MOLECULAR AND CELLULAR BIOLOGY, July 1997, p. 3469–3476 Vol. 17, No. 7
0270-7306/97/$04.00+0

The Branchpoint Residue Is Recognized during Commitment Complex Formation before Being Bulged out of the U2 snRNA–pre-mRNA Duplex

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Received 2 January 1997/Returned for modification 24 February 1997/Accepted 8 April 1997

We have analyzed the mechanism of branchpoint nucleotide selection during the first step of pre-mRNA splicing. It has previously been proposed that the branchpoint is selected as an adenosine residue bulged out of an RNA helix formed by the U2 snRNA–pre-mRNA base pairing. Although compatible with this bulge hypothesis, available data from both yeast and mammalian systems did not rule out alternative structures for the branch nucleotide. Mutating the residue preceding the branchpoint nucleotide in our reporter construct conferred a splicing defect that was suppressed in vivo by the complementary U2 snRNA mutants. In contrast, substitutions on the 3′ side of the branchpoint could be suppressed by complementary U2 snRNA mutants only in a weakened intron context. To test why the identity of the branch nucleotide was important for its selection, we analyzed the effect of substitutions at this position on spliceosome assembly. We observed that these mutations block the formation of one of the two commitment complexes. Our results demonstrate that yeast branchpoint selection occurs in multiple steps. The nature of the branch residue is recognized, in the absence of U2 snRNA, during commitment complex formation. Then, base pairing with U2 snRNA constrains this residue into a bulge conformation.

Splicing of pre-mRNAs occurs in two distinct steps: (i) a first transesterification links the guanosine of the 5′ splice site to the branchpoint nucleotide through a 2′-5′ bond, leading to the formation of lariat intermediate and free 5′ exon; and (ii) a second transesterification leads to the cleavage of the junction between the intron and the 3′ exon sequence and the ligation of the two exons. Both steps involve the contribution of different proteins and snRNPs (small nuclear ribonucleoproteins) (reviewed in references 18, 28, and 31) that associate with the pre-mRNA in a stepwise manner. The sequential interactions between the pre-mRNA and the snRNPs can be reproduced in vitro and analyzed by nondenaturing gel electrophoresis, gradient sedimentation, or affinity chromatography (3, 9, 10, 17, 33, 34). In *Saccharomyces cerevisiae* extracts, recognition of the 5′ splice site by U1 snRNP occurs during the formation of an early complex named CC1 (commitment complex 1). The formation of the second commitment complex, CC2, requires in addition the recognition of the branchpoint region of the pre-mRNA. However, CC2 formation occurs in the absence of U2 snRNP (reviewed in reference 38). Analysis of *cis*- and *trans*-acting mutants suggest that CC1 and CC2 bear a precursor-product relationship (1, 44). A prespliceosomal complex is formed by addition of U2 snRNP to CC2. This prespliceosome matures in a full spliceosome with the association of the three other snRNPs involved in splicing, U4, U5, and U6.

The accuracy of the splicing reaction needs to be very high to allow the formation of functional messages, especially in the case of genes containing numerous introns. The necessity for high accuracy seems, however, in contradiction with the poor conservation of the 5′ splice site, branchpoint, and 3′ splice site sequences of metazoan introns (47). On the other hand, there is rarely more than one intron per yeast transcript, and these have highly conserved 5′ splice site (G/GUAUGU), branchpoint (UACUAACA), and 3′ splice site (PyAG) sequences (15, 50). Several studies have shown that the 5′ splice site is recognized through base pairing with the U1, U5, and U6 snRNAs (16, 20, 30, 40, 46, 52; reviewed in reference 31). These interactions are at least partly responsible for splicing accuracy.

In yeast, the selection of the branchpoint nucleotide is also particularly accurate, with the penultimate adenosine of the UACUAACA sequence being chosen for branching. For most metazoan introns, an A residue is also mainly selected for branching. The sequence of the branchpoint region is important for splicing even though it appears much more degenerate (29, 37). Nevertheless, determination of the consensus branchpoint sequence through competition experiments has demonstrated that the optimal branchpoint sequence is UACUAAC for metazoan introns (54). Ares proposed that following the cloning of the yeast U2 snRNA, the nucleotide preceding and following the branchpoint could base pair with the U2 snRNA (2). This hypothesis has received some experimental support in both yeast (32) and mammalian (51, 54) systems with the demonstration that mutation of some positions preceding the branch nucleotide can be suppressed by complementary mutations in the U2 snRNA. It was thus proposed that the branch nucleotide is selected as the residue bulged out of the U2 snRNA–pre-mRNA base pairing. (Throughout this report, a nucleotide bulged out of an RNA helix refers to a nonpaired nucleotide flanked by two paired residues. The bulged base can either stack in the helix or protrude from it.)

Recently, branchpoint selection has been extensively analyzed in human cell nuclear extracts in vitro (35, 36). Analysis of the splicing behavior of pre-mRNAs carrying a site-specific substitution at the branchpoint supports the bulge hypothesis for branchpoint selection. Indeed, substitutions of the ribose group favoring a bulged conformation positively affect branchpoint selection. These analyses also indicated that either of the last two A's of a UGCUAAC sequence can be used for branching. Finally, cross-linking studies demonstrated that the branch-

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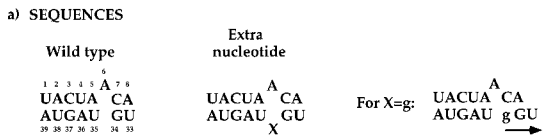
point region interacts specifically with proteins in the absence of U2 snRNA (4, 7, 22).

These data do not, however, provide a complete explanation of the mechanism of branchpoint selection. Indeed, it has not been demonstrated that the pre-mRNA nucleotides located 3′ to the branchpoint do base pair with U2 snRNA. It therefore remains possible that the branchpoint nucleotide is not maintained in a bulged conformation. Furthermore, the selection of the branchpoint as the nucleotide bulged out of the U2 snRNA–pre-mRNA helix does not explain the strong selection for an A residue at this position. Finally, analysis of the branchpoint selected in vivo in yeast indicates that the penultimate adenosine of the UACUAACA sequence was nearly exclusively selected (see reference 43, however). This contrasts with the results of in vitro analyses using human cell extracts (35). Given these gaps in our understanding of branchpoint selection, we decided to reinvestigate this process in yeast. Our results indicate that the selection of the branchpoint occurs in multiple steps: the nature of the branch nucleotide is recognized during commitment complex formation, while its bulged configuration results from the U2 snRNA–pre-mRNA pairing.



b)

U2 snRNA	G34A	G34C	G34U	U35G	U35C	U35A	UU	UA	UC	UG
Viability on FOA	NO	NO	NO	NO	NO	NO	NO	NO	NO	YES

FIG. 1. Proposed structure of the U2 snRNA-branchpoint duplex and U2 snRNA mutant phenotypes. (a) Potential base-pairing structures formed between the branchpoint and wild-type or mutant U2 snRNAs. A8 is only partially conserved in natural introns, and therefore base pairing with U33 is not essential for pre-mRNA splicing. (b) Phenotypes of U2 snRNA mutants on 5-fluoro-orotic acid (FOA) plates.

MATERIALS AND METHODS

Plasmids. The different U2 snRNA mutants were constructed by site-directed mutagenesis (19) using plasmid pBS144 as the template. The portion of the U2 snRNA gene (2) located upstream of the *Bst*BI restriction site was sequenced to ascertain the presence of the desired mutation and absence of unwanted changes. A *Bst*BI-*Sal*I fragment from the mutagenized clones was then transferred in a *LEU2* centromeric yeast backbone (pBS296) already containing the wild-type U2 snRNA sequence located downstream of the *Bst*BI site (38a). The following constructs were made in this study: pBS513 (UU), pBS692 (UA), pBS693 (UG), pBS694 (UC), pBS857 (U35A), pBS858 (G34U), pBS859 (G34A), pBS860 (G34C), pBS914 (U35G), and pBS915 (U35C).

The reporter gene mutants were obtained by site-directed mutagenesis of the RP51 intron inserted in plasmid pBS7 (40). The presence of the desired mutation was ascertained by DNA sequencing. The following constructs were made in this study: pBS898 (C7G), pBS899 (C7A), pBS900 (C7U), pBS901 (A5U), pBS902 (A5G), pBS903 (A5C), pBS1034 (A6C), pBS1035 (A6G), and pBS1036 (A6U). (The branchpoint spans positions 334 to 341 of the RP51 intron.)

The fragments harboring the desired intron mutation were then subcloned into two different vectors. (i) The *Sac*I-*Sal*I fragment of the mutated DNA was ligated to a 2μm yeast plasmid bearing an *ADE2* marker. In this construct, the expression of the reporter gene is under control of the *CUP1* promoter. The following constructs were made: pBS745 (wild-type intron), pBS1027 (G5A and C340G), pBS1028 (G5A and C340A), pBS1029 (G5A and C340U), pBS1030 (G5A), pBS1031 (C340G), pBS1032 (C340A), and pBS1033 (C340U). (ii) The *Sal*I-*Ase*I fragment of the mutated DNA was subcloned into plasmid pHZ18 (48) bearing the *URA3* marker. The expression of RP51 is under the control of the galactose promoter. The following constructs were made: pBS908 (C340G), pBS909 (C340A), pBS910 (C340U), pBS1084 (A339C), pBS1085 (A339G), and pBS1086 (A339U).

All plasmids were propagated in *Escherichia coli* MC1066.

Yeast strains and genetic techniques. All yeast transformations were done by the lithium acetate method (12). Media were prepared by using standard protocols (45).

Strain BSY325 (*MAT***a** *ade2 arg4 leu2-3,112 trp1-289 ura3-52 snr20*::*TRP1*, pBS499 [*CEN URA3 SNR20*]) (41) was used to test if U2 snRNA mutants were able to complement a U2 snRNA gene disruption. The U2 mutants to be tested were introduced into this strain, and transformants were selected on Leu-deficient plates. Four independent clones for each construct were transferred on 5-fluoro-orotic acid plates and assayed for growth at 30°C.

The wild-type strain BSY17 (*MAT***a** *ade2 arg4 leu2-3,112 trp1-289 ura3-52*) (40) was cotransformed with U2 snRNA constructs and *GAL*- or *CUP*-driven reporters. Transformants were grown in selective medium without inducer. When the optical density of the culture reached 0.3 to 0.5, expression of the reporter genes was induced by addition of galactose (to a final concentration of 2%) or by the addition of $CuSO_4$ (to a final concentration of 500 μM). Alternatively, U2 snRNA mutants and *CUP*-driven reporters were introduced in strain BSY88 (*MAT***a** *ade2 arg4 leu2-3,112 trp1-289 ura3-52 snr20*::*URA3*, pBS129 [*CEN TRP1 GAL-U2*]) (41). In this strain, expression of the wild-type U2 snRNA gene located on a *TRP1*-marked plasmid is under the control of the *GAL10-CYC1* promoter (11). Cells were grown in medium lacking Ade, Leu, and Trp and containing 3% galactose, 1% sucrose, and 0.05% glucose before being transferred to medium lacking Ade, Leu, and Trp and containing 2% glucose for 23 h to allow depletion of the wild-type U2 snRNA. Then, expression of the reporter constructs was induced for 1 h by addition of $CuSO_4$ to the culture medium to a final concentration of 500 μM.

β-Galactosidase assays. β-Galactosidase assays were performed in duplicate as described previously (16). We report the values of duplicate assays using two different transformants. The average and an error equal to the half difference are indicated. When the two measurements were identical, the error was set as the lowest half difference observed in the same experiment.

Total yeast RNA preparation. Ten milliliters of each induced culture was spun at 4°C for 20 min at 2,500 rpm in the GH3.7 rotor of a GPR centrifuge (Beckman). RNA extractions were made essentially as described previously (40). Briefly, cells were washed in 1 ml of RNA extraction buffer (100 mM LiCl, 1 mM EDTA, 100 mM Tris-Cl [pH 7.5]) and spun as described above; 500 μl of RNA extraction buffer, 10 μl of 10% sodium dodecyl sulfate, 500 μl of acid-washed siliconized glass beads, and 500 μl of PCI (phenol-chloroform-isoamyl alcohol, 25:25:1) were added to the cell pellets. Cells were then disrupted by vigorous shaking for 1 min in a cooled mixer (B. Braun). The aqueous phase was recovered after centrifugation for 5 min at 4°C. It was further extracted twice with PCI, and RNAs were recovered by ethanol precipitation. RNAs were analyzed by primer extension (40) using oligonucleotide RB1 priming in exon 2 (48).

Analysis of commitment complex formation. Strains BSY17 and BSY88 (see above) were grown for 20 h in YP medium containing 2% glucose. Forty milliliters of culture was used to prepare yeast extracts as described previously (41). Wild-type and branchpoint mutant pre-mRNAs were prepared by in vitro transcription using DNA from plasmids pBS7, pBS1034 (A6C), pBS1035 (A6G), and pBS1036 (A6U) cut with *Dde*I as templates. RNA transcribed from plasmid pBS87 carrying 5′II mutation (13) was used as a control for the specificity of the reaction (44). Radiolabeled transcripts were gel purified (41). Commitment complex formation reaction mixtures were incubated for 30 min at 25°C and contained 5 mM $MgCl_2$, 6% polyethylene glycol 8000, 120 mM potassium phosphate (pH 7.0), 4 μl of extract, and 20,000 cpm of radiolabeled pre-mRNA in a final volume of 10 μl. Reactions were transferred to ice and stopped by the addition of 10 μl of buffer R and 1 μl of total yeast RNA (10 μg/μl) as described previously (41, 44). After 10 min of incubation on ice, 5 μl of loading buffer was added and the samples were loaded on a nondenaturing acrylamide-agarose gel. Gels were run for 16 h at 80 V in a cold room before being dried and exposed (41, 44).

RESULTS

U2 snRNA mutants at positions 34 and 35 do not support growth. The model that has been proposed (2) for the structure of the U2 snRNA–pre-mRNA duplex is depicted in Fig. 1a. Throughout this report, the U2 snRNA nucleotides will be denoted by their positions from the 5′ end of the molecule. The conserved UACUAACA intronic residues will be collectively named the branchpoint and numbered from U1 to A8, with A6 being the branch residue. Previous experiments with yeast have demonstrated that positions C3 and U4 of the branchpoint base pair with positions G37 and A36, respectively, of the yeast U2 snRNA (32). Similar experiments have demonstrated base pairing between positions C3, U4, and A5 of the branchpoint and the complementary nucleotides U34, A35, and G36 of U2 snRNA in mammalian systems (51, 53). Furthermore, substitutions in the branchpoint binding region of U2 snRNA confer a recessive lethal phenotype and domi-

nant slow-growth phenotype in yeast (27, 32). In contrast, most single substitutions at the pre-mRNA branchpoint reduce but do not completely abolish splicing (6, 13, 49).

To test if U2 snRNA residues U35 and G34 base pair with positions A5 and C7, respectively, of the branchpoint, we first constructed all possible substitution mutants in the U2 snRNA gene at positions U35 and G34 (see Materials and Methods). We also constructed mutant U2 snRNA genes harboring all possible single-nucleotide insertions between positions U35 and G34 (U2 UU, UA, UC, and UG). These mutants should alter the proposed interaction with the branchpoint sequence either by extending the U2 snRNA–pre-mRNA base pairing to the branch residue (U2 UU) or by inserting a nonpaired nucleotide opposite the branch residue (U2 UA, UC and UG [Fig. 1a]).

We first tested whether these mutants were able to support growth by using a standard plasmid shuffling strategy (24). None of the U2 snRNA mutants with substitutions of U35 or G34 are able to support growth (Fig. 1b). This experiment indicated that U35 and G34 are essential for viability. We conclude that this region of the U2 snRNA is important for either snRNP assembly or function (see below).

Similarly, U2 snRNA insertion mutants U2 UU, U2 UA, and U2 UC did not support growth (Fig. 1b). Primer extension analyses indicated that the elongated U2 snRNA levels were similar to those of the endogenous wild-type U2 snRNA (data not shown). It is therefore likely that these mutations affect snRNP function through an alteration of the U2 snRNA-branchpoint duplex structure rather than by preventing snRNP assembly and stability. Surprisingly, a U2 snRNA mutant carrying an extra G between nucleotides 34 and 35 (U2 UG) was fully viable. We believe that in this mutant strain, C7 of the branchpoint sequence base pairs with the inserted G rather than with G34 (Fig. 1a). Consistent with the result of a previous in vitro analysis (25), our data indicate that the three-residue linker connecting the domains of the U2 snRNA interacting with U6 snRNA (positions 26 to 30) (23) with the one interacting with the branchpoint (positions 34 to 39) is flexible and can be structurally altered.

U2 snRNA U35 base pairs with A5 of the branchpoint. To determine whether U2 snRNA position 35 base pairs with the branchpoint A5, we constructed all possible substitutions at this position in our reporter construct. This construct contains the yeast RP51A intron inserted in the *E. coli lacZ* gene. Accurate removal of this intron is required for β-galactosidase production, making it possible to analyze the efficiency of splicing of each construct by assaying β-galactosidase activity. The reporter plasmids and U2 snRNA mutants at position 35 were introduced into the wild-type yeast strain BSY17 by cotransformation. As a control, we also cotransformed this strain with the reporter plasmids and a plasmid harboring the wild-type U2 snRNA gene. Each strain, therefore, expresses a wild-type chromosomal copy of the U2 snRNA gene in addition to the plasmid-encoded one. Expression of the reporter gene in these transformants was induced by addition of galactose and β-galactosidase assays were performed.

In the strains containing the reporter construct and an extra copy of the wild-type U2 snRNA gene on a plasmid, we observed that mutation of branchpoint position 5 resulted in a lower β-galactosidase activity (Table 1). This effect was more marked for the A5C and A5U mutants, with A5G being only moderately affected. This result is consistent with a previous analysis which demonstrated that substitution of the branchpoint A5 affects splicing efficiency in yeast (6). Surprisingly, the levels of β-galactosidase produced by the wild-type reporter were significantly reduced by the coexpression of the U35A, U35G, or U35C U2 snRNA mutant along with the wild-type gene (Table 1). These semidominant phenotypes probably reflect the ability of the mutant U2 snRNAs to compete with the wild-type U2 snRNA for assembly on wild-type pre-mRNA even though the mutant U2 snRNAs are, in this case, unable to efficiently carry out later steps of the splicing reaction. This observation is consistent with earlier observations which suggested that the U2 snRNA–pre-mRNA base pairing was not necessary for the U2 snRNP–pre-mRNA association (27, 40).

TABLE 1. Results of β-galactosidase assay for the A5 branchpoint mutants expressed in wild-type strain BSY17 in combination with wild-type or U35 mutant U2 snRNA

Mutant	Mean β-galactosidase activity (U) ± SE)			
	Wild-type U2 snRNA	U2 U35A	U2 U35G	U2 U35C
UACUAAC	641.4 ± 10.7	403.6 ± 4	284.1 ± 13.3	295.9 ± 1
UACUuAC	300 ± 25.9	424.4 ± 5.1	393.3 ± 1.2	259.3 ± 6.5
UACUgAC	510.6 ± 1.2	334 ± 15.4	346.6 ± 3.9	459.6 ± 18
UACUcAC	259.7 ± 5.7	152.8 ± 0.05	435.2 ± 1.7	59.5 ± 0.05

Interestingly, the β-galactosidase activity of the A5G reporter was significantly higher in the strains harboring the wild-type U2 snRNA or the U2 U35C mutant than in the strains harboring the U2 U35A and U35G mutants; similarly, the amount of β-galactosidase produced by the A5C mutant was strikingly and specifically enhanced in the presence of the U35G U2 snRNA (Table 1). Finally, the A5U construct was most efficiently expressed in cells containing either the U35A or U35G U2 snRNA mutant (Table 1). A direct analysis of the RNA present in these cells confirmed that increased β-galactosidase production reflected the enhancement of the first step of pre-mRNA splicing (data not shown).

These effects strictly correlate with the ability of the U2 snRNA position 35 to form a Watson-Crick (A-U or G-C) or a wobble G-U base pair with the branchpoint position 5. Therefore, we conclude that U2 snRNA U35 base pairs with the branchpoint A5. The efficient suppression of the reporter mutation by the complementary U2 snRNA mutants further suggests that U2 snRNA U35 and branchpoint A5 are probably not involved in any other strong specific interaction rate limiting for splicing.

C7 mutants affect splicing efficiency but are not efficiently suppressed by complementary U2 snRNA G34 mutants. The base pairing between the branchpoint sequence and U2 snRNA has to extend on both sides of the branch nucleotide to create the proposed bulged structure (Fig. 1a). To check if U2 snRNA G34 was involved in a base-pairing interaction with the pre-mRNA, we constructed all possible substitutions at position C7 in our reporter construct. These plasmids were cotransformed with either the wild-type U2 snRNA or derivatives carrying substitutions of G34 into the wild-type yeast strain BSY17. The splicing behavior of each construct was monitored by measuring the production of β-galactosidase after transcriptional induction with galactose. The results obtained in the presence of wild-type U2 snRNA indicate that the C7U mutant had only a mild effect on β-galactosidase production, while the C7A and C7G mutants reduced β-galactosidase levels by a factor of 2 (data not shown; see also reference 6). This is quantitatively similar to the effects observed for substitutions of the branchpoint A5 (Table 1). Surprisingly, however, the expression of the reporters harboring the C7 mutants was only weakly affected by the presence of mutant U2 snRNAs (data not shown). These negative results did not allow us to

TABLE 2. Results of β-galactosidase assay for the C7 branchpoint mutants combined with the 5′ splice site G5A mutation expressed in wild-type strain BSY17 in combination with wild-type or G34 mutant U2 snRNA

Mutant	Mean β-galactosidase activity (U) ± SE				
	Wild-type U2 snRNA	U2 G34U	U2 G34A	U2 G34C	No extra U2 snRNA
UACUAAC	128.3 ± 0.01	43 ± 0.01	148.5 ± 0.04	151.5 ± 0.01	131.5 ± 0.01
UACUAAg	0.4 ± 0.02	1.37 ± 0.04	2.71 ± 0.01	20.5 ± 0.03	0.3 ± 0.03
UACUAAa	1.7 ± 0.05	6.5 ± 0.02	1.8 ± 0.01	1.8 ± 0.01	1.3 ± 0.04
UACUAAu	18.4 ± 0.02	19.6 ± 0.01	42.8 ± 0.01	7.7 ± 0.02	22.1 ± 0.01

confirm or exclude the possibility of a base-pairing interaction between C7 and G34. We therefore decided to repeat these experiments in a more sensitive background.

In a weakened intron, U2 snRNA G34 mutants suppress the C7 mutants. The experiments described above were repeated with a pre-mRNA that is only weakly spliced, since we have previously shown that in a weakened intron background the effect of U snRNA–pre-mRNA base pairing is more apparent (8, 39, 42). For this purpose, we chose to combine the branchpoint C7 mutants with a G-to-A transition at position 5 of the 5′ splice site (see Materials and Methods). The substitution of a G by an A at 5′ splice site position 5 has been extensively analyzed with this reporter (13, 40, 43). The U2 snRNA and reporter constructs were cotransformed in the wild-type strain BSY17, the reporter was induced by the addition of copper, and β-galactosidase assays were performed. In the presence of a wild-type branchpoint, the 5′ splice site mutation reduced β-galactosidase production by a factor of 5, while combining

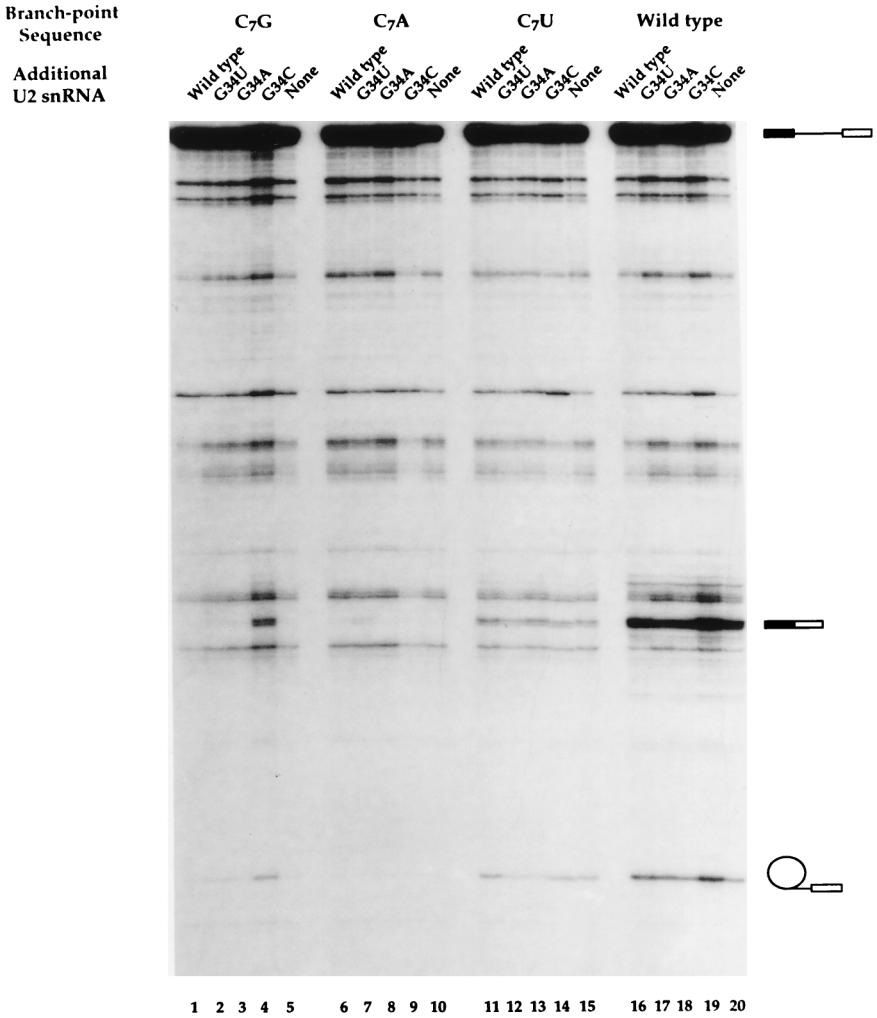


FIG. 2. Primer extension analysis of the constructs carrying the C7 branchpoint mutants combined with the 5′ splice site G5A mutation. Constructs were expressed in wild-type strain BSY17 in combination with wild-type or G34 mutant U2 snRNA. RNAs were extracted 1 h after induction of the reporter by the addition of 500 μM copper to the culture medium. The oligonucleotide RB1 used for primer extension hybridized to exon 2 of the RP51-LacZ pre-mRNA. Lanes: 1 to 5, C7G reporter; 6 to 10, C7A reporter; 11 to 15, C7U reporter; 16 to 20, wild-type reporter; 1, 6, 11, and 16, wild-type U2 snRNA; 2, 7, 12, and 17, G34U U2 snRNA; 3, 8, 13, and 18, G34A U2 snRNA; 4, 9, 14, and 19, G34C U2 snRNA; 5, 10, 15, and 20, no additional U2 snRNA.

the 5′ splice site and branchpoint mutations resulted in a 30- to 100-fold drop in β-galactosidase levels (data not shown). This result suggests that the nucleotide present at the branchpoint position 7 has a more critical role in the context of the weakened intron (see also reference 42). Expression of the construct containing the 5′ splice site mutant and a wild-type branchpoint was identical in strains expressing only the chromosomal copy of the U2 snRNA gene or the chromosomal and plasmid-encoded wild-type U2 snRNA or when either the U2 G34A or G34C mutant was expressed along with the wild-type chromosomal U2 snRNA; however, the expression of this reporter was reduced by the U2 snRNA G34U mutant (Table 2). For the reporter harboring the C7U branchpoint substitution combined with the 5′ splice site position 5 mutation, the G34A U2 snRNA enhanced its expression compared to wild-type U2 snRNA, while the G34C mutant reduced its expression (Table 2). When the C7G mutant was combined with the 5′ splice site mutation, we observed extremely low levels of β-galactosidase. Expression of this mutant was, however, strongly and specifically stimulated by the U2 snRNA G34C substitution; similarly, expression of the reporter combining the C7A substitution with the 5′ splice site mutation was specifically stimulated in the presence of the U2 snRNA G34U mutant (Table 2).

To confirm that these observations resulted from a splicing effect, we extracted RNA from these cells and analyzed them by primer extension using an exon 2-specific oligonucleotide (Fig. 2). As expected, high levels of pre-mRNA accumulated for these poorly spliced pre-mRNAs. Only low levels of lariat intermediate and mRNA accumulated, confirming that the mutations block pre-mRNA splicing before the first step. In agreement with the results of the β-galactosidase assay, the levels of mRNA were higher for the constructs harboring the wild-type branchpoint and only partly reduced for the C7U branchpoint sequence (Fig. 2, lanes 1, 6, 11, and 16). These levels were only slightly affected by the presence of U2 snRNA mutants, in agreement with the result of the β-galactosidase assay (Table 2). For the construct harboring the C7A mutation, we specifically observed traces of mRNA in the presence of the complementary G34U mutant U2 snRNA (Fig. 2, lane 7). For the C7G mutant, mRNA was detected only in the presence of the complementary G34C mutant (Fig. 2, lane 4). Overall, these results support the model that G34 of the U2 snRNA base pairs with the branchpoint position 7.

To confirm these results, we repeated this experiment with yeast strain BSY88, in which the chromosomal copy of the wild-type U2 snRNA gene has been disrupted. This mutation is complemented by a plasmid-borne wild-type U2 snRNA under the control of a galactose-inducible promoter. This promoter allows the induction of the U2 snRNA gene in the presence of galactose and its repression in the presence of glucose. We reasoned that a stronger effect of the base pairing between the last nucleotide of the branchpoint and U2 snRNA position 34 would be detected in the absence of the background splicing supported by the wild-type U2 snRNA expressed from the chromosomal gene. To perform this experiment, we transformed wild-type and mutant reporter genes inserted behind a copper-regulated promoter alongside U2 snRNA constructs in strain BSY88. Transformants were grown in galactose selective medium and then transferred to glucose selective medium for 23 h before the induction of the reporter gene by copper. This procedure allowed for the efficient depletion of the *GAL*-driven wild-type U2 snRNA, leaving only the constitutively expressed U2 snRNA in the cell at the time of reporter RNA synthesis. As before, reporter RNA splicing was monitored both by measuring β-galactosidase production (Fig. 3) and by primer extension (Fig. 4). The β-galactosidase

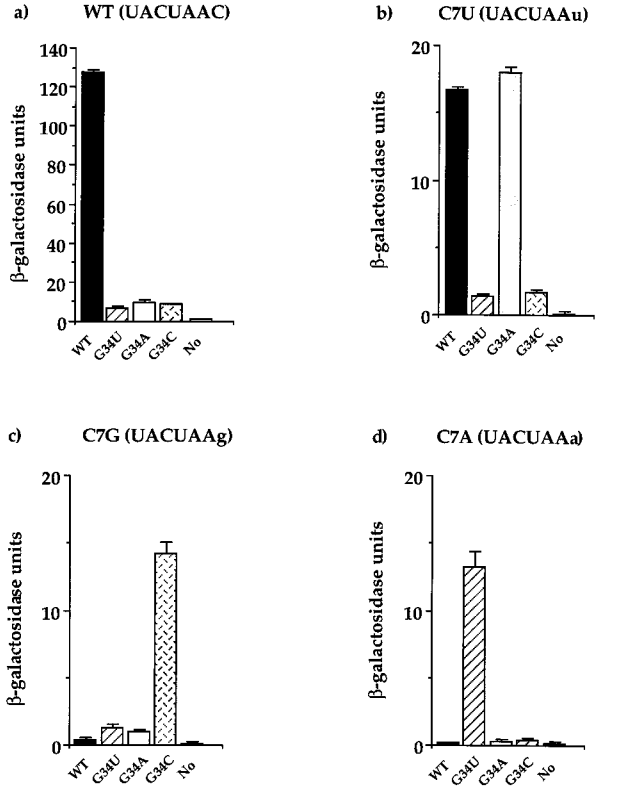


FIG. 3. Results of the β-galactosidase assay for the C7 branchpoint mutants combined with the 5′ splice site G5A mutation in the presence of either the wild-type or G34 mutant U2 snRNA. Constructs were expressed in strain BSY88 after depletion of the *GAL*-regulated wild-type U2 snRNA. The four panels depict the four different branchpoints: (a) wild type (WT); (b) C7U; (c) C7G; (d) C7A. The bars indicate the U2 snRNA cotransformed.

assay revealed that the construct containing the wild-type branchpoint is expressed most efficiently in the presence of the wild-type U2 snRNA (Fig. 3a), the C7U construct is expressed most efficiently in the presence of the complementary wild-type and G34A U2 snRNAs (Fig. 3b), the C7G construct is expressed most efficiently in the presence of the G34C U2 snRNA (Fig. 3c), and the C7A construct is expressed most efficiently in the presence of the G34U U2 snRNA (Fig. 3d). Although we could have anticipated that the G34U U2 snRNA would also suppress the C7G mutant through a wobble interaction, this does not appear to be the case (Fig. 3c). It is possible that the mutant U of the G34U U2 snRNA base pairs preferentially with the branch adenosine rather than with C7G, thereby preventing suppression (see also reference 27). The results of the β-galactosidase assay were fully supported by a direct analysis of the corresponding RNAs by primer extension (Fig. 4).

In summary, our results demonstrate a clear base pairing interaction between U2 snRNA G34 and the branchpoint C7. Taken together with the results described above, this finding strongly suggests that the branch nucleotide is constrained into a bulged configuration by the base pairing of U2 snRNA with flanking nucleotides.

The branchpoint adenosine is recognized prior to U2 snRNP addition. Our results suggest that the branch nucleotide is positioned to attack the 5′ splice site phosphodiester bond through the interaction of the branchpoint with U2 snRNA. However, these results do not explain why substitution of the branch A residue reduces the efficiency of the first

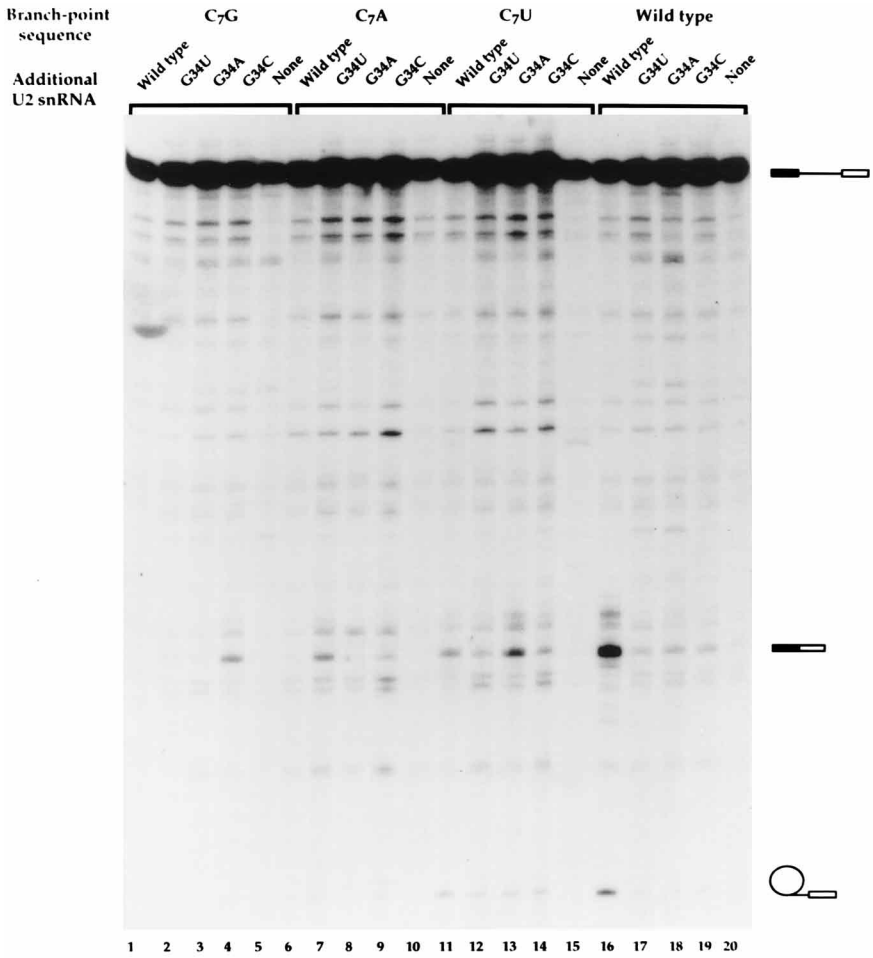


FIG. 4. Primer extension analysis of the constructs carrying the C7 branchpoint mutants combined with the 5′ splice site G5A mutation in the presence of either the wild-type or G34 mutant U2 snRNA. Constructs were expressed in strain BSY88 after depletion of the *GAL*-regulated wild-type U2 snRNA. RNA were extracted 1 h after induction of the reporter by the addition of 500 μM copper to the culture medium. The oligonucleotide RB1 used for primer extension hybridized to exon 2 of the RP51-LacZ pre-mRNA. Lanes: 1 to 5, C7G reporter; 6 to 10, C7A reporter; 11 to 15, C7U reporter; 16 to 20, wild-type reporter; 1, 6, 11, and 16, wild-type U2 snRNA; 2, 7, 12, and 17, G34U U2 snRNA; 3, 8, 13, and 18, G34A U2 snRNA; 4, 9, 14, and 19, G34C U2 snRNA; 5, 10, 15, and 20, no additional U2 snRNA. The mRNA levels depicted here covary with the β-galactosidase activity expressed from each construct (Fig. 3). However, there is not a proportional relationship between them because they assay different populations of RNA.

splicing step. To analyze this problem, we constructed all possible substitution mutants at branchpoint position A6 in the RP51 intron (see Materials and Methods). We then generated ^{32}P-labeled mutant and wild-type pre-mRNAs by in vitro transcription. These RNAs were incubated in U2 snRNA-depleted extracts or complete yeast extracts, and spliceosome assembly was monitored by native gel electrophoresis (41). Previous analyses using this technique have shown that during spliceosome assembly, U1 snRNP interacts first with the pre-mRNA 5′ splice site, leading to the formation of a first complex, CC1. This is followed by interaction of unidentified factors with the branchpoint in CC2, association of U2 snRNP in the prespliceosome, and the joining of the U4/U6 · U5 triple snRNP in the mature spliceosome. The results of the analysis of a U2-depleted extract are presented in Fig. 5 (lanes 6 to 10). These results indicate that the wild-type RNA forms mostly CC2, with only a trace of CC1 under these conditions (Fig. 5, lane 6). In contrast, we observed only CC1 formation for the A6G, A6U, and A6C mutants (Fig. 5, lanes 7 to 9). No commitment complex is formed with the negative control RNA carrying a 5′ splice site mutation (Fig. 5, lane 10) (44). We controlled that the complexes observed truly represent an association of the pre-mRNA with factors present in the extracts, rather than alternative conformations of the RNAs induced by the mutation, by incubating the same RNAs in buffer alone (Fig. 5, lanes 1 to 5). In a complete extract, we observed spliceosome formation for the wild-type pre-mRNA (Fig. 5, lane 11), while the RNAs carrying a substitution of the branch residue again accumulated in CC1 (data not shown).

These results demonstrate that the branch residue is recognized during the transition from CC1 to CC2. As this step is independent of U2 snRNA (Fig. 5) (21, 41), we conclude that the branch residue is preselected during commitment complex formation in the absence of U2 snRNP.

DISCUSSION

We have reinvestigated the mechanism of branchpoint selection in yeast introns. It has previously been proposed that the branch residue is selected as the nucleotide left unpaired by the U2 snRNA–pre-mRNA association. Previous studies using yeast had shown that U2 snRNA does indeed base pair

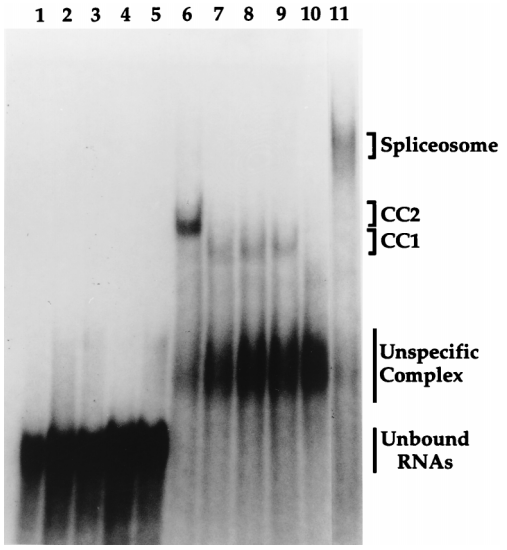


FIG. 5. Analysis of splicing complex assembly for the branchpoint A6 mutants. Five different RNAs were used: wild-type RP51 (lanes 1, 6, and 11), A6C branchpoint mutant (lanes 2 and 7), A6G branchpoint mutant (lanes 3 and 8), A6U branchpoint mutant (lanes 4 and 9), and G5A 5′ splice site mutant (lanes 5 and 10). Complexes were assembled in extract depleted of U2 snRNA (lanes 6 to 10), complete extract (lane 11), or control buffer (lanes 1 to 5). We cannot rule out that the complexes observed with the A6C, A6G, and A6U mutant RNAs represent an aberrantly migrating form of CC2. This is unlikely, however, because previous experiments have shown that a mutation upstream of the branchpoint does not affect the mobility of CC1 (44).

with positions C3 and U4 of the branchpoint sequence. For mammalian introns, it had been shown in addition that U2 snRNA base pairs with A5. Therefore, evidence that U2 snRNA engages in base pairing on both sides of the branch residue was lacking. Query et al. previously showed that substituting the branch nucleotide ribose group by arabinose enhanced its usage as a branchpoint (35). Because arabinose reduces base-pairing capability, this result was taken as indicating that the branch residue was bulged. However, the same result would be expected if the branch nucleotide was not flanked by a base-paired residue on its 3′ side. Our data demonstrate that U2 snRNA does indeed base pair with the nucleotides preceding (position A5) and following (position C7) the branch residue. These results indicate that the branch residue is constrained into a bulged conformation by the base pairing of U2 snRNA. This structure is important for the first step of pre-mRNA splicing; however, it has not yet been demonstrated that the branch residue is bulged during the first catalytic step of pre-mRNA splicing. It is nevertheless likely that the bulge structure is required to properly align the attacking 2′OH group of the ribose ring with the phosphodiester bond at the 5′ splice site. While we could easily detect a Watson-Crick base pairing between U2 snRNA and the nucleotide on the 5′ side of the branchpoint, the base pairing between the U2 snRNA and the nucleotide on the 3′ side of the branchpoint was more difficult to observe. This required a weakened intron context and was most obvious when endogenous wild-type U2 snRNA was deleted from the host strain. This finding suggests that base pairing on the 3′ side of the branchpoint has a less crucial role for splicing than on the 5′ side, at least for our reporter. As up to five pre-mRNA nucleotides preceding the branchpoint base pair with U2, while this is true for only one or two nucleotides following it, it is not unlikely that the former interaction would be mediated solely through base pairing while the second would be stabilized by other factors. This would explain why splicing is less dependent on base pairing of the pre-mRNA C7 with U2 snRNA G34. In this vein, it is noteworthy that the branch nucleotide of self-splicing group II intron is also an adenosine that is often strained in a bulged conformation by the folding of a stem-loop structure. Interestingly, base pairing appears to be more stringently conserved on one side of the branch nucleotide than on the other in this case also (26).

While our results demonstrate that U2 snRNA base pairs on both sides of the branch nucleotide, these interactions are not absolutely essential for splicing, at least for our reporter. Indeed, substitutions in the pre-mRNA at the positions flanking the branch nucleotide still allow accurate splicing, albeit with reduced efficiency. For these constructs, it is likely that other protein-RNA and RNA-RNA interactions maintain the U2 snRNA and the pre-mRNA in the correct conformation in the absence of one of these single base pairs. In contrast, U2 snRNA mutants harboring substitutions at the positions involved in these base-pairing interactions are unable to complement a U2 snRNA gene disruption. It is unlikely that these nucleotides are essential for snRNP assembly or interaction with a splicing factor, as U2 snRNA mutants at these positions were able to rescue complementary pre-mRNA mutants (Table 2 and Fig. 2 to 4). This observation suggests that this base pairing might be absolutely required for the splicing of at least one pre-mRNA encoding an essential function. Alternatively, these positions might be essential to sustain a general level of splicing compatible with vegetative growth.

In yeast, the nucleotide preceding the branch residue is also a highly conserved adenosine. The presence of this adenosine adds to the complexity of the mechanism of branchpoint selection. Indeed, based solely on base-pairing interactions, one could expect that either the branch residue or the nucleotide preceding it would be equally well forced into a bulged configuration. Nevertheless, only the second one is used as a branchpoint. Query et al. have reported that for pre-mRNA carrying the UGCUAGC branch region, the last A and G residues are equally selected for branching in human cell extracts (35). They proposed that either of these two nucleotides could be bulged out of the U2 snRNA–pre-mRNA pairing and therefore selected for branching. Our analysis of the substitutions of the adenosine preceding the branchpoint in yeast indicates that this does not promote its use as a branch residue (data not shown). In yeast, the activation of this position for branching was detected only concomitantly with the activation of an aberrant 5′ splice site (43). It is therefore likely that for wild-type introns, even if several residues can potentially adopt bulged configurations, only one is properly positioned to act as the nucleophile for the first splicing step.

The bulged configuration of the branch residue does not explain why an adenine is selected in most cases at this position. Our experiments indicate that the nature of the branch nucleotide is preselected during commitment complex assembly in yeast. Previous experiments have shown that pre-mRNAs with a U4A mutation are unable to assemble into the CC2 complex (44). These results suggest that a factor, possibly associated with the U1 snRNP, specifically recognizes the branchpoint region in the absence of U2 snRNP. Experiments using mammalian systems have also indicated that the branch nucleotide is recognized by proteins prior to the addition of the U2 snRNP onto the pre-mRNA (4, 7, 22). The identification and characterization of similar proteins from yeast should give us further insight into the precise mechanism of branchpoint selection. Our results demonstrate that yeast branchpoint selection occurs in multiple steps before the first splicing step.

However, the identity of the branch residue will further affect pre-mRNA splicing by affecting the second transesterification reaction (5, 6, 14, 49).

ACKNOWLEDGMENTS

We are grateful to D. J. Ecker for the gift of the *CUP* promoter-containing plasmid and S. Kandels-Lewis for participation at an early stage of this project. We thank C. Allmang, T. Dandekar, M. Luukkonen, I. Mattaj, P. Mitchell, B. Rutz, J. Salgado-Garrido, D. Tollervey, and J. Valcarcel for fruitful discussion and/or constructive comments on the manuscript. The expert assistance of the EMBL services is gratefully acknowledged.

E.P. was supported by AFRT and a network fellowship of the European Community (CHRX-CT93-0191).

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