Yeast U1 snRNP–pre-mRNA complex formation without U1snRNA–pre-mRNA base pairing

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ABSTRACT

Base pairing between the 5' end of U1 snRNA and the conserved 5' splice site of pre-mRNA is important for commitment complex formation *in vitro*. However, the biochemical mechanisms by which pre-mRNA is initially recognized by the splicing machinery is not well understood. To evaluate the role of this base pairing interaction, we truncated U1 snRNA to eliminate the RNA–RNA interaction and surprisingly found that U1 snRNP can still form a nearly normal RNA–protein complex and maintain sequence specificity. We propose that some feature of U1 snRNP, perhaps one or more protein factors, is more important than the base pairing for initial 5' splice site recognition. In addition, at least five sets of interactions contribute to complex formation or stability. Only one of these is base pairing between the 5' splice site and the 5' end of U1 snRNA, without which the U1 snRNP–pre-mRNA complex is less stable and has a somewhat altered conformation.

Keywords: 5' splice site; base pairing; stability; yeast commitment complex

INTRODUCTION

Pre-mRNA splicing plays an essential role in eukaryotic cell metabolism. This process removes intervening sequences (introns) from a precursor mRNA and ligates the resulting exons together. The splicing process is very similar in yeast and higher eukaryotes, and introns in both systems have three conserved sequence regions: a 5' splice site region (5'ss), a branch point region, and a 3' splice site region (3'ss) (Moore et al., 1993; Nilsen, 1998; Burge et al., 1999). These regions are the sites of covalent bond cleavage and formation during the splicing reaction and also serve to recruit splicing factors, which constitute the spliceosome. Each spliceosome consists of a large RNA–protein complex, containing pre-mRNA, five small nuclear ribonucleoprotein particles (snRNPs) and many non-snRNP proteins (Moore et al., 1993; Reed, 1996; Kramer, 1996; Will & Lührmann, 1997; Burge et al., 1999). Based on *in vitro* experiments, spliceosome formation is believed to occur in a stepwise fashion: the first step is an ATP-independent recognition of the pre-mRNA 5'ss by U1 snRNP, forming what is called the commitment complex in yeast (Séraphin & Rosbash, 1989) or the E complex in mammals (Michaud & Reed,

1991). In yeast, a base-pairing interaction between the 5' end of U1 snRNA and the pre-mRNA 5'ss is important for commitment complex formation and is required for all subsequent steps of spliceosome assembly, for example, the base-pairing interaction between U2 snRNP and the branchpoint region, the replacement of U1snRNP at the 5'ss by the U4/5/6 tri-snRNP, and ultimately splicing (Zhuang & Weiner, 1986; Steitz, 1992; Moore et al., 1993; Reed & Palandjian, 1997; Nilsen, 1998; Staley & Guthrie, 1999).

In mammalian systems, however, the situation is more complicated. SR proteins can compensate for U1 snRNP activity and promote efficient splicing of some pre-mRNAs in HeLa nuclear extracts depleted of U1 snRNP or blocked by an anti-U1 snRNA antisense oligonucleotide (Crispino et al., 1994; Tarn & Steitz, 1994). In fact, some pre-mRNA substrates do not even require U1 snRNP or excess concentrations of SR proteins to splice *in vitro* (Crispino et al., 1996). And U2 snRNP can bind to the branch point region of certain model substrates in the absence of a 5'ss and any detectable U1 snRNA–pre-mRNA base-pairing interaction (Ruskin & Green, 1985; Chiara & Reed, 1995; Query et al., 1997). This is consistent with observations that some exonic enhancer elements can recruit U2 snRNP to the branch point independent of a pre-mRNA 5'ss (Wang et al., 1995; Zuo & Maniatis, 1996). In these *in vitro* U1 snRNP-bypass reactions, therefore, a protein factor or factors are promoting the association of U2 snRNP

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with the branch point independent of U1 snRNP recognition of the pre-mRNA 5'ss. The pre-mRNA 5'ss is subsequently recognized in a rate-limiting reaction by the U4/U5/U6 tri-snRNP (Staley & Guthrie, 1998; Burge et al., 1999). These results demonstrate that U1 snRNP is not absolutely required for spliceosome assembly or for splicing catalysis.

In yeast, however, the present view is that U1 snRNP is essential for pre-mRNA splicing. First, mutations in either the pre-mRNA 5'ss region or the 5' end of U1 snRNA cause a dramatic decrease in commitment complex formation and inhibit subsequent steps in spliceosome assembly (Sraphin et al., 1988). Second, no U1 snRNP-independent commitment to splicing has been described in yeast system. Third, nearly all U1 snRNP proteins are essential. Although this could indicate an additional function for these proteins, it is consistent with the simple view that U1 snRNP is essential for pre-mRNA splicing. Relatively few yeast genes have introns, and they have more highly conserved splice site and branch point regions than in vertebrates. Furthermore, most yeast intron-containing genes have a single intron, and it is usually located near the 5' end of the gene (Rymond & Rosbash, 1992; Spingola et al., 1999). This relatively simple nature of yeast introns parallels the lack of evidence for SR protein activity in yeast intron/exon definition or in yeast prespliceosome assembly. In addition, yeast U1 snRNP is more complex than mammalian U1 snRNP. First, yeast U1 snRNA is considerably larger than vertebrate U1 snRNA (Kretzner et al., 1987; Siliciano et al., 1987). Although the yeast-specific regions of U1 snRNA can be deleted without affecting cell viability, they still contribute to splicing and commitment complex formation (Liao et al., 1990; Siliciano et al., 1991). Second, there are at least six yeast U1 snRNP proteins with no well-described vertebrate counterparts within the core U1 snRNP particle (Neubauer et al., 1997; Gottschalk et al., 1998; Fortes et al., 1999a). These observations have led to the view that yeast U1 snRNP functions relatively autonomously, without cofactors like SR proteins.

Although base pairing between the pre-mRNA 5'ss and the 5' end of U1 snRNA plays an important role in splice site recognition and commitment complex formation, it is probably insufficient for exon/intron recognition and pre-mRNA commitment to the splicing pathway. Protein-protein and protein-pre-mRNA interactions also play an important role (Siliciano & Guthrie, 1988; Heinrichs et al., 1990; Kohtz et al., 1994; Will & Lhrmann, 1997), and previous work from this and another laboratory has identified eight proteins with direct contacts to the pre-mRNA 5'ss within the commitment complex (Zhang & Rosbash, 1999; Puig et al., 1999). To address this issue further, we used oligonucleotide-directed RNase H degradation to remove the 5' end of U1 snRNA in yeast extracts. Surprisingly, base pairing between the 5' end of U1 snRNA and the pre-mRNA

5'ss was not required for U1 snRNP-pre-mRNA complex formation although the base-pairing was required for splicing. Moreover, the same proteins interacted with the pre-mRNA 5'ss region in the presence or absence of base pairing. We furthermore showed that the absence of base pairing affects the complex stability and conformation, suggesting that this is why there is no subsequent spliceosome formation. These findings indicate that base pairing between the 5' arm of U1 snRNA and the pre-mRNA 5'ss is not essential for 5'ss recognition or for complex formation but for events downstream of this initial recognition process in yeast splicing.

RESULTS

Base pairing between the pre-mRNA 5'ss and the U1 snRNA 5' arm is not required for complex formation

To address the importance of the canonical 5'ss-U1 snRNA base pairing interaction to commitment complex formation in yeast, we eliminated the 5' end of U1 snRNA with oligonucleotide-directed RNase H digestion (see Materials and Methods). A DNA oligonucleotide, shown in Figure 1A and complementary to the first 14 nt of U1 snRNA, was used (Fig. 1A; OHD41). Primer extension indicated that removal of the 5' end of U1 snRNA was more than 90% complete (Fig. 1B). Yet there was nearly complete recovery of a digestion product 10 nt shorter than intact U1 snRNA (Fig. 1C). There was also no detectable pre-mRNA/U1 snRNA interaction in the RNase H-treated extracts by psoralen crosslinking, consistent with absence of the U1 snRNA 5' arm (Fig. 1D, lane 2).

To test for U1 snRNP-pre-mRNA complex formation in the absence of the canonical base pairing interaction, biotinylated pre-mRNA substrate was prebound to streptavidin beads and then incubated with wild-type or RNase H-treated extracts under splicing conditions. Association of U1 snRNA with the substrate was assayed by subsequent primer extension. Because the 72-nt substrate has no branch point region sequence, primer extension for U2 snRNA served as a negative control. In this assay, biotinylated substrate interacts with U1 snRNP, not only in untreated extract but also in the treated extract (Fig. 2A, lanes 2 and 6). Although there is only about 30% as much signal in the treated extract as in the untreated extract, this is much greater than the values obtained by native gel electrophoresis or by psoralen crosslinking (Fig. 1C; data not shown). This 30% value is not due to a background problem; in addition to the lack of U2 snRNA binding, there is little or no U1 snRNA binding to a nonbiotinylated substrate (Fig. 2A, lane 7) or to a substrate with three 5'ss mutations (GUAUGU $\rightarrow$ AUGUAU; Fig. 2A, lane 8). These observations indicate that the truncated U1 snRNP not

FIGURE 1. Cleavage of 5' end of U1 snRNA. **A:** Schematic representation of *Saccharomyces cerevisiae* U1 snRNA. The oligonucleotide DT2133 complementary to the positions 114–135 of U1 snRNA was used for primer extension. The 5' end of U1 snRNA, shown base paired with DNA oligonucleotide OHD41, is cleaved by RNase H. **B:** Primer extension. RNA was either extracted from wild-type extract (lane 1) or from RNase H-treated extract (lane 2) and used in reverse transcription reactions with ³²P-labeled DNA oligonucleotides complementary to U1 and U2 snRNA. Primer extension was terminated by adding loading buffer and boiling 2 min at 90 °C. Samples were analyzed on 6% denaturing acrylamide gels. U1, U2, and truncated U1 are indicated by arrows. **C:** High-resolution primer extension. RNA from either wild-type (lane 5) or RNase H-treated extract (lane 6) was analyzed by primer extension using primer DT2133. Sequencing of plasmid snR19 (Kretzner et al., 1987) with primer DT2133 is shown in lanes 1–4. **D:** Psoralen UV crosslinking. ³²P-labeled RNA substrate was incubated with wild-type (lane 1) or RNase H-treated extract (lane 2). ATM-psoralen was added to the reaction and the mixture was irradiated for 10 min at 4 °C to crosslink the double-stranded RNA formed between U1 snRNA and pre-mRNA. Proteins were removed by proteinase K treatment and phenol extraction. RNA was ethanol precipitated, resolved on a 6% denaturing polyacrylamide gel and visualized by autoradiography.

only interacts with an RNA substrate but also retains 5'ss specificity. The reduced recovery of U1 snRNA from the treated extracts was confirmed by a titration experiment: More substrate was required to detect signal in the treated extract than in the untreated extract (Fig. 2B).

Protein crosslinking with a 4-thioU labeled RNA substrate was another indication that the truncated U1 snRNP interacts with the 72-nt substrate (Fig. 3A). Un-

der standard conditions, there are eight proteins that crosslink to this substrate: seven U1 snRNP proteins and CBP80, the large subunit of the cap-binding complex (CBC) (Zhang & Rosbash, 1999). The same eight proteins were visualized with the treated extract, suggesting that truncated U1 snRNP interacts with substrate in a quasi-normal fashion. To confirm that these eight proteins are those identified previously, extracts were made from strains in which epitope-tagged ver-

FIGURE 2. Yeast U1 snRNP-pre-mRNA complex can be formed in the absence of base pairing. **A:** Wild-type (lanes 1–4) and RNase H-treated splicing extracts (lanes 5–8) were incubated with 10 ng/ μ l biotinylated pre-mRNA or nonbiotinylated control pre-mRNA (prebound to streptavidin beads) under standard splicing conditions. RNA was extracted from the beads and assayed by reverse transcription with specific oligonucleotides complementary to U1 and U2 snRNA. U1 and U2 snRNA are indicated by arrows. Lanes 1 and 5: RNA was extracted directly from splicing extract. **B:** Titration experiment. Repeat of **A**, except different amounts of biotinylated pre-mRNA substrate were used in the commitment complex formation reaction. The amounts are indicated at the top of each lane.

sions of the genes had been used to rescue endogenous gene disruptions. If a band corresponds to a tagged protein, it should disappear from its wild-type position and appear with a new lower mobility (Zhang & Rosbash, 1999). For example, the identity of U1-70K was confirmed by this approach (Fig. 3B, compare lanes 1 and 2 with lanes 3 and 4). In this way, we verified the identity of the other seven proteins (data not shown). We further verified that the effects of RNase H are due to digestion of U1 snRNA prior to substrate addition rather than subsequent to complex formation, by adding EDTA to inactivate RNase H activity prior to assaying complex formation (data not shown).

The band intensity in the treated extracts is approximately four- to fivefold less than in the control extracts

(Fig. 3A), consistent with the reduced efficiency of U1 snRNA recovery in the streptavidin pull-down assay (Fig. 2A). Importantly, the intensity of the Sm D1 and D3 proteins is even further reduced in the treated extracts relative to the other proteins (Fig. 3A, lane 2), indicating that qualitative features of complex formation are also affected in the absence of the 5'ss-U1 snRNA base-pairing interaction. As the three Sm proteins have been previously shown to crosslink within or very near the 5'ss region, it is not surprising that these signals are more prominently reduced in the absence of the base-pairing interaction.

To address additional aspects of complex formation with truncated U1 snRNP, we used uncapped substrate RNA. In untreated extracts, uncapped pre-mRNA sub-

FIGURE 3. UV crosslinking protein profiles of yeast U1 snRNP-pre-mRNA complexes. **A:** Complex formed with 4-thioU-labeled pre-mRNA substrate in a standard commitment complex formation reaction, UV crosslinked at 365 nm, and immunoprecipitated with an anti-Prp40p antibody. Labeled proteins were subsequently visualized on a 4–20% linear gradient SDS-PAGE gel by autoradiography. Molecular size markers are indicated on the left. Lane 1 shows the proteins interacting with the 5'ss region in wild-type extract; lane 2 shows proteins in the absence of base pairing. **B:** UV irradiation, immunoprecipitation, and other analyses were performed identically to those in **A**. Lanes 1 and 2: wild-type splicing extract was used; lanes 3 and 4: U1-70K HA-tagged splicing extract was used. Lanes 1 and 3: mock-treated splicing extract; lanes 2 and 4: RNase H-treated splicing extract. **C:** The complex formation is cap dependent in the absence of base pairing. 4-thio-UTP-labeled pre-mRNA, either capped (lanes 1, 3, 4, and 6) or uncapped (lanes 2 and 5), and using WT-72 (lanes 1, 2, 4, and 5) or 5'ss mutant substrate (lanes 3 and 6) was incubated with wild-type splicing extract (lanes 1–3) or RNase H treated extract (lanes 4–6) in a standard commitment complex formation reaction.

strate forms less commitment complex than the usual capped RNA substrate (Colot et al., 1996; Zhang & Rosbash, submitted). Protein crosslinking is also less efficient with uncapped substrate RNA, and there is almost a complete absence of the CBP80 band (Fig. 3C, compare lanes 1 and 2). This is not unexpected and reflects the fact that CBP80 interacts not only with the pre-mRNA exon region and with U1 snRNP proteins but also indirectly with the cap structure (Izaurralde et al., 1994; Fortes et al., 1999b; Zhang & Rosbash, 1999). In the treated extract, protein crosslinking to the uncapped substrate was virtually undetectable. We interpret this to indicate that complex formation is extremely weak in the absence of both a capped RNA substrate and the 5'ss region–U1 snRNA base-pairing interaction. The result suggests that the CBC–cap interaction and the base pairing contribute in a complementary fashion to complex formation or stability: At least one interaction is required for reasonable levels of stable complex formation.

Complex stability is reduced in the absence of base pairing

To directly test the effect of U1 snRNP truncation on complex stability, we performed chase experiments. After complex formation with the biotinylated substrate, an excess of nonbiotinylated RNA was added and the incubation continued before the streptavidin pulldown. In control extract, the complex is very stable: after 60 min incubation with excess cold substrate RNA, the signal only decreased by 40% (Fig. 4A, lanes 4–8). This is consistent with previous results indicating that the U1 snRNP-pre-mRNA complex is very stable (Pikielny et al., 1986; Puig et al., 1999; Zhang & Rosbash, submitted). In the absence of base pairing, however, the complex was much less stable, as the signal was almost absent after 10 min of incubation under chase conditions and completely absent after 30 min (Fig. 4A, lanes 11–13). This experiment clearly illustrated that base pairing contributes to RNA–protein complex sta-

FIGURE 4. U1 snRNP-pre-mRNA complex stability is decreased without base pairing. **A:** Competition experiment. Biotinylated pre-mRNA was incubated with splicing extract in a standard commitment complex reaction for 30 min at 4°C as described in Figure 2. A large molar excess (400-fold) of nonbiotinylated pre-mRNA was then added to each reaction. Incubation was continued at 4°C for 0 min (lanes 4 and 11), 10 min (lanes 5 and 12), 30 min (lanes 6 and 13), or 60 min (lanes 7 and 14). Lanes 1–7: wild-type splicing extract was used; lanes 8–14: RNase H-treated splicing extract was used. Relative intensity of each band was plotted against time from two independent experiments. **B:** Native gel analysis of complexes. Capped, labeled pre-mRNA substrate was synthesized in an *in vitro* transcription reaction. The labeled RNAs were incubated with either wild-type (lane 1) or RNase H-treated extract (lane 2) under standard conditions for commitment complex formation and analyzed on a native gel. Commitment complex (CC) and free pre-mRNA are indicated.

bility. Indeed, the complex formed in the absence of base pairing is undetectable by native gel electrophoresis, suggesting that it is too unstable to withstand electrophoresis (Fig. 4B). Taken together, the results show that base pairing between the U1 snRNA 5' arm and the pre-mRNA 5' ss is important for complex stability.

To map the positions of the crosslinked proteins onto the RNA substrate, we employed the previous strategy of varying the number and positions of U residues, thereby altering the locations of photoreactive 4-thioU residues (Zhang & Rosbash, 1999). Two substrates, one that eliminated all exon Us and another that elim-

inated all intron Us, were used as described previously; in both cases, the three Us in the 5' ss are present. In the absence of the exon Us, proteins previously shown to bind to the intron (Nam8p and Snu56p) and to the 5' splice site (U1-C) were readily apparent; there was no difference between the control and the U1-truncated patterns (Fig. 5, lanes 3 and 4). In the absence of the intron Us, however, little or no signal was apparent in the RNase H-treated extract (Fig. 5, compare lanes 5 and 6). These observations have mechanistic implications for the residual interactions without base pairing between the 5' ss and U1 snRNA (see Discussion).

FIGURE 5. Mapping the binding locations of crosslinked proteins. The substrates used in this experiment either eliminated all the exon Us or all the intron Us (labeled exon U6A and intron U6A in the figure, respectively), to map proteins interacting with the 5'ss and its upstream exon region, or with the 5'ss and its downstream intron region, as previously described (Zhang & Rosbash, 1999). Wild-type pre-mRNA substrate Δ -72 and the two mutant substrates were incubated with wild-type splicing extract (lanes 1, 3, and 5) or RNase H-treated extract (lanes 2, 4, and 6) in a standard commitment complex formation reaction. The experiment was performed as in Figure 3.

DISCUSSION

Pre-mRNA splicing is an extraordinarily precise reaction in eukaryotes, which depends on correct recognition of a few sequence elements. These are primarily within the intron, near the 5' and 3' splice sites. Based on *in vitro* experiments, the first step in intron recognition and spliceosome assembly is the interaction of U1 snRNP with the pre-mRNA substrate. Base pairing between the 5'ss and the 5' end of U1 snRNA was thought to be an essential component of this interaction. We discovered, however, that it is not required for yeast U1 snRNP-pre-mRNA complex formation, at least *in vitro*. Without base pairing, U1 snRNP proteins still interact with a pre-mRNA 5'ss to form nearly normal RNA-protein complex. This observation led to a characterization of the complexes without the usual RNA-RNA pairing.

We first examined U1 snRNA and U1 snRNP after oligonucleotide-directed RNase H treatment (Fig. 1). In general, RNA without a 5' cap is readily degraded by 5' $\rightarrow$ 3' exonucleases in extract (Beelman et al., 1996; Caponigro & Parker, 1996). We were therefore somewhat surprised that U1 snRNA is apparently intact and quantitatively unaffected by digestion of its 5' arm. Because the truncated U1 snRNA is 10 nt shorter than U1 snRNA (Fig. 1C), we presume that either stem-loop 1 (which begins at nt 11; Fig. 1A) and/or U1 snRNP proteins protect the rest of the molecule from digestion. To verify that the truncated U1 snRNP was largely intact, we used anti-Prp 40 to immunoprecipitate U1 snRNP

from treated extracts. Truncated U1 snRNA could be specifically recovered, suggesting that U1 snRNP did not disassociate after oligonucleotide-directed RNase H truncation. Anti-Nam8p antibodies and anti-HA antibodies with tagged extracts gave similar results (data not shown).

We next studied the properties of complexes in the absence of base pairing. They still form, as indicated by the near-normal protein crosslinking characteristic of their protein-RNA interactions. But these complexes are much less stable than control complexes with a normal base-pairing interaction (Fig. 4). Consistent with this observation, the truncated complexes are not detected if the substrate does not contain a cap structure at its 5' end (Fig. 3C, lane 5). The cap-CBC interaction is known to contribute to commitment complex stability (Colot et al., 1996; Lewis et al., 1996a, 1996b; unpubl. observations). We interpret these observation to indicate that the complexes are sufficiently stable to detect without a single interaction but not without both interactions. This does not preclude an additional effect of base pairing or of the cap-CBC interaction on complex formation.

Our data suggest that Nam8p also contributes to complex formation and/or stability. When all intron Us were mutated, none of the eight pre-mRNA-interacting proteins were detected in the truncated extract (Fig. 5, lane 6). This presumably reflects the fact that Nam8p binds to intron sequences and favors U-rich RNA (Puig et al., 1999). Because this mutant substrate forms commitment complex normally with control U1 snRNP, the argument is analogous to the dual contribution of the cap-CBC and base-pairing interactions: Either a normal Nam8p-pre-mRNA interaction or the base-pairing interaction is required for protein crosslinking. These data therefore indicate at least three contributions to commitment complex stability: the U1 snRNA-5'ss base pairing, a cap-CBC interaction, and the Nam8p-intron interaction. Recent observations indicate that the basic tails of three Sm proteins make a fourth set of interactions that contribute to complex stability (Zhang & Rosbash, submitted). Finally, the failure of the truncated U1 snRNP to interact detectably with a 5'ss mutant substrate suggests at least a fifth contact contributing to complex formation or stability (Fig. 2A).

This surprising result implies further that there is sequence-specific recognition of the 5'ss sequence by some feature of U1 snRNP or some protein factor other than the U1 snRNA 5' arm. The failure to observe complex formation without this contact(s) indicates that it contributes either to complex stability or formation; without additional data, we cannot distinguish between these two possibilities. Based on our previous characterization of protein contacts to the 5'ss within commitment complex, however, we favor at least some contribution to complex stability. Both U1C and three Sm proteins crosslink within and/or very close to the 6-nt 5'ss sequence (Zhang & Rosbash, 1999) and are good can-

didates for sequence-specific recognition factors. For the Sm proteins, there is direct evidence that the C-terminal tails not only crosslink to pre-mRNA but also contribute to complex stabilization (Zhang & Rosbash, submitted). Based on the positions of the crosslinks, we presume that this is through an effect on duplex stabilization, at least in part (Zhang & Rosbash, 1999). Quantitation of crosslinking with the treated extracts is consistent with this hypothesis, especially for Sm D3. Truncation of U1 snRNP reduces levels of immunoprecipitated pre-mRNA four- to sixfold, indicating a modest effect on complex stability and perhaps even formation. However, crosslinking of SmD3 is reduced more than tenfold and is the most affected crosslink in the truncated U1 snRNP pattern. This is consistent with the notion that at least Sm D3 binds preferentially to the RNA–RNA duplex within the commitment complex.

Although the association of Sm proteins with the duplex contributes to the stability issue, it does not address sequence specificity. On the contrary, it is difficult to understand how the same proteins can associate in a sequence-specific manner with this RNA duplex and, in the absence of the 5' end of U1 snRNA, with the single-stranded 5'ss sequence. Moreover, the Sm proteins may play a similar duplex-binding role for many snRNPs, most of which probably do not have targets with the same sequence as a 5'ss. A possible resolution is suggested by the same quantitation of commitment complex crosslinking, focusing in this case on U1C. Crosslinking of this protein is reduced only two- to threefold; by comparison to the four- to sixfold effect on total complex formation, U1C crosslinking is relatively enhanced by the U1 snRNA truncation. This suggests that U1C preferentially binds to the single-stranded 5'ss. Site-specific labeling within the 5'ss confirms that U1C crosslinks to this region of pre-mRNA in a U1 snRNA-truncated extract (data not shown). In a normal extract, the U1C–pre-mRNA contacts might be different, that is, these contacts could be predominantly to the duplex phosphate backbone rather than to the 5'ss bases. Indeed, a role for U1C in duplex stabilization is suggested by genetic experiments in which U1C mutants suppress the effects of mutants in PRP28 (T. Chang, pers. comm.). In addition, U1C–pre-mRNA crosslinking in normal commitment complexes could even reflect a mixed population: fully base-paired complexes as originally imagined (Zhang & Rosbash, 1999) as well as complexes in which the 5' end of U1 snRNA and the 5'ss are partially or completely single stranded. The latter could even be precursors to the fully base-paired complex. In this view, the putative sequence specificity of U1C for the single-stranded version of the 5'ss sequence serves to recognize and recruit pre-mRNA into commitment complex: perhaps a more prominent contribution to complex formation than to complex stabilization. Previous experiments from this laboratory suggested that yeast U1C potentiates base pairing be-

tween a 5'ss and U1 snRNA, but did not distinguish between a role in complex formation from one in complex stabilization (Tang et al., 1997).

Are there other unexpected protein factors that might also have sequence-specific recognition features relevant to commitment complex formation? Recent observations in mammalian and nematode systems show that the U4/U5/U6 tri-snRNP directly interacts with a pre-mRNA 5'ss independently of U2 snRNP. The Prp8 protein of U5 snRNP forms a strong crosslink to the phosphate between the 5'ss positions 1G and 2U (Maroney et al., 2000). Crosslinking of Prp8, like canonical U2 snRNP-dependent tri-snRNP addition, requires ATP. These authors also present suggestive evidence that U1 snRNP and the U4/5/6 triple snRNP occupy the 5'ss simultaneously. Because Prp8 has been previously shown to crosslink to the GU of a 5'ss, these recent experiments raise the intriguing possibility that U5 snRNP and more specifically yeast Prp8p might provide the sequence-specific recognition properties reported here. However, ATP depletion prior to substrate addition had no effect on the crosslinking pattern of control or of truncated extracts. Moreover, there was no prominent crosslinking signal at a molecular weight appropriate for Prp8p, even when the substrate was labeled only between positions 1G and 2U (data not shown). We therefore believe that Prp8p and U5 snRNP are unlikely to be responsible for the sequence-specific properties of the truncated U1 snRNP reported here. This does not preclude a subsequent or even parallel role of Prp8p in 5'ss identification.

Although not investigated in detail, we suspect that the unstable RNA–protein complex formed in the absence of base pairing is not biologically active. Little or no subsequent spliceosome assembly takes place, and splicing efficiency is also dramatically reduced (data not shown). This may reflect the fact that protein crosslinking with 4-thioU-labeled RNA substrates and the biotinylated RNA pull-down assay are very sensitive. But base pairing between the 5'ss and the 5' end of U1 snRNA is probably required for subsequent steps in spliceosome assembly, for example, a conformational change that promotes the transition from commitment complexes to active spliceosomes. This is consistent with the genetic experiments of Staley and Guthrie (1999).

The experiments in this report are remarkably consistent with prior studies in mammalian systems. They indicate a prominent role of U1C in potentiating E complex formation and base pairing between a 5'ss and U1 snRNA (Heinrichs et al., 1990; Jamison et al., 1995; Will et al., 1996). There is even one study suggesting that U1C is involved in 5'ss recognition prior to base pairing (Rossi et al., 1996). A key prediction is that recombinant U1C has sequence-specific recognition features for single-stranded RNA, and we are attempting to verify this notion.

MATERIALS AND METHODS

Constructs and splicing extracts

The plasmids used in this paper have been described previously (Zhang & Rosbash, 1999). Briefly, a wild-type pre-mRNA substrate containing the first 72 nt of rp51A pre-mRNA (Δ -72) was generated by in vitro transcription of plasmid BT81. The 5' splice site mutant RNA substrate was generated by in vitro transcription of plasmid pDZ32, in which GTATGT was mutated to ATGTAT in the 5' splice site region. In plasmids pHD18 and pHD19, the templates for exonU6A and intronU6A RNAs, all thymidines have been mutated to adenines in either the exon or intron region, respectively, using synthesized DNA oligonucleotides as described previously (Zhang & Rosbash, 1999). Splicing extracts were prepared from the wild-type strain Y59 and tagged strains using a modified miniextract protocol (Umen & Guthrie, 1995).

RNase H digestion assay

Oligonucleotide-directed RNase H digestion was performed as described previously, with minor modifications. Seventy-five units of RNase H and 75 μ g of an oligonucleotide complementary to the first 14 nt of U1 snRNA (OHD41, CTTAAGGTAAGTAT) were added to 400 μ L splicing reaction and incubated at 30 °C for 20 min under splicing conditions. RNase H digestion was stopped by placing the reaction directly on ice or by adding 2.5 μ L 0.5 M EDTA, pH 8.0. Subsequent commitment complex formation was initiated by addition of in vitro-transcribed pre-mRNA substrate for UV crosslinking, biotinylated RNA for streptavidin pulldown, or native gel electrophoresis.

RNA extraction and primer extension

RNA extraction and primer extension were carried out according to standard procedures (Pikielny & Rosbash, 1986). Primer DT2133 (GACCAAGGAGTTTGCATCAATG) is complementary to nt 114–135 of U1 snRNA. Primer DT58 (GCCAAAAAATGTGTATTGTAAC) is complementary to nt 100–121 of U2 snRNA. AMV reverse transcription products were analyzed by 6% denaturing polyacrylamide gel electrophoresis and autoradiography. Band intensities were quantitated using molecular analyst software.

Psoralen crosslinking and protein crosslinking assays

Psoralen crosslinking was performed as described previously (Tarn & Steitz, 1996). Commitment complexes were formed under splicing conditions in 10 μ L and placed on ice, AMT (4'-aminomethyl-4,5',8-trimethyl) psoralen (HRI Associates, Concord, California) was added to 20 μ g/mL, and samples irradiated with 365 nm UV light for 10 min. Samples were deproteinized by addition of 10 μ L PK mix (20% SDS, 20 mM EDTA, pH 8.0, freshly added Proteinase K to 1 mg/mL) and incubation at 65 °C for 10 min prior to extraction with phenol/chloroform and ethanol precipitation. Samples were analyzed by 6% denaturing polyacrylamide gel electrophoresis and autoradiography. 4-thioU UV crosslinking and im-

munoprecipitation were performed as described previously (Zhang & Rosbash, 1999).

In vitro commitment complex assembly

Native gel electrophoresis was performed according to standard protocols (Séraphin & Rosbash, 1989). Radiolabeled RNA was incubated in yeast extract under standard splicing reaction condition at room temperature. Samples were loaded onto a 3% native polyacrylamide gel (3% acrylamide(60:1), 0.5% agarose, 0.5 $\times$ TBE, 5% glycerol). Electrophoresis was at 70 V 15 h, and complexes visualized by autoradiography.

Biotinylated RNA pulldowns and competition assay

Biotinylated RNA was transcribed in vitro using a biotin/NTTP mixture (3.5 mM Biotin-16-UTP/6.5 mM UTP). Biotinylated RNA was prebound to 20 μ L streptavidin magnetic beads in Binding and Washing (B&W) buffer (5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1.0 M NaCl) at 4 °C for 30 min and washed twice with 100 μ L D buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 20% glycerol). Fifty microliters wild-type or RNase H-treated splicing extract was incubated with pre-bound biotinylated RNA under standard commitment complex formation conditions at 4 °C for 30 min and washed three times with 100 μ L NET150 (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% NP-40). RNA was recovered in 100 μ L splicing dilution buffer (100 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% SDS, 150 mM NaCl, 300 mM NaOAc) by phenol-chloroform extraction and ethanol precipitation. For competition experiments, a 400-fold molar excess of nonbiotinylated RNA was added prior to washing with NET150, and the incubation continued at 4 °C for different times. U1 snRNA primer extension products were analyzed by 6% denaturing polyacrylamide gel electrophoresis and autoradiography.

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