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The yeast branchpoint sequence is not required for the formation of a stable U1 snRNA-pre-mRNA complex and is recognized in the absence of U2 snRNA

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Commitment complexes contain U1 snRNP as well as pre-mRNA and are the earliest functional complexes that have been described during *in vitro* spliceosome assembly. We have used a gel retardation assay to analyze the role of the yeast pre-mRNA *cis*-acting sequences in commitment complex formation. The results suggest that only a proper 5′ splice site sequence is required for efficient U1 snRNA-pre-mRNA complex formation. A role for the highly conserved UACUAAC branchpoint sequence is indicated, however, by competition experiments and by the direct analysis of branchpoint mutant substrates, which cannot form one of the two commitment complex species observed with wild-type substrates. The results suggest that the formation of a U1 snRNP-pre-mRNA complex is not dependent upon the presence of a branchpoint sequence but that the branchpoint sequence is recognized prior to U2 snRNP addition during *in vitro* spliceosome assembly.

Key words: commitment complexes/intron recognition/spliceosome/splice sites/*Saccharomyces cerevisiae*

Introduction

Numerous analyses have shown that *in vitro* spliceosome assembly occurs in multiple steps which are likely to reflect the ordered addition of snRNPs and protein factors to the pre-mRNA (Frendewey and Keller, 1985; Konarska and Sharp, 1986, 1987; Pikielny *et al.*, 1986; Bindereif and Green, 1987; Cheng and Abelson, 1987; Lamond *et al.*, 1988; Blencowe *et al.*, 1989). U1 snRNA was first suggested to be a splicing factor a decade ago (Lerner *et al.*, 1980; Rogers and Wall, 1980). Although it was subsequently shown to be required for splicing (Krämer *et al.*, 1984; Zhuang and Weiner, 1986), it was not observed associated with spliceosomes in these early studies.

In higher eukaryotes, formation of U2 snRNP-containing complexes (or pre-spliceosomes) also seemed to occur in the absence of functional U1 snRNP (Frendewey *et al.*, 1987; Krämer, 1987; Hamm *et al.*, 1989). This was the case even in partially purified systems (Krämer, 1988; Zamore and Green, 1989). Pre-spliceosome formation also takes place on substrates lacking a 5′ splice site (Frendewey and Keller, 1985; Konarska and Sharp, 1986; Bindereif and Green, 1987; Lamond *et al.*, 1987). Because the 5′ splice site had been shown to interact with U1 snRNA by base pairing (Zhuang and Weiner, 1986), these other observations reinforced the suggestion that U1 snRNP was not involved in U2 snRNP addition to the pre-mRNA. Very recent experiments indicate to the contrary, i.e. that U1 snRNP is involved in the formation of the U2 snRNP-pre-mRNA complex, although no interaction with the 5′ splice site is required (Barabino *et al.*, 1990). Another recent study reports the identification of a U1 snRNP-pre-mRNA complex during mammalian spliceosome formation (Reed, 1990). Further studies will be required to clarify the exact role of U1 snRNP in mammalian pre-spliceosome formation.

In contrast, a relatively consistent picture of the early steps of spliceosome assembly has emerged from *in vitro* studies in yeast (*Saccharomyces cerevisiae*) extracts. Both the 5′ splice site and branchpoint region of the pre-mRNA are required for pre-spliceosome formation (Pikielny and Rosbash, 1986; Cheng and Abelson, 1987; Rymond and Rosbash, 1988). The 5′ splice site requirement is due in part to a requirement for a proper 5′ splice site-U1 snRNA base pairing interaction before or concomitant with U2 snRNP binding (Séraphin *et al.*, 1988).

In the absence of active U2 snRNP, pre-mRNA is assembled into a functional stable complex, committed to the spliceosome and splicing pathway (a 'commitment complex'—Legrain *et al.*, 1988). With the development of effective genetic procedures to prepare snRNP-depleted extracts, it was shown that commitment complex formation required U1 snRNP but took place in the absence of U2 snRNP (Séraphin and Rosbash, 1989). Further insight into commitment complex assembly was obtained with a novel native gel electrophoresis procedure. This technique revealed that commitment complexes could be fractionated into two sub-species, both of which contained U1 snRNP (Séraphin and Rosbash, 1989). Finally, indirect competition assays indicated that optimal commitment complex formation required two pre-mRNA *cis*-acting sequences, the 5′ splice site (GUAUGU) and branchpoint region (UACUAAC) (Legrain *et al.*, 1988; Séraphin and Rosbash, 1989).

Similar conclusions were independently drawn by Ruby and Abelson (1988). Although the affinity chromatography procedure used in that study could not address the functional relevance of the observed pre-mRNA-containing complexes, U1 snRNP binding seemed to precede and was necessary for subsequent U2 snRNP binding. Also, direct assays of mutant substrates showed that the formation of the U1 snRNP-pre-mRNA complex required a proper branchpoint sequence as well as a proper 5′ splice site.

In this communication, we have used our native gel electrophoresis procedures and U2 snRNP-depleted extracts to analyze directly the role of pre-mRNA *cis*-acting sequences in the formation of functional U1 snRNP complexes, i.e. commitment complexes (Seraphin and Rosbash, 1989). U1 snRNP binding required a proper 5′ splice site but did not require a branchpoint sequence.

However, one of the two commitment complex sub-species was absolutely dependent on a wild-type branchpoint sequence, consistent with the notion that this *cis*-acting sequence is recognized prior to U2 snRNP binding during spliceosome assembly.

Results

Construction of mutant pre-mRNA substrates

To study the effects of intron *cis*-acting sequences on commitment complex formation, we constructed a set of mutant pre-mRNA substrates. The starting plasmid was a T7 promoter-containing vector into which the WT-Δ2 intron had been inserted (see Materials and methods). Because the WT-Δ2 intron contains a pseudo-branchpoint sequence (UACaAAC, Figure 1) that might complicate interpretation, this region was mutagenized and changed into an *Nsi*I restriction site. The resulting construct was named WT-B. A 5′ splice site mutation (GUAUaU, formerly called 5′II, Jacquier *et al.*, 1985) and three mutations in the branchpoint region (UACaAAC; ΔUACUAAC and Δ2-3B) were then independently introduced into the WT-B background (Figure 1).

Complex formation using 5′ splice site and branchpoint mutant substrates

Full length RNA substrates were incubated in a U2 snRNP-depleted (U2-depleted) extract and the complexes resolved by native gel electrophoresis (Figure 2A). The two U1 snRNP-containing commitment complex bands previously described (Seraphin and Rosbash, 1989) were observed with both the WT-Δ2 and the WT-B substrates (lanes 1 and 2). Interestingly, the ratio of the two bands (i.e. the amount of faster mobility complex, CC1, compared with the amount of slower mobility complex, CC2) differed slightly between the two 'wild-type' substrates. This is consistent with some interference by the pseudo-branchpoint sequence of the WT-Δ2 construct with formation of the more mature CC2 complex (see below).

With the 5′ splice site mutant substrate, only trace amounts of complex were observed (lane 3), consistent with the idea that a proper 5′ splice site is important for stable U1 snRNP binding. With all three branchpoint mutants, large amounts of commitment complex formation took place (lanes 4–6), but only a single band that comigrated with CC1 was detected. We will refer to this complex also as CC1 although we do not know that this complex is identical to the CC1 complex formed with the wild-type substrates. Complex formation with the branchpoint mutant substrates occurred in the absence of added ATP but required U1 snRNP (data not shown, see also Legrain *et al.*, 1988; Séraphin and Rosbash, 1989).

In a second set of experiments, complex formation was assayed after incubating the same substrates in a complete whole cell extract (Figure 2B). Spliceosome formation was observed with both the WT-Δ2 and WT-B substrates (lanes 1 and 2, respectively), whereas only traces of splicing complexes were detected when the 5′ splice site GUAUaU mutant substrate was used (lane 3) as expected from previous observations (Séraphin *et al.*, 1988). With the three branchpoint mutant substrates, only a single complex comigrating with CC1 was observed (lanes 3–5). As these results were indistinguishable from those obtained with the branchpoint mutant substrates in the U2-depleted extract (Figure 2A), we conclude that they were not due to the depletion procedure but that these mutant substrates were unable to proceed past this early stage of the spliceosome assembly pathway.

To examine further the role of the 3′ splice site region in commitment complex formation, we generated truncated pre-mRNA substrates by cleaving the DNA templates with the restriction enzyme *Nsi*I before *in vitro* transcription (see Figure 1). Control transcripts were synthesized after cleavage of the templates at the *Dde*I site 32 nucleotides downstream of the 3′ splice site. After incubation in extract, the complexes were resolved by native gel electrophoresis (Figure 2C).

The WT-B substrate formed spliceosomes in a complete extract and commitment complexes in a U2 snRNP-depleted extract, with or without a 3′ splice site (compare lane 1 with lane 2, and lane 3 with lane 7, respectively; see also Rymond and Rosbash, 1985; Rymond *et al.*, 1987). Similarly, the

Fig. 1. Primary sequences of the various substrates. Sequences encompassing the 5′ splice site, branchpoint region and 3′ splice site of the RP51A derivatives used in this study are depicted. The names of constructs are shown preceding the corresponding sequences. The pseudo-branchpoint sequence present in the WT-Δ2 construct is indicated; in the other constructs this sequence has been replaced by an *Nsi*I restriction site. Dots indicate deleted nucleotides.

branchpoint deletion mutant substrate formed CC1 with or without a 3′ splice site (lanes 5 and 9). These results indicate that CC1 formation can occur in the absence of both a branchpoint sequence and a 3′ splice site, suggesting that the only highly conserved pre-mRNA *cis*-acting sequence required for formation of a U1 snRNP-containing complex is the 5′ splice site. As expected, little or no specific complexes were detectable with the full length or truncated 5′ splice site GUAUaU mutant substrate (lanes 4 and 8) or with non-specific substrates of similar lengths (lanes 6 and 10).

In summary, a 5′ splice site mutant blocked commitment complex formation, whereas deletion of the 3′ splice site did not affect this process. An intermediate situation obtains for the branchpoint sequence, as it was not required for formation of the CC1 complex but was required for CC2 formation.

Commitment complex formation using actin substrates

The observations described above were consistent with our previous competition experiments that indicated a role for the branchpoint as well as the 5′ splice site prior to U2 snRNP addition (e.g. Séraphin and Rosbash, 1989). The branchpoint independence of commitment complex formation (CC1 formation) was surprising, however, because Ruby and Abelson (1988) had reported that U1 snRNP binding to the pre-mRNA requires a wild-type UACUAAC sequence. Because actin gene-derived substrates were used in that study, we analyzed commitment complex formation using the same substrates (Figure 3). The wild-type actin substrate formed spliceosomes in a complete extract (lane 6) and the two expected commitment complex bands in a U2-depleted extract (lane 2). In both a U2-depleted extract and a complete extract, the actin branchpoint mutant (A257,

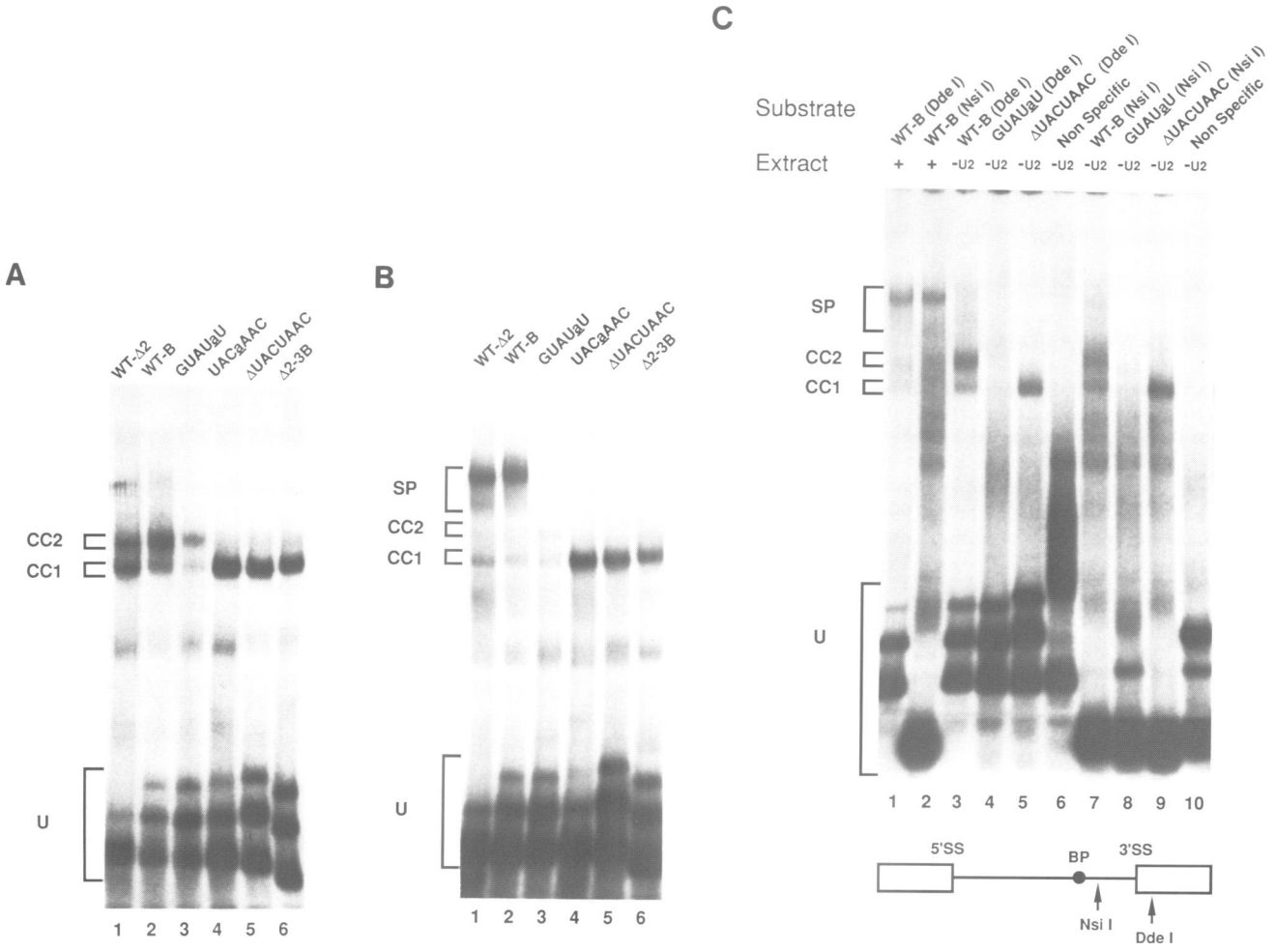

Fig. 2. Effect of the pre-mRNA sequence elements on commitment complex assembly. (**A**) Complexes formed after incubation of wild-type, 5′ splice site and branchpoint mutant pre-mRNAs in a U2-depleted extract. Complexes were resolved by native gel electrophoresis. The names of the substrates are shown at the tops of the corresponding lanes. CC1 and CC2 indicate the two forms of commitment complex, while U denotes non-specific complexes and un-complexed pre-mRNA. (**B**) Complexes formed in a complete extract. The same substrates as those used in Figure 2A were incubated in a complete extract and the complexes formed resolved by gel electrophoresis. Substrates and complexes are labeled as in Figure 2A, with S indicating spliceosomes. (**C**) Effect of the 3′ splice site sequence on commitment complex assembly. Complex formation was analyzed using pre-mRNA substrates lacking the 3′ splice site (*Nsi*I) or control full-length pre-mRNA (*Dde*I). The diagram at the bottom of the figure indicates the location of the *Nsi*I and *Dde*I restriction sites used to cleave the DNA templates with respect to the *cis*-acting sequence elements. The WT-B (wild-type), GUAUaU (5′ splice site mutant) and ΔUACUAAC (branchpoint mutant) were used in this experiment. Non-specific substrates are derived from transcription of the pTZ19R vector. The plasmid DNA was cleaved with *Bgl*I to give RNA of a size similar to full length pre-mRNAs or with *Pvu*II to give RNA of size similar to pre-mRNAs lacking the 3′ splice site. RNAs were incubated either in complete extract (lanes 1 and 2 labeled +) or in U2-depleted extract (lanes 3−10 labeled −U2). Complexes formed are labeled as in (A) and (B). The heterogeneity of the non-specific complexes (U) results from the use of substrates of different lengths.

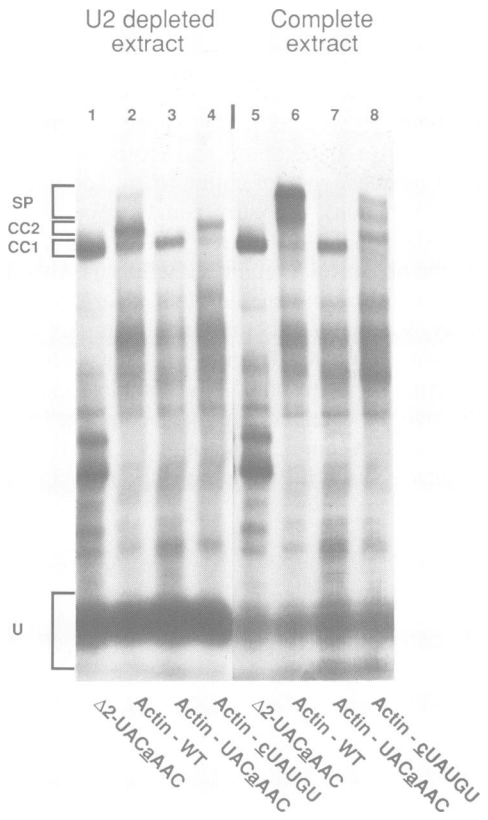

Fig. 3. Complex formation using actin substrates. The wild-type actin substrate (Actin-WT), a 5′ splice site mutant derivative (Actin-cUAUGU, C1 in Vijayraghavan *et al.*, 1986) and a branchpoint mutant (Actin-UACaAAC, formerly A257) were used to study complex formation in U2-depleted and complete extracts. The UACaAAC mutant derived from the RP51A gene was used as a positive control as well as a size marker (lane 1). Complexes are labeled as in Figure 2.

UACaAAC) formed only a single complex that comigrated with CC1 (compare lanes 3 and 7 with lanes 1 and 5). In a U2-depleted extract, the actin 5′ splice site mutant (C1, cUAUGU) formed a reduced but significant amount of commitment complex, most of which comigrated with CC2 (lane 4). In a complete extract, this 5′ splice site mutant gave rise to a reduced level of commitment complexes and trace amounts of spliceosomes (lane 8), indicating that this mutant may affect U2 snRNP addition as well as commitment complex formation (see Discussion).

In conclusion, the results with the actin substrates were almost identical to those obtained with the WT-Δ2 (RP51A gene-derived) substrates.

Effect of the branchpoint sequence on the kinetics of commitment complex formation and commitment complex stability

We considered that the branchpoint mutants might have pronounced effects on the rate of formation or stability of commitment complexes. A representative time course of commitment complex formation for both a WT-B and a Δ2-3B substrate is shown in Figure 4A. Quantitative results, obtained by scintillation counting of the relevant portions of the gel, show that the relative rates of commitment complex formation are indistinguishable for the two substrates (Figure 4B).

To assess the relative stability of the different commitment complexes under splicing conditions we first incubated the

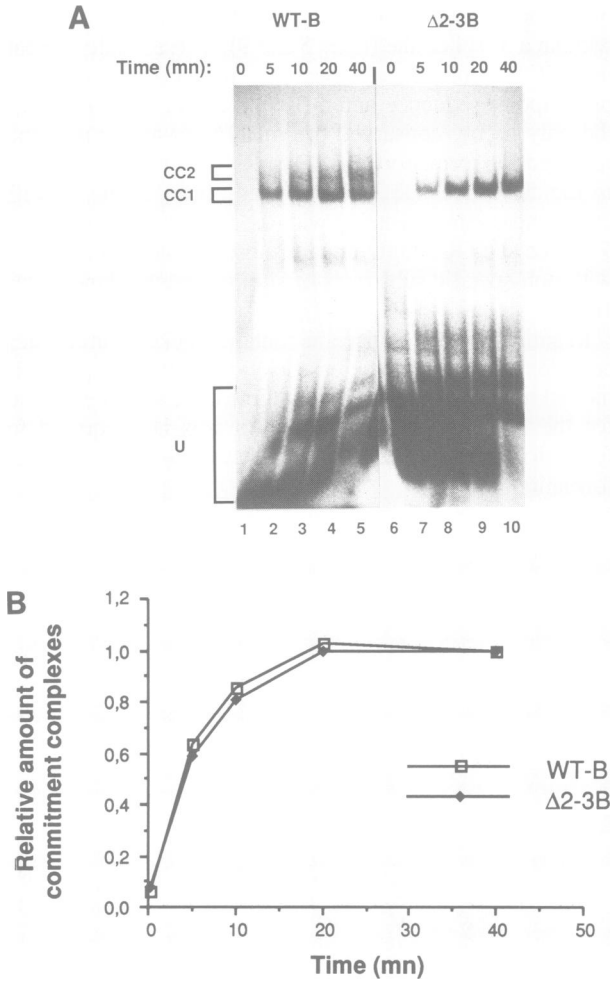

Fig. 4. Kinetics of commitment complex assembly. (**A**) Determination of the kinetics of commitment complex formation in a U2-depleted extract for either a WT-B substrate (lanes 1–5) or a Δ2-3B substrate (lanes 6–10). Samples were taken after 0 (lanes 1 and 6), 5 (lanes 2 and 7), 10 (lanes 3 and 8), 20 (lanes 4 and 9) and 40 (lanes 5 and 10) min of incubation. Complexes are labeled as in Figure 2. (**B**) Plot of the amount of commitment complexes formed as a function of time. The regions corresponding to the commitment complexes were excised from the gel shown in (A) and the radioactivity present in the relevant bands was determined by scintillation counting. The background, determined by counting a 'non-radioactive' portion of the gel, was subtracted. The values are expressed as a fraction of the commitment complexes formed at 40 min.

various labeled substrates for 20 min in a U2-depleted extract to form commitment complexes. The assembly reactions were then quenched by the addition of a large excess of cold WT-B pre-mRNA. The incubation was continued and samples taken at various times after the addition of the cold RNA. The results of such experiments indicated that the commitment complexes formed by wild-type or branchpoint mutant substrates were very stable (Figure 5, lanes 1–3, 7–9 and 10–12). Only the trace amounts of commitment complex formed by the 5′ splice site mutant were visibly unstable (Figure 5, lanes 4–6). We conclude that the branchpoint mutations do not measurably affect the rate of formation or the stability of commitment complexes.

Competition analyses

Although the experiments presented above showed that rapid and stable U1 snRNP binding does not require a pre-mRNA branchpoint sequence, we performed more sensitive

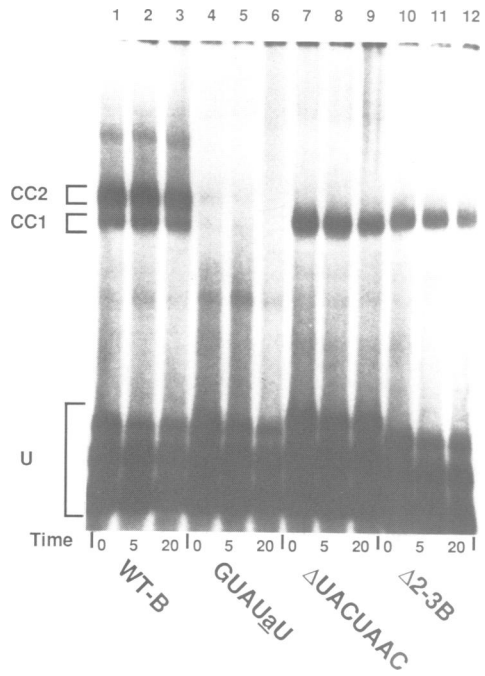

Fig. 5. Stability of commitment complexes. Radioactively labeled wild-type and mutant pre-mRNAs were incubated in a U2-depleted extract for 20 min. A large molar excess (50-fold) of cold wild-type pre-mRNA was then added to each reaction. Samples were then withdrawn 0 min lanes 1, 4, 7 and 10), 5 min (lanes 2, 5, 8 and 11) and 20 min (lanes 3, 6, 9 and 12) after addition of the cold pre-mRNA and analyzed by native gel electrophoresis. Complexes are labeled as in Figure 2.

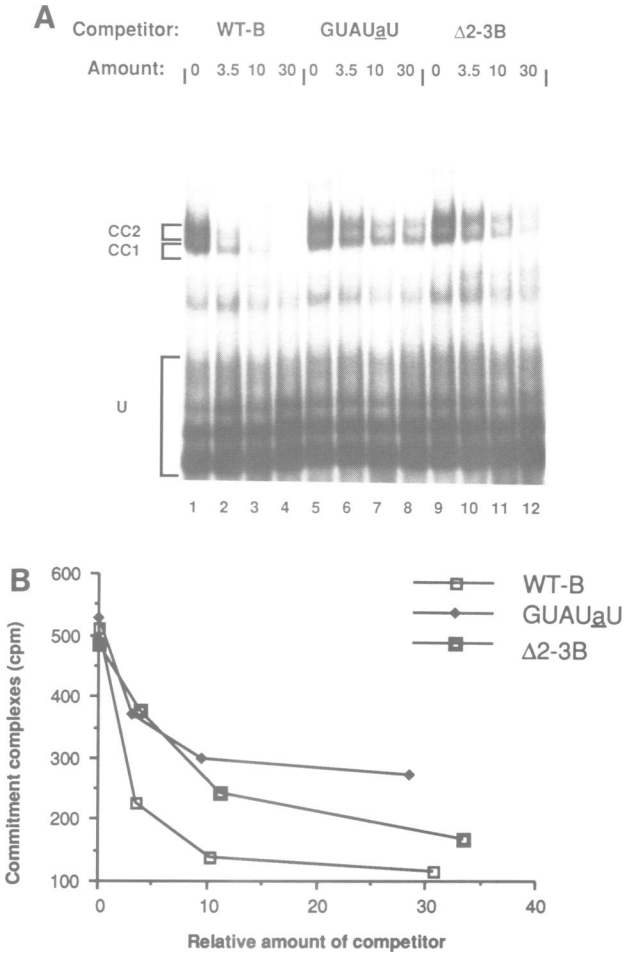

Fig. 6. Competition analyses. (**A**) Increasing amounts of cold wild-type or mutant pre-mRNA were mixed with labeled wild-type pre-mRNA. RNA was incubated for 20 min at 25°C after addition of U2-depleted extract and analyzed by native gel electrophoresis. The competitors used were WT-B, the GUAUaU 5′ splice site mutant and the Δ2-3B branchpoint deletion mutant. The amount of added competitor is indicated at the top of each lane; a value of 1 is equal to the amount of labeled pre-mRNA. Complexes are labeled as in Figure 2. (**B**) Quantification of the competition experiments. Bands corresponding to the commitment complex region of the gel shown in (A) were excised and the radioactivity determined by scintillation counting. The background value was determined and subtracted (see Figure 4). The values (in c.p.m.) are plotted as a function of the amount of competitor RNA.

competition experiments to test for an effect of the branchpoint sequence on commitment complex formation. In preliminary experiments, we empirically determined the amount of pre-mRNA required to saturate the commitment complex assembly reaction. Then a fixed and saturating amount of labeled WT-B pre-mRNA was mixed with increasing amounts of non-radioactive WT-B, 5′ splice site mutant GUAUaU, or the branchpoint deletion mutant Δ2-3B competitor RNA before incubation in the U2-depleted extract. Commitment complex formation was assayed by gel electrophoresis, and quantitative data were obtained by excising the relevant bands and scintillation counting (Figure 6).

The results indicate that the wild-type RNA was the most effective competitor. Competition with this substrate generated a curve corresponding to the result expected for isotopic dilution, confirming that the reaction was saturated with labeled pre-mRNA substrate (Figure 6B). In contrast, both a branchpoint deletion mutant and a 5′ splice site mutant were relatively poor competitors, although the former was slightly stronger than the latter. We conclude that commitment complex formation is affected by the branchpoint sequence, despite its not being required for the formation of U1 snRNP–pre-mRNA complexes.

Discussion

The experiments presented in this report extend and deepen our view of the early steps of spliceosome assembly. Our previous report indicated that the pre-mRNA 5′ splice site, the pre-mRNA branchpoint sequence, U1 snRNP, and a putative factor X interact to form a commitment complex that is the substrate for U2 snRNP addition to the pre-mRNA (Figure 7, CC2). That previous communication did not directly address the precise relationship between the branchpoint sequence and U1 snRNP binding, and it noted that commitment complex formation may consist of two or more steps of undetermined order and requirements (Séraphin and Rosbash, 1989). Indeed, the data presented here are most easily interpreted by suggesting that commitment complex formation occurs in at least two steps (Figure 7a). The first step, CC1 formation, results from an interaction between U1 snRNP and the pre-mRNA 5′ splice site. A second interaction that requires the branchpoint sequence and factor X then takes place and gives rise to CC2. Although X is depicted as an independent factor, it is also possible that X is part of U1 snRNP. In this case the mobility difference between CC1 and CC2 might involve a conformational change.

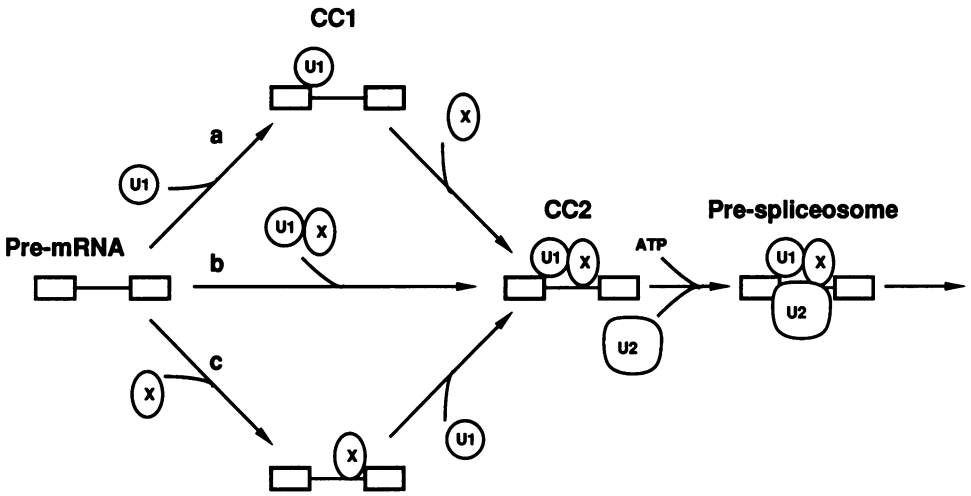

Fig. 7. Models for commitment complex assembly. Three potential pathways for commitment complex assembly are depicted (**a**–**c**). The pathway most compatible with the experiments described in the text is pathway a, where U1 snRNP interacts first with the 5′ splice site to form complex CC1. Then an unidentified factor X interacts with the branchpoint to give CC2. We cannot exclude the possibility that CC2 assembly also occurs to some extent through pathways b or c—see test. ATP-dependent binding of U2 snRNP to CC2 then leads to pre-spliceosome formation.

It is important to note that this model is only tentative because we have not shown that CC1 is a functional precursor to CC2 [e.g. with chase procedures (Legrain *et al.*, 1988)]. Thus either of the two commitment complexes may be a gel artefact. However, this possibility seems unlikely for several reasons. First, both complexes contain U1 snRNP (Seraphin and Rosbash, 1989). Second, they are affected differentially by substrate mutations, indicating that they represent two distinct molecular entities. Third, the two complexes can be chased into spliceosomes (Seraphin and Rosbash, 1989), suggesting that they both represent functional intermediates. In any case, we will use the two step model shown in Figure 7a to discuss the role(s) of the substrate *cis*-acting sequences during commitment complex assembly.

Both the RP51A (GUAUaU) and the actin-C1 (cUAUGU) 5′ splice site mutants dramatically reduced the amount of commitment complex, indicating that a proper 5′ splice site sequence is required for CC1 formation. Despite the low level of commitment complexes, these mutants formed a normal ratio of CC1 to CC2 (Figures 2A and 3), suggesting that the 5′ splice site sequence has little or no effect on the step that converts CC1 into CC2. In contrast, a proper 5′ splice site appears important for the CC2 to pre-spliceosome transition (Figure 7), as incubation of the actin-C1 (cUAUGU) substrate in complete extract resulted in inefficient spliceosome formation and the accumulation of commitment complexes (Figure 3, lane 8; compare with the wild-type control in lane 6). These data show that the 5′ splice site is recognized (at least) twice during splicing, once for commitment complex formation and again for efficient U2 snRNP addition (Figure 7). Based on independent experiments, we have previously suggested that multiple and sequential recognition of the 5′ splice site sequence is an important feature of the spliceosome assembly process and contributes to specificity (Séraphin and Rosbash, 1990).

The branchpoint mutations had a very different effect on commitment complex formation. All tested point mutants (AUCaAAC, UAuUAAC) and deletion mutants (ΔUAAC, ΔUACUAAC, Δ2-3B) did not form CC2 (Figure 2 and data not shown). Similar results have been obtained by modification interference procedures (H.V.Colot, personal communication). This was also true for the actin derivatives (Figure 3), thus demonstrating a strict requirement for the branchpoint sequence for converting CC1 into CC2. The results confirm that the branchpoint sequence can be recognized prior to pre-spliceosome formation, i.e. before U2 snRNP addition (Legrain *et al.*, 1988).

In both yeast and mammals, U2 snRNP has been shown to interact with the branchpoint sequence by base pairing (Parker *et al.*, 1987; Wu and Manley, 1989; Zhuang and Weiner, 1989). The observations reported here suggest that this pairing interaction is not the primary determinant of branchpoint recognition. This notion might contribute to an explanation of the relatively weak suppression of branchpoint mutants by compensating U2 suppressor snRNAs as well as their dominant phenotype (Parker *et al.*, 1987). As it is even possible that the U2 snRNA–pre-mRNA pairing interaction occurs subsequent to the association of U2 snRNP with the branchpoint region, further experiments will be required to determine at what point during spliceosome assembly this base pairing interaction takes place. In any case, our results indicate that recognition of the branchpoint region, like recognition of the 5′ splice site region, occurs more than once.

In contrast to their strong effects on CC2, the branchpoint mutations had little influence on CC1. Because the 3′ splice site deletion had no effect on commitment complex assembly and because we observed CC1 formation on substrates lacking both a branchpoint sequence and a 3′ splice site (Figure 2C), only the 5′ splice site appears to be absolutely required for U1 snRNP binding. We note, however, that other constructs containing only a 5′ splice site were inefficiently assembled into CC1 (data not shown). Although negative results of this nature suggest the existence of additional *cis*-acting sequence requirements for CC1 formation, other explanations are possible, e.g. that the secondary structures of those substrates were incompatible with U1 snRNP association.

To complement these direct assays, we have also performed competition experiments to assess the effects of mutations on commitment complex formation. This indirect approach is useful because the effect of a mutation on protein–nucleic acid complex formation (e.g. Dorn *et al.*,

1987; Revzin, 1989) can be quantified even if the corresponding complex is relatively unstable or otherwise less suitable for direct analysis, for example, in a gel shift assay.

The 5′ splice site mutant substrate competes poorly for both CC1 and CC2 formation (Figure 6), in perfect agreement with the direct assay. All the data obtained with the yeast system are consistent with a requirement for a U1 snRNA−5′ splice site interaction (most likely including base pairing) before the assembly of the U2 snRNP−pre-mRNA complex. In the HeLa system, cleavage or masking of the U1 snRNA 5′ end does not inhibit pre-spliceosome formation (Frendewey *et al.*, 1987; Krämer, 1987; Barabino *et al.*, 1990), yet U1 snRNP is involved in this process (Barabino *et al.*, 1990). It would appear, therefore, that U1 snRNP is required in the early events of spliceosome assembly in both yeast and mammals, but that a substantial difference between these systems lies in the extent of interaction of U1 snRNP with the pre-mRNA 5′ splice site.

The branchpoint deletion substrate Δ2-3B was a less effective competitor than the wild-type substrate but more effective than the 5′ splice site mutant (Figure 6), confirming our previous conclusions (Séraphin and Rosbash, 1989). These observations indicate that the branchpoint sequence is less important than the 5′ splice site for commitment complex formation, consistent with the results of the direct assays. Although more detailed interpretations are difficult, the fact that the branchpoint mutants do not compete as well as wild-type RNA for CC1 formation (Figure 6 and data not shown) is also consistent with the direct assays where we often observed slightly less total commitment complex with the branchpoint mutant substrates than with the wild-type substrates (data not shown). This suggests that the branchpoint region may also be relevant to the assembly of this complex, e.g. there may be other factors that contact the branchpoint and stabilize or aid an initial U1 snRNP−5′ splice site interaction. Alternatively, the order of addition of the two *trans*-acting factors indicated in Figure 7 may not be absolutely fixed, so that factor X might be able to interact with the branchpoint region before U1 snRNP interacts with the 5′ splice site (Figure 7c). U1 snRNP and X might even be able to interact with each other before binding simultaneously to the pre-mRNA substrate (Figure 7b). Although the direct assays favor the two-step pathway (CC1 to CC2; Figure 7a), these alternative assembly routes might coexist to some extent *in vitro* and help explain certain features of the competition data.

Ruby and Abelson (1988) have previously reported that, with an affinity chromatography assay, they detected either strongly reduced or only background U1 snRNP binding to several actin branchpoint mutant substrates. We were unable to confirm this conclusion, as we consistently observed efficient commitment complex formation with RP51A substrates containing point mutations and even deletions of the branchpoint sequence. Furthermore, identical observations were made with the actin substrates used in that study. We have ruled out the possibility that this discrepancy reflects abnormal characteristics (instability or different kinetics of formation) of the CC1 complex assembled on the various branchpoint mutant substrates, but it remains possible that it results from differences in extract preparation and/or differences in the two assays for U1 snRNP−pre-mRNA complex formation.

Several PRP gene products are required for the formation of U2 snRNP−pre-mRNA complex *in vitro* (Lin *et al.*, 1987; Abovich *et al.*, 1990). In the case of PRP9 (Abovich *et al.*, 1990), this is consistent with an *in vivo* study showing that this gene product is required to prevent pre-mRNA escape from the nucleus (Legrain and Rosbash, 1989). A PRP gene of this nature might code for the putative factor X. There are also several reports on mammalian protein factors required for a U2 snRNP−pre-mRNA interaction (e.g. Krämer, 1988; Ruskin *et al.*, 1988). In the case of U2AF, it has been shown that this factor recognizes and interacts with the polypyrimidine tract of the pre-mRNA prior to U2 snRNP binding to the branchpoint region (Zamore and Green, 1989). As mammalian U1 snRNP interacts with the pre-mRNA branchpoint/polypyrimidine region (Tatei *et al.*, 1984, 1987; Zillman *et al.*, 1987) and is necessary for pre-spliceosome formation (Barabino *et al.*, 1990), it is conceivable that, like the putative yeast factor X (Figure 7), U2AF or another splicing factor interacts simultaneously with the 3′ splice site region and U1 snRNP during the early steps of mammalian spliceosome assembly. The evolutionary conservation of the later steps of the spliceosome assembly pathway (e.g. Pikielny *et al.*, 1986; Cheng and Abelson, 1987; Lamond *et al.*, 1988) might then extend to the earlier steps, i.e. commitment complex formation.

Materials and methods

Plasmid construction

Plasmids were constructed according to standard protocols (Maniatis *et al.*, 1982) and propagated in *E.coli* strain JM101. The pBS117 plasmid contains a *Hin*dIII−*Sac*I fragment overlapping the wild-type Δ2 intron (Pikielny *et al.*, 1983; hereafter referred to as WT-Δ2) inserted between the *Hin*dIII and *Sac*I sites of the pTZ19R vector (US Biochemical). Both *Hin*dIII sites had been filled with the Klenow enzyme before ligation. The pseudo-branchpoint sequence (UACAAAC) of pBS117 was changed into an *Nsi*I restriction site by *in vitro* mutagenesis according to the method of Kunkel (1985) using the DT694 oligonucleotide (5′ GAATTGCATTCATGCATTTTTTATTTTG 3′). The resulting construct, pBS195, contains the WT-B intron. The ΔUACUAAC mutant is identical to the WT-B construct except that the 7 nucleotide branchpoint sequence has been removed by site-directed mutagenesis using oligonucleotide DT636 (5′ TTGATATCAGTAAAGTTGAATTGC 3′). The GUAUaU (formerly 5′II, Jacquier *et al.*, 1985) and Δ3B (Pikielny *et al.*, 1983) mutations were transferred from the HZ18 background to the WT-B intron background by standard cloning procedures. Unless otherwise stated, DNA templates were linearized at the *Dde*I site located in exon 2 before *in vitro* transcription. SP6−actin constructs (Vijayraghavan *et al.*, 1986) were obtained from S.Ruby and J.Abelson. DNA was phenol extracted and ethanol precipitated before *in vitro* transcription with either T7 RNA polymerase (Promega Biotec or US Biochemical) or SP6 polymerase (Biolabs). Labeled RNAs were prepared according to Pikielny and Rosbash (1986) while cold RNAs had a 500-fold reduced specific activity.

In vitro splicing complex assembly

Splicing extracts were prepared according to the mini-extract procedure of Séraphin and Rosbash (1989). U2 snRNA-depleted extracts were prepared from strain BS-Y88 (MATa, leu2-3, leu2-112, ura3-52, trp1-289, arg4, ade2, snr20::URA3, pBS129) after growth for 16 h in glucose-containing medium. The isogenic wild-type strain, BS-Y46, was used for wild-type extract preparation.

In vitro splicing reactions contained ~0.5 ng of labeled pre-mRNA, 2 mM ATP, 2.5 mM $MgCl_2$, 3% PEG 8000, 60 mM potassium phosphate, pH 7.0 and 3 or 4 μl of splicing extract in a 10 μl final volume unless otherwise stated. Reaction were typically incubated at 25°C for 20 min. Competitor RNA in 1 μl of water was mixed with the other components before starting the reaction. Reactions were stopped by the addition of 10 μl of cold R* buffer (2 mM $Mg(OAc)_2$, 50 mM HEPES, pH 8.0) and 10 μg (1 μl) of total yeast RNAs. 5 μl of dyes were added before loading (Pikielny *et al.*, 1986).

Native gel analysis

Native gels were according to Séraphin and Rosbash (1989) and contained 3% acrylamide (60:1), 0.5% agarose, 0.5 × TBE and 5% glycerol. Addition

of glycerol in the gel resulted in sharper bands (data not shown). Gels were run in the cold room for 15 h at 100 V, dried and autoradiographed.

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