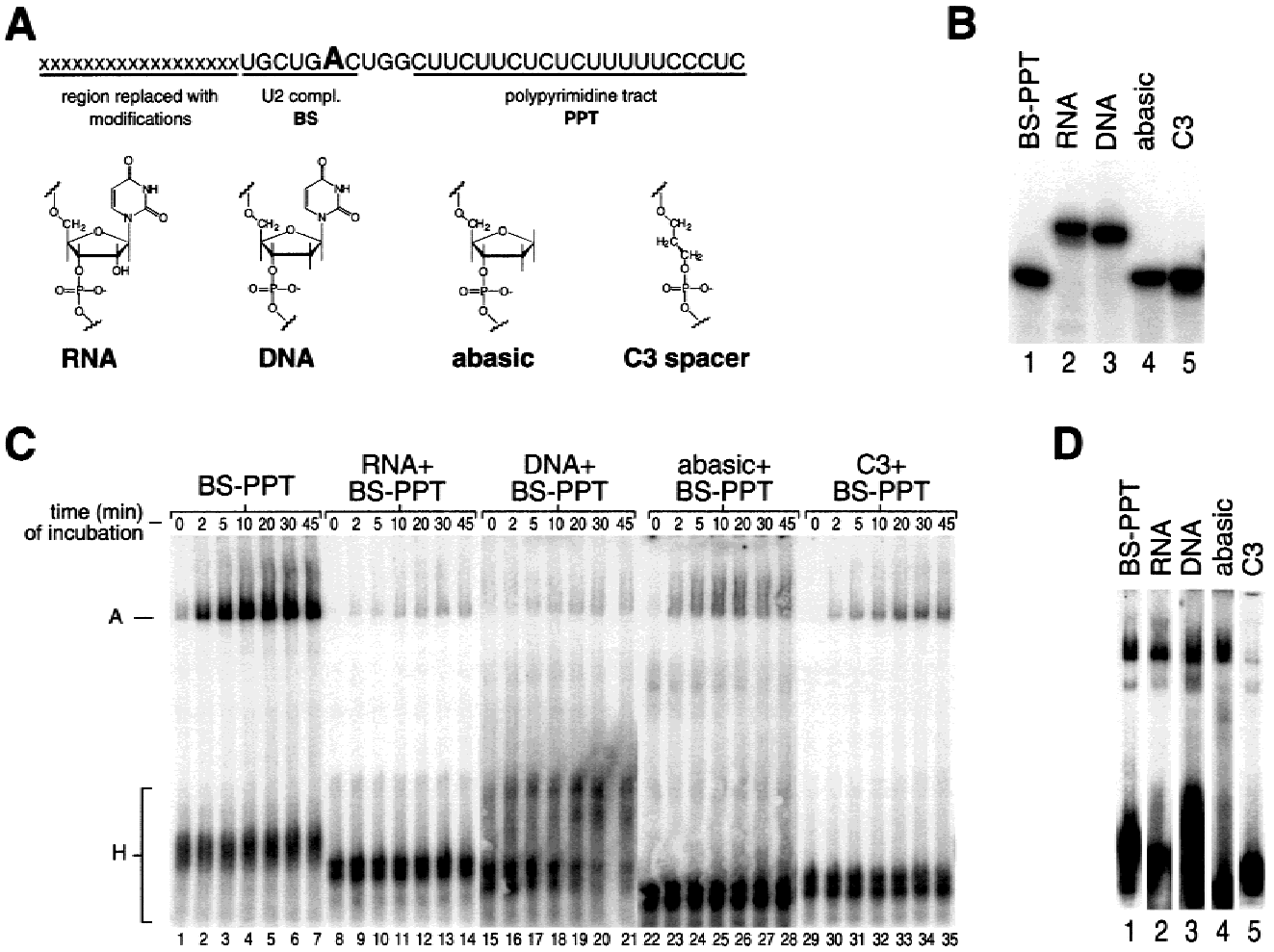

FIGURE 6. Role of nucleobases and sugar–phosphate backbone. **A:** Schematic representation of modified RNAs. Upper: sequence of the RNAs used, with the region coupled to ATP dependence, which was replaced by modified residues, indicated by x. Lower: representation of the modified nucleotides. For the RNA and DNA, only a uracil nucleobase is shown, although all four nucleobases were represented in the actual sequence (as in Fig. 1A). **B:** Denaturing gel analysis of the modification-containing RNAs, described in **A** and used in **C** and **D**. RNAs were prepared by chemical synthesis and template-mediated RNA ligation, as described in Material and Methods, and analyzed on a denaturing 20% polyacrylamide (19:1) gel. All modified RNAs migrated as expected according to their mass/charge ratio. **C:** Formation of U2-containing complex in the absence of ATP on RNAs containing modifications 5′ to the BS-PPT. RNAs contained either no sequence 5′ to the branch region (BS-PPT RNA; lanes 1–7), or 18 nt of RNA (RNA+BS-PPT RNA; lanes 8–14), 18 nt of DNA (DNA+BS-PPT RNA; lanes 15–21), 18 abasic residues (abasic+BS-PPT RNA; lanes 22–28), or 18 residues of C3 spacer (C3+BS-PPT RNA; lanes 29–35). These RNAs were incubated in HeLa nuclear extract at 30 °C for the times indicated, adjusted to 0.5 mg of heparin per mL, and separated on a native 4% polyacrylamide gel. A: U2 snRNP complexes; H: nonspecific complexes. **D:** Formation of complexes in the presence of ATP on modified RNAs as in **C**.

DISCUSSION

Analysis of an ATP-dependent step

Rearrangements of snRNAs and snRNP proteins are required for formation of the active spliceosome. Biochemical systems recapitulating each of these events will be useful for understanding the underlying mechanisms. Previously, several spliceosome rearrangement events have been studied by manipulating the protein components required for or likely to mediate them. These include (1) an event between the two catalytic steps of splicing that results in changes in 3′ splice site protection, dependent upon the DExH/D protein Prp16p and ATP (Schwer & Guthrie, 1992); (2) the reformation of U4/6 di-snRNP complexes postsplicing, dependent on the RNA-binding protein Prp24p (Raghunathan & Guthrie, 1998b); and (3) the dissociation of U4/6 base airs dependent upon the DExH/D protein Brr2 (Raghunathan & Guthrie, 1998a). These studies have specifically tied function of these proteins to these events. Here, we manipulate the pre-mRNA to identify an RNA region that imposes the ATP requirement of U2 snRNP addition; in its absence the ATP-dependent step is bypassed for access of U2 snRNP to the branch region.

The addition of U2 snRNP to the pre-mRNA to form complex A is the first ATP-dependent step of spliceosome assembly. On short BS-PPT substrates, however, addition of U2 is ATP independent, thus arguing that a region of the pre-mRNA (external to the branch region or PPT) must be linked to this ATP-requirement or event. In this study, we compared the ATP-independent substrate to ATP-dependent substrates in order to identify this region, which we located immediately 5′ to the branch sequence. Addition of sequences as short as 6 nt to the 5′ region results in a significant ATP requirement, and 18 nt recapitulate nearly complete dependence on ATP. Because the ATP-dependent and -independent substrates differ in a minimal way, this provides a simple system to study one of the early ATP-dependent events of spliceosome assembly.

No sequence dependence was detected for this U2 addition reaction, consistent with the general absence of sequence bias within this region in introns (Burge et al., 1999) and with other studies that altered this sequence without affecting the ability to form complex A (Gozani et al., 1996). It has previously been shown that blocking this region, either by binding to an antisense 2′-*O*-Me oligonucleotide in vitro or to proteins in vivo, results in failure to bind U2 snRNP and to undergo splicing (Gozani et al., 1996; Kanopka et al., 1996, 1998). This is consistent with the recent observation that certain purine-rich sequences within this region 5′ to the branch site do not support complex A formation in a *trans*-splicing system (Ast et al., 2001). Thus, there may exist a variety of ways in which this region may be used to block spliceosome assembly, for example, by protein binding or RNA structure, that are specific to individual intron sequence.

The 5′ region imposes the ATP requirement

What does an ATP requirement imposed by the region 5′ to the branch site mean? This can be envisioned in two different mechanistic scenarios: (1) an ATP-dependent action is required on the substrate RNA or RNA–protein complex to make it available for U2 binding; or (2) an ATP-dependent action upon U2 snRNP is required to allow it to interact with this region.

The first of these could be either the ATP-dependent disruption of RNA structure involving the 5′ region or the ATP-dependent removal of a protein. Because no specific sequence is required here, nor are nucleobases, it seems highly unlikely that RNA structure is involved. It also seems unlikely that a protein could be stabilized by interactions with the bases. The significant possibility that remains is that a protein bound to the branch region, such as SF1/BBP (Berglund et al., 1997), is stabilized by interactions with phosphates within the 5′ region and must be removed. This would have to occur in such a way that without these phosphate interactions, removal of the protein could occur independent of ATP, but with these additional phosphate interactions, an ATP-dependent event would be necessary. Evidence from yeast mutant in the Cus2p-component of U2 snRNPs, as described below, argues against this possibility. However, there is no direct evidence for or against this possibility in mammalian systems, and additional studies will be required to resolve this issue.

The second scenario seems more likely (Fig. 7). Here, action of ATP binding or hydrolysis may alter the structure of U2 snRNP in such a way that allows U2 binding to RNA containing the region 5′ to the branch site. For example, without ATP, components of U2 that normally engage this region may be in a “closed” state and sterically block stable binding to RNAs containing the 5′ region. In this closed state, U2 may bind to RNA lacking any sequence 5′ to the branch region, because the 5′ region is not present to sterically interfere. With ATP, these components may adopt a different, “open” conformation, now without the steric block to binding the 5′ region. This conformation would also allow protein–RNA interactions to contribute to binding. This scenario is consistent with the description of two different states of U2 snRNPs [two different RNA conformations in yeast (Ares & Igel, 1990; Yan & Ares, 1996) and increased access to RNase H cleavage in the presence of ATP in both yeast and mammals (Black et al., 1985; O’Day et al., 1996; Wiest et al., 1996)] and with the ultimate deposition of protein components of U2 snRNP (SF3 proteins) on the 5′ region (Gozani et al., 1996). Furthermore, this second scenario (and not the first) is supported by the formation of ATP-independent complex A in yeast extracts made from deletion mutants in the U2 protein Cus2p (Perriman & Ares, 2000); that is, an alteration in U2 structure, either directly or indirectly due to absence of Cus2p, can allow U2 addition to pre-mRNA without ATP. This model is similar to and consistent with that proposed for two different states of yeast U2 snRNPs based on access to the branch-pairing region (O’Day et al., 1996; Wiest et al., 1996).

However, the role of the region 5′ to the branch region also seems more complex. As discussed above, the data of Figure 6 argue that the presence of any modification used in this region resulted in the inability to form complexes with U2 snRNP in the absence of ATP, consistent with the steric block model in Figure 7. But, removal of the sugar backbone (C3-spacer) resulted in a substrate not responsive to the presence of ATP. Thus, the ribose-phosphate backbone may play a requisite role in the ATP-dependent process (e.g., stimulating or stabilizing), but further studies will be needed to dissect this role.

What may use the ATP?

Factors that may use ATP include phosphorylases and DExD/H ATPases. The DExD/H proteins couple ATP

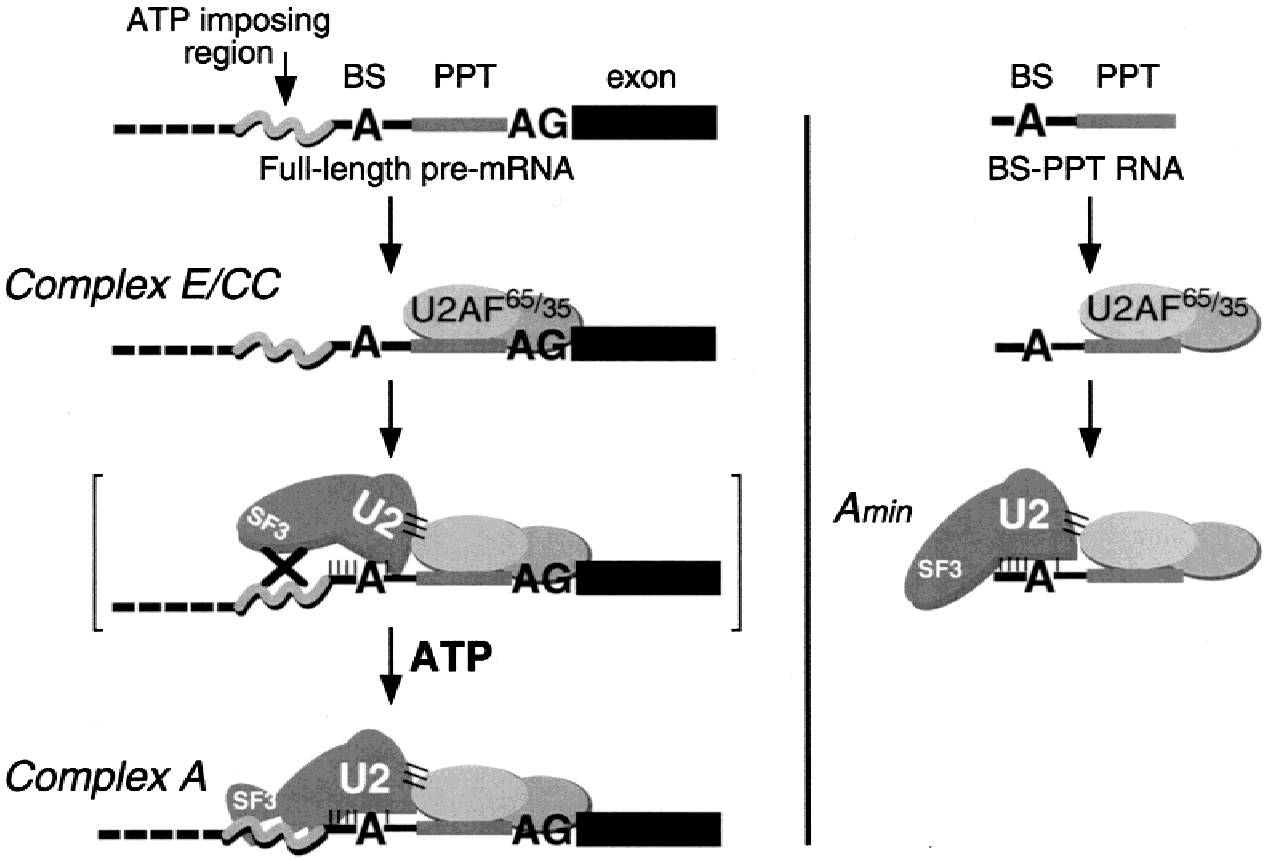

FIGURE 7. Model for ATP-dependence of U2 addition to pre-mRNA. A schematic comparison of interactions between U2 snRNP and the region surrounding the branch site is shown for ATP-dependent (left) and ATP-independent (right) complexes. The region 5′ to the branch site (shown as wavy line) imposes a requirement of ATP (left), and in its absence, the ATP requirement is bypassed (right). Bracketed complex indicates components that interfere (indicated by the ×) or that cannot interact in the absence of ATP. See text for details of this and alternative models. Other components required for CC/E formation, such as U1 snRNP, SF1/BBP, and SR proteins, are not shown for simplicity. Arrangement of proteins is illustrative and not intended to imply known spatial order. BS: branch site; PPT: polypyrimidine tract; CC/E: commitment or early complex; A: prespliceosomal complex A containing U2 snRNP and pre-mRNA; Amin: minimal substrate RNA-U2 snRNP complex.

hydrolysis with nucleic acid unwinding, or RNP "remodeling," and are ideal candidates for mediating the changes that must occur for the assembly of the functional spliceosome (reviewed in Schwer, 2001; Will & Lührmann, 2001). For example, Prp28p, for which the most information is available, is implicated in the switch of pairing partners between U1/5′SS and U6/5′SS duplexes; Prp28p also crosslinks to 5′SS, making the 5′SS or 5′SS/U1 duplex a likely substrate for its action (Staley & Guthrie, 1999; Chen et al., 2001; Ismaïli et al., 2001). However, the targets of most of the other DExD/H proteins during splicing are not known. U2 snRNA undergoes several structural rearrangements, most notably the exchange of intramolecular base pairing for U2/U6 helices (Staley & Guthrie, 1998). Exactly what kind of remodeling in U2 structure may be required prior to or concomitant with complex A formation is not known, but may include an RNA secondary structure transition as described for yeast U2 (Ares & Igel, 1990; Yan & Ares, 1996). Two potential DExD/H RNA helicases are required for U2 addition in yeast—Prp5p and Sub2p/yUAP56—and their human orthologs could reasonably be associated with the activity described here.

In yeast, Sub2/yUAP56 is required both before and after U2 addition (Kistler & Guthrie, 2001; Libri et al., 2001; Zhang & Green, 2001). In mammals, UAP56 is associated with U2AF^{65} and required for complex A assembly (Fleckner et al., 1997). Because Mud2 deletion allows viability of a sub2-deletion strain, Sub2/UAP56 action is suggested to be a removal or repositioning of Mud2/U2AF^{65} (Kistler & Guthrie, 2001). Prp5p is also required for U2 snRNP addition (Dalbadie-McFarland & Abelson, 1990). It interacts genetically with U2 snRNA, with Prp9p, Prp11p, and Prp21p (yeast homologs to the human SF3a components), and with the U2 protein Cus2p (Ruby et al., 1993; Wells & Ares, 1994; Perriman & Ares, 2000). In addition, the ATPase activity of Prp5p can be mildly stimulated by U2 snRNA (O'Day et al., 1996). However, the specific role of Prp5p remains to be identified. Although orthologs of Prp5p

have not been reported in species outside of *Saccharomyces cerevisiae*, we have recently identified a potential human homolog, hPrp5, that appears to be associated with U2 snRNP (C.M. Newnham and C.C. Query, unpubl.). Together with the above-stated genetic interaction with U2 components, this protein will be a good candidate for the activity observed in this study. As argued above, activity would not be involved in substrate rearrangements, but rather in changes in structure of the U2 snRNP that would enable U2 snRNP binding to the substrate. However, there is no direct evidence linking either Prp5p or Sub2p/UAP56 to the ATP-dependence of U2 addition, and additional studies will be required to distinguish between these possibilities. Either of these proteins may be required structurally rather than catalytically prior to complex A formation—as has been demonstrated to be the case for Prp22p in yeast, which is required structurally for splicing step two and catalytically later, for mRNA release (Schwer & Gross, 1998; Schwer & Meszaros, 2000). The system presented here will likely help separate and elucidate the roles of factors involved in the ATP-dependent process for U2 snRNP addition.

MATERIALS AND METHODS

Construction of RNA substrates

Sequences of RNAs used in this study were derived from the splicing substrate PIP85.B, which encodes the following 234-nt sequence: 5′-GGGCGAAUUCGAGCUCACUCUCUUCCGCAUCGCUGUCUGCGAGGUACCCUACCAG↓GUGAGUAUGGAUCCCUCUAAAAGCGGGCAUGACUUCUAGAGUAGUCCAGGGUUUCCGAGGGUUUCCGUCGACGAUGUCAGCUCGUCUCGAGGG**UGCUGACUGGCUUCUUCUCUCUUUUUCCCUC**AG↓GUCCUACACAACAUACUGCAGGACAAACUCUUCGCGGUCUCUGCAUGCAAGCU-3′. Arrows indicate the 5′ and 3′ splice sites, and the underlined A indicates the branch site. The bold sequence represents RNA(149–179), or BS-PPT RNA, and the double-underlined region represents the 18 nt 5′ to the BS that were modified in this study. Transcription of this full-length pre-mRNA, of derivatives thereof derived by PCR, and of other RNAs were performed under standard conditions.

Two-way RNA ligation reactions and gel purification of products were performed as described previously (Moore & Query, 2000). Briefly, oligo-ribonucleotides containing a branch sequence and polypyrimidine tract [BS-PPT RNA: RNA(149–179)] were prepared by joining a branch region heptamer [RNA(149–155): 5′-UGCUGAC-3′] and a 5′-^{32}P-phosphorylated polypyrimidine tract [RNA(156–179): 5′-UGGCUUCUUCUCUCUUUUUCCCUC-3′] using T4 DNA ligase (USB) and a bridging oligonucleotide [cDNA(169–136): 5′-GAGAGAAGAAGCCAGTCAGCACCCTCGAGACGAG-3′]. 6+BS-PPT, 11+BS-PPT, 18+BS-PPT, and 22+BS-PPT RNAs were similarly prepared by joining RNA(146–155), RNA(143–155), RNA(138–155), RNA(131–155), or RNA(127–155), respectively, to 5′-^{32}P-PPT RNA. 14+BS-PPT, 28+BS-PPT, and 44+BS-PPT RNAs were prepared by joining RNA(135–145), RNA(121–145), or RNA(105–145), respectively, to RNA(146–155) and 5′-^{32}P-PPT RNA. Altered sequence RNAs 18-2+BS-PPT RNA and 18-3+BS-PPT RNA were prepared by joining 5′-CAGUCGUCCCUCGAGACGUGCUGAC-3′ and 5′-GUUUCCUUGAAGCUUUCGUGCUGAC-3′, respectively, to 5′-^{32}P-PPT RNA. DNA-, abasic-, and C3 spacer-containing RNAs were prepared by joining 5′-dGdTdCdAdGdCdTdCdGdTdCdTdCdGdAdGdGdG-UGCUGAC-3′, 5′-[abasic]$_{18}$-UGCUGAC-3′, and 5′-[C3]$_{18}$-UGCUGAC-3′, to 5′-^{32}P-PPT RNA, respectively. Ligation products were purified on 15% polyacrylamide (29:1), 8 M urea gels run in 0.5× TBE (44.5 mM Tris-borate, 1 mM EDTA). All-RNA, abasic-containing, and carbon spacer oligomers were prepared by chemical synthesis on an Expedite 8909 oligonucleotide synthesizer using CE (β-cyanoethyl) phosphoramidites with 2′ TBDMS (*t*-butyl-dimethylsilyl) protecting groups (Glen Research), were base and 2′ deprotected as described (Wincott et al., 1995), and were purified by gel filtration through Bio-Gel P-2 resin (Bio-Rad) and on 20% polyacrylamide 8 M urea gels where necessary prior to RNA ligations.

Native gel analysis of splicing complexes

Nuclear extracts were prepared from HeLa cells as described by Dignam et al. (1983). Cells were obtained from the National Cell Culture Center (Minneapolis, MN). To form splicing complexes, RNAs were incubated under standard splicing conditions (Grabowski et al., 1984) using HeLa nuclear extracts; or, for ATP-depleted reactions, ATP and creatine phosphate were omitted from the mixes, which were preincubated for 15 min at 30 °C to deplete endogenous ATP and, in some cases, then adjusted to 2 mM EDTA. RNAs were then added and incubated at 30 °C for the times indicated. For competition experiments, RNA oligonucleotides were added to nuclear extract and mixed prior to substrate addition. Reactions were adjusted to 0.5 mg/mL heparin and separated by electrophoresis in 50 mM Tris-glycine through nondenaturing 4% (80:1) polyacrylamide gels (Konarska & Sharp, 1986, 1987). Polyacrylamide gels were dried and quantitated using a Molecular Dynamics PhosphorImager and ImageQuant software, Macintosh version 1.11.

RNase H analysis

ATP was depleted from reactions as described above but without the addition of EDTA, and labeled substrates (BS-PPT and 18nt-BS-PPT) were incubated for 30 min at 30 °C. After this incubation, a DNA oligonucleotide (5′-CCCTCGAGACGAGCTGAC-3′) complementary to the region 5′ to the branch site was added to a concentration of 10 μM. RNasin, 2 mM $MgCl_2$, and RNase H (2 U/μL; Promega) were added and reactions were incubated for an additional 30 min at 30 °C. Mock RNase H treatments contained RNase H, 2 mM $MgCl_2$, and RNasin, but with no oligonucleotide added or with a control oligonucleotide (5′-CTACACTTGATCTTAGCC-3′). Reactions were adjusted to 0.5 mg heparin per mL, prior to native gel analysis. Samples were removed from the initial 30-min incubation and the RNase H incubations, purified by phenol/chloroform extraction and ethanol precipitation, and the RNA analyzed on a 20% poly-

acrylamide (acrylamide-bisacrylamide 19:1)-8 M urea gel run in 0.5× TBE.

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