U1 snRNP can influence 3'-splice site selection as well as 5'-splice site selection

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To address the mechanisms that underlie splice site selection and splice site partner assignment, we analyzed the splicing of yeast (*Saccharomyces cerevisiae*) transcripts containing splice site region duplications. When the 5'-splice site region was duplicated, both sites were utilized to the same extent, indicating little or no influence of proximity on 5'-splice site choice. However, the effect of a 5'-mutant site was greatly enhanced by the presence of an adjacent wild-type site, and this effect was reversed by the restoration of base-pairing with U1 snRNA. 3'-Splice site choice was apparently influenced by proximity, as the site closest to the 5'-splice site was greatly preferred. Studies with strains carrying some U1 snRNA mutations showed an increase in the use of the distal 3'-splice site, indicating a role for U1 snRNP in 3'-splice site selection. The data are compared with those from mammalian splice site choice experiments and suggest mechanisms that influence differential splice site choice as well as exon skipping.

[Key Words: Splice site selection; U1 snRNP; *S. cerevisiae*]

Received March 21, 1991; revised version accepted May 21, 1991.

Splicing of nuclear pre-mRNA and group II mitochondrial RNAs follows a conserved two-step pathway of cleavage–ligation reactions. First, 5'-splice site cleavage occurs concomitant with lariat formation. Second, 3'-splice site cleavage occurs concomitant with exon ligation (for review, see Green 1986; Padgett et al. 1986). Some mitochondrial group II intron-containing RNAs can undergo self-splicing in vitro, indicating that catalysis can take place without any protein or RNA cofactors (Peebles et al. 1986; Van der Veen et al. 1986). On the contrary, nuclear splicing has been observed only within the large, multicomponent spliceosome (Brody and Abelson 1985; Frendewey and Keller 1985; Grabowski et al. 1985). Four small nuclear ribonucleoprotein particles (U1, U2, U5, and U4/6 snRNPs), as well as a number of protein factors, are components of the spliceosome and/or are required for nuclear splicing in both yeast (*Saccharomyces cerevisiae*) and mammalian cells (for review, see Maniatis and Reed 1987; Guthrie and Patterson 1988; Steitz et al. 1988).

Despite much progress in elucidating the highly ordered, ATP-dependent spliceosome assembly pathway (Konarska and Sharp 1986, 1987; Pikielny et al. 1986; Cheng and Abelson 1987; Lamond et al. 1988; Séraphin et al. 1988; Séraphin and Rosbash 1989), relatively little is known about the rules and mechanisms that underlie

splice site selection. We define splice site selection as the early events that lead to selection of a splice site region without the later events also required for accurate splice site cleavage (Séraphin and Rosbash 1990). In mammalian cells, the current view is that the 5'-splice site is chosen as the best match to the 5'-splice site consensus sequence within an appropriate sequence context, and this choice reflects, at least in part, the best available pairing interaction with the U1 snRNA component of U1 snRNP (Eperon et al. 1986; Zhuang and Weiner 1986). Mammalian 3'-splice site choice is probably more complicated, as it involves three closely spaced *cis*-acting sequence elements, that is, the branch-point region, a polypyrimidine sequence, and the 3'-splice site AG (e.g., Reed 1989; Smith et al. 1989). These three elements interact with snRNPs (e.g., Wu and Manley 1989; Zhuang and Weiner 1989) and other protein factors, among which U2AF is the best characterized (Ruskin et al. 1988; Zamore and Green 1989, 1991). The situation in yeast is similar (Parker et al. 1987; Séraphin et al. 1988; Siliciano and Guthrie 1988), except that the absolutely conserved yeast branchpoint region (UAC-UAAC) probably plays a more important role and the polypyrimidine sequence and 3'-splice site play less important roles than in mammalian cells (Rymond et al. 1987; Rymond and Rosbash 1988). Also, as yet, there are no identified yeast proteins that interact directly with the 3'-splice site sequence elements.

An understanding of splice site selection must also explain how 5'- and 3'-splice site partners are assigned in

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pre-mRNAs that contain multiple introns. There is some indication that splice site proximity might play an important role in splice site choice *in vitro*, that is, the closest 5' and 3' sites are significantly favored over more distant sites (Reed and Maniatis 1986). More recently, a protein factor required for *in vitro* splicing has been shown to influence the choice of the most proximal 5'-splice site *in vitro* (Ge and Manley 1990; Krainer et al. 1990). However, it is also possible that there are certain aspects of splice site partner assignment that cannot be addressed by *in vitro* splicing experiments, as spliceosome assembly and splicing occur on some nascent transcripts (Beyer and Osheim 1988; LeMaire and Thummel 1990).

If splice site selection and splice site partner assignment involve features of the basic splicing machinery, we reasoned that it would be interesting to address these issues in yeast where the 5'-splice site and branchpoint regions are so highly conserved. To this end, splice site selection was assayed *in vivo* on yeast substrates that contained duplicated splice site regions. Splicing of these substrates was also examined in the presence of several U1 mutants. The results suggest that proximity plays a role in yeast 3'-splice site selection, as shown for mammalian systems (Reed and Maniatis 1986). They also indicate a role for U1 snRNP in 3'-splice site selection as well as its more expected role in 5'-splice site choice.

Results

5'-Splice site duplication

To gain some insight into the rules of splice site selection in yeast, we generated a duplication of the 5'-splice

site region. The duplication was made by inserting a 75-bp 5'-splice site-containing fragment into the wild-type *rp51A* intron inserted in a reporter gene construction (Teem and Rosbash 1983). The splicing machinery could therefore choose between two identical, adjacent 5'-splice sites. Only mRNAs derived from the upstream 5'-splice site could give rise to β -galactosidase expression. Parallel constructions were also made where the upstream, the downstream, or both 5'-splice sites contained a mutation (HZ12 or 5'II; GUAUGU $\rightarrow$ GUAUaU; Jacquier et al. 1985). The β -galactosidase assays showed that the presence of the additional 5'-splice site led to a decrease in β -galactosidase expression, suggesting that the downstream site was properly recognized and interfered with use of the upstream 5'-splice site (Fig. 1A; cf. VG18-18 with HZ18). Consistent with this interpretation, a mutant downstream site (VG18-12) gave rise to a relative increase in β -galactosidase activity (85% is not significantly different from 100% in this assay). When only the upstream 5' site was mutant (VG12-18), β -galactosidase was strongly affected compared to the control wild-type duplication (cf. VG12-18 with VG18-18). This decrease was also much more prominent than that for the double mutant construct in which both splice sites contained the 5'II mutation (VG12-12) or that for the α 5'II mutation when assayed in a standard single intron reporter gene construct (HZ12; Jacquier et al. 1985).

To directly assay 5'-splice site selection, RNAs were also analyzed by primer extension (Fig. 1B). The control gene HZ18 gave rise to a prominent set of mRNAs corresponding to the multiple starts of transcription of the *CYC1* gene (Fig. 1B; Faye et al. 1981; Teem and Rosbash

Figure 1. Splicing of the 5'-splice site duplications. (A) β -Galactosidase analysis. Open boxes represent splicing consensus sequences; solid boxes represent the exon sequences. (X) The 5'-splice site mutation 5'II (Jacquier et al. 1985). β -Galactosidase activities were assayed in wild-type cells transformed by the different 5'-splice site duplication plasmids and are reported as a percentage of the β -galactosidase expression generated by the wild-type reporter gene HZ18. Arrows indicate where splicing should occur to produce mRNAs in frame for β -galactosidase expression. (B) RNA analysis by primer extension. RNAs were extracted from the different yeast strains and analyzed by primer extension with the oligonucleotide RB1 [complementary to sequences located at the beginning of the second exon; Teem and Rosbash 1983]. Resulting products were separated on a 6% denaturing polyacrylamide gel. Brackets indicate the positions of cDNAs corresponding to mRNAs resulting from splicing at the downstream 5'-splice site (D) and at the upstream 5'-splice site (U). (P) Pre-mRNA species.

Table 1. Measurements of β -galactosidase activities conferred by the 5'-splice site duplications in the U1-4U strain compared to the wild-type strain

Plasmid	Strain (%)	
	WTU1	U1-4U
HZ18	100	100
HZ12	50	60
VG18-18	50	50
VG18-12	85	48
VG12-18	0.2	30
VG12-12	15	22

1983). As suggested by the β -galactosidase results, the wild-type duplication construct gave rise to two sets of RNAs that corresponded to the use of both 5'-splice sites (VG18-18). With this assay, use of the downstream 5'-splice site appeared less prominent than indicated by the β -galactosidase results. We attribute this finding to the fact that transcripts generated by splicing at the downstream site are out of frame and may have a shorter cytoplasmic half-life (Legrain and Rosbash 1989). For the double mutant construct (VG12-12), splicing was less efficient, but both splice sites were still used to a similar extent. When one of the two 5'-splice sites was wild type and the other mutant, we could only clearly identify a single set of mRNAs corresponding to splicing at the wild-type site (VG18-12, VG12-18), consistent with the β -galactosidase values obtained for these constructs.

In this particular duplication construct, we interpret the data to indicate that there is competition between the two 5'-splice sites but no obvious preference for either the upstream or downstream 5'-splice site. The strong preference for a wild-type site, when adjacent to a mutant site, indicates that the effect of a mutation is enhanced dramatically by the presence of a competing wild-type splice site (see Discussion).

U1 snRNA is important for 5'-splice site selection

It is known that the 5' end of U1 snRNA base-pairs with the 5'-splice site region in both mammalian cells and in yeast (Zhuang and Weiner 1986; Séraphin et al. 1988; Siliciano and Guthrie 1988). Differences in the strength of mammalian 5'-splice sites have been attributed to differences in pairing potential to the U1 snRNA component of U1 snRNP (Eperon et al. 1986; Zhuang et al. 1987; Nelson and Green 1988, 1990; Lear et al. 1990); in one case, this has been directly demonstrated (Zhuang and Weiner 1986). To examine the role of U1 snRNP in 5'-splice site selection in yeast, we introduced our 5'-duplication constructs into a strain containing only a mutant U1 snRNA gene (Séraphin et al. 1988). In this viable strain (U1-4U), position 4 of U1 snRNA has a C $\rightarrow$ U change that can still form a base pair (G : U) with the G at position 5 of the wild-type 5'-splice site and can

also form a base pair (A : U) with the mutant A at position 5 of the 5'II 5'-splice site.

Consistent with previous experiments (Séraphin et al. 1988), the U1-4U background effected a partial rescue of the 5'II mutation (single intron) phenotype (Table 1). Also, the frequency of use of the wild-type site upstream of the mutant site (VG18-12) was decreased, and the use of the mutant site upstream of the wild-type site (VG12-18) was dramatically rescued. In the constructs where the two sites were the same (VG18-18, VG12-12), the enzyme activities in the U1-4U strain were similar if not identical to activities observed in the wild-type strain.

These results were confirmed by primer extension analyses (Fig. 2). For all of the different 5'-splice site duplications, we observed a similar use of both sites in the U1-4U strain. Most importantly, the very poor use of the 5'II 5'-splice site when adjacent to a wild-type 5'-splice site in a wild-type strain (Fig. 1B; VG12-18 and VG18-12) was rescued in the U1-4U strain. We conclude that a major factor that discriminates between these two different 5'-splice sites is their relative ability to pair with U1 snRNA.

3'-Splice site duplication

The same strategy was applied to the analysis of 3'-splice site selection. A 95-bp fragment from the *rp51A* gene

Figure 2. Splicing of the 5'-splice site duplications in the U1-4U strain. RNAs were analyzed by primer extension as described in Fig. 1B. The positions of the cDNAs corresponding to pre-mRNA species (P) and to sets of mRNAs generated by splicing at the upstream 5'-splice site (U) and at the downstream 5'-splice site (D) are indicated.

that contained the branchpoint (TACTAAC box) and the intron/exon junction was inserted into the wild-type reporter gene construct so that a duplication of the 3'-splice site region was generated. As a consequence, this 3'-splice site region duplication gene contains a short intron, similar to the efficiently spliced $\Delta 2$ intron described previously (Pikielny et al. 1983), followed by an additional 3'-splice site region. The duplicated 3'-splice site regions are separated by 250 bp, and only splicing at the downstream site can generate β -galactosidase activity. A parallel construct was made with a strong branchpoint mutation at the upstream 3'-splice site (UACUAAC $\rightarrow$ UACUAcC; Jacquier and Rosbash 1986) so that this branchpoint would be inactivated with minimal effect on the overall structure of the RNA.

The β -galactosidase values indicate that use of the downstream 3'-splice site was infrequent, suggesting a predominant choice of the upstream 3' site (Fig. 3A). The control construct with the strong branchpoint mutation at the upstream 3'-splice site gave rise to high levels of β -galactosidase, showing that the downstream 3'-splice site was not intrinsically defective and could be used efficiently if the upstream site was inactivated.

Primer extension analyses were in agreement with these conclusions. The major mRNAs from the wild-type duplication construct were derived from splicing at the upstream site (Fig. 3B, lane 2). The mRNAs from the mutant construct were smaller and identical to what was generated from the single intron-containing gene, showing that the splicing machinery used the down-

stream 3'-splice site. In summary, the data indicate that the upstream 3'-splice site was highly favored.

U1 snRNA can influence 3'-splice site selection

There is in vitro evidence that an interaction between the branchpoint and U1 snRNP takes place early in spliceosome assembly (Zillman et al. 1987; Legrain et al. 1988; Ruby and Abelson 1988; Séraphin et al. 1988; Séraphin and Rosbash 1989, 1991; Barabino et al. 1990). Because the branchpoint probably plays an important role in yeast 3'-splice site selection, we decided to test whether U1 snRNP might also influence 3'-splice site selection. To this end, several strains harboring U1 snRNA mutants were examined for an in vivo effect on 3'-splice site selection with the 3'-splice site region duplication reporter gene construct.

Of the several U1 snRNA mutants tested, three showed a strong and reproducible effect on β -galactosidase activity by increasing use of the downstream 3'-splice site (Table 2). The Δ YC mutation, a 95-nucleotide deletion located in the U1 snRNA yeast-specific region (Fig. 4; Liao et al. 1990), showed a fourfold increase in β -galactosidase activity. Similar results were obtained when the Δ YC deletion was combined with a point mutation in the universally conserved A loop, thus creating a more severe U1 mutant (Δ YC/LII-27A; Liao et al. 1990). A third viable U1 mutant with a deletion of helices V-X (Fig. 4) had an even stronger effect on β -galactosidase expression (Table 2). To directly assay 3'-

Figure 3. Splicing of the 3'-splice site duplications. (A) β -Galactosidase analysis. (Open boxes) The branchpoint region; (solid boxes) the exonic sequences. (X) The 3'-splice site mutation 3'III (Jacquier and Rosbash 1986). Only splicing that takes place at the downstream 3'-splice site can produce mRNAs in frame for β -galactosidase expression. (B) RNA analysis by primer extension. After extraction, RNAs were analyzed by primer extension with the oligonucleotide RB1 (see Fig. 1B) and electrophoresed on a 6% denaturing polyacrylamide gel. (Lane 1) Control reporter gene, HZ18; (lane 2) wild-type 3'-splice site duplication, HZ1-18; (lane 3) duplication construction with a branchpoint mutation in the upstream 3'-splice site HZ2-18. (Right) The positions of cDNAs corresponding to pre-mRNAs of the duplicated constructions (P) and to mRNAs resulting from splicing at the downstream 3'-splice site (D) or at the upstream 3'-splice site (U).

Table 2. Measurements of β -galactosidase activities conferred by the 3' splice site duplication in strains harboring different U1 snRNA mutations

Vector	U1 snRNA			
	wild type	Δ YC	Δ YC/LII-27A	Δ V-X
HZ18	100%	100%	100%	100%
HZ1-18	0.7%	3%	2.9%	17%
Ratio compared to wild type	1	4	4	24

Data from wild-type, Δ YC, and Δ YC/LII-27A strains were averaged from six or seven experiments, ranging from 0.3% to 0.9%, 2.6% to 3.4%, and 2% to 3.8%, respectively. Data from the Δ V-X strain was average from five experiments ranging from 15% to 18%.

splice site selection, RNA was extracted from the strain showing the highest effect on β -galactosidase expression (Δ V-X deletion). Primer extension analysis revealed an additional set of mRNAs resulting from splicing at the downstream 3'-splice site, in contrast to that observed with RNA extracted from a strain containing wild-type U1 snRNA (Fig. 5).

Among the various U1 mutants tested, only large deletions in the yeast-specific region had an influence on 3'-splice site selection. A smaller deletion that removed helices VII–VIII was without effect (Fig. 4). Other U1 mutants containing a deletion of the conserved A loop (Δ loopII, Δ loopII/HVII-m3; Liao et al. 1990) or the U1-4U mutation (Sraphin et al. 1988) had no effect on 3'-splice site selection, and there was no general correlation between the severity of the U1 mutant on splicing or cell growth with its effect on 3'-splice site selection (data not shown). As the U1 snRNP mutants decreased overall splicing efficiency (Liao et al. 1990), these measurements may even underestimate the effect on 3'-splice site choice. In summary, the results indicate that U1 snRNP plays a direct or indirect role in 3'-splice site selection.

Discussion

The experiments presented in this report were designed to provide an initial glimpse into the process of splice site selection in yeast. The strategy was to present the splicing machinery with a choice between two identical splice sites in a single pre-mRNA, a problem that it does not normally confront.

To examine 5'-splice site selection, we constructed duplications of the 5'-splice site region within a single intron. To minimize the effect of sequence context on relative splice site strength (Pikielny and Rosbash 1985; Reed and Maniatis 1986; Furdon and Kole 1988; Nelson and Green 1988), the duplications were constructed so that both 5'-splice sites, separated by 75 nucleotides, would be situated in the same local sequence environment. The fact that there was no difference in the choice

of the two sites indicates the absence of any obvious proximity or polarity features of the selection system.

We imagine that both 5' sites are initially recognized by two sets of factors (including two U1 snRNPs). Then, only one complex is stabilized and selected by a subsequent interaction with the single 3'-splice site region. This interpretation is consistent with in vitro observations in mammalian systems (Nelson and Green 1988, 1990) and parallels observations in yeast systems that formation of pre-spliceosome complex requires both 5'- and 3'-splice site regions (Pikielny and Rosbash 1986; Rymond and Rosbash 1986, 1988).

The presence of a mutant 5'-splice site adjacent to a wild-type site had the expected result, as it has been shown that conformity to the highly conserved yeast consensus sequence is a major contributor to splice site strength (Woolford 1989). More surprising, however, was the extent to which the presence of a wild-type splice site depressed the frequency with which the mutant site was selected. The 5'II mutation (GUAUGU $\rightarrow$ GUAUaU) in a standard single intron reporter gene construct has only a modest effect on mRNA levels (Jacquier et al. 1985; Table 1). In the presence of an adjacent wild-type splice site, however, selection of the mutant site was further reduced by a factor of 10^2 – 10^3 . Because the reduction in the choice of the mutant site is reversed almost completely by the presence of U1-4U snRNA, pairing at position 5 of the 5'-splice site with position 4 of U1 snRNA must make a major contribution to 5'-splice site strength and 5'-splice site region selection.

The observations suggest that the presence of an adjacent wild-type site makes 5'-splice site selection at the mutant site limiting for product formation. Assuming that equilibrium calculations are appropriate, this allows an estimate of the energetic consequences of the mutation in vivo. The absence of the wild-type G and the presence of an A at position 5 of the 5'-splice site gives rise to a $\Delta\Delta G$ of ~ 2.8 – 4.2 kcal/mole (1.4 kcal/mole for each factor of 10 in relative K_d). These $\Delta\Delta G$ values should reflect the difference in binding energy between a GC pair and an AC mismatch between position 5 of the 5'-splice site and position 4 of U1 snRNA. This estimate is considerably greater than the 1.5 kcal/mole that is calculated for the replacement of the GC base pair with an AC mismatch within the yeast 5'-splice site/U1 snRNA pairing region (Freier et al. 1986; D. Turner, pers. comm.). We note that the helix stability values of mismatched duplexes are based on a number of untested assumptions, and the in vivo milieu is impossible to compare with the in vitro conditions used to establish these values. Nonetheless, the discrepancy suggests that this pairing interaction has structural consequences far in excess of what is contributed by formation of the few RNA base pairs in the canonical U1 snRNA/5'-splice site interaction. U1 snRNP proteins (Heinrichs et al. 1990) and/or non-snRNP proteins (Ge and Manley 1990; Krainer et al. 1990) might be important in this context.

This perspective offers an explanation for the apparent "all or none" effect at cryptic splice sites (Eperon et al. 1986; Lear et al. 1990). The $\Delta\Delta G$ between complex for-

Figure 4. Secondary structure and mutations in U1 snRNA. The region removed by the $\Delta V-X$ deletion (L. Kretzner and B. Séraphin) is shown inside the largest box. The lightly shaded area indicates the region removed in the ΔYC mutant (Liao et al. 1990); the darkly shaded area indicates the deletion removing helices VII–VIII (X. Liao., unpubl.).

mation at a cryptic site and at a wild-type site is substantial despite a seemingly small difference in 5'-splice site sequence. In the absence of splice site competition, the (even substantially) increased $\Delta\Delta G$ for complex formation at a mutant site may be much less apparent or even invisible as this step in spliceosome assembly may not be rate limiting for product formation (Pikielny and Rosbash 1985). Consistent with this perspective is the fact that the 5'II mutation has a much greater effect

when the substantial increase in precursor RNA levels is included in the splicing efficiency calculation (Jacquier et al. 1985; Pikielny and Rosbash 1985).

The same arguments should apply to the reverse situation, that is, when the real 5'-splice site is less strong and contributes less binding energy than adjacent sequence elements that resemble 5'-splice sites and compete for factors and complex formation. 5'-Splice site cleavage need not occur at these adjacent sites, but they

Figure 5. Splicing of the 3'-splice site duplication in the $\Delta V-X$ strain. RNAs were analyzed by primer extension as described in Fig. 1B. Indicated are the positions of the cDNAs corresponding to pre-mRNA (P) and mRNAs resulting from splicing at the downstream 3'-splice site (D) or at the upstream 3'-splice site (U). (A) Wild-type strain; (B) $\Delta V-X$ strain.

might still sequester factors (such as U1 snRNP) and interfere with productive complex formation and splicing at the proper site (Nelson and Green 1988; Siebel and Rio 1990).

The 3'-splice site region duplication gave rise to entirely different results, as the upstream site was greatly preferred. The controls showed that the downstream 3'-splice site was not inherently defective. Also, the analysis of several additional constructs, in which the same 3'-splice site duplication was located at different distances from the 5'-splice site, showed that the upstream wild-type 3'-splice site was always the preferred site (data not shown). These observations indicate a proximity effect on 3'-splice site selection.

Our study shows that some U1 snRNA mutants can affect 3'-splice site selection *in vivo*, indicating that U1 snRNP interacts, directly or indirectly, with the 3'-splice site region. It lends support to previous *in vitro* observations, in both yeast and mammalian systems, that U1 snRNP interacts with the 3'-splice site region (Zillmann et al. 1987; Legrain et al. 1988; Ruby and Abelson 1988; Séraphin et al. 1988; Séraphin and Rosbash 1989, 1991; Barabino et al. 1990). Among several different U1 snRNA mutants tested, only those that contained large deletions in the yeast-specific portion of the molecule showed an effect on 3'-splice site selection (Table 2) and no effect on 5'-splice site selection (data not shown). This is paralleled by a comparable *in vitro* effect, namely, that extracts derived from the ΔYC strains are deficient in U2

snRNP binding but normal for commitment complex formation (U1 snRNP binding; X. Liao, unpubl.). These considerations suggest that yeast U1 snRNP might have two separable domains, one that interacts with the 5'-splice site and another that interacts with the 3'-splice site region. As the V-X domain is absent from the much smaller vertebrate U1 snRNA molecule, the results reported here may reflect RNA-RNA interactions (e.g., U1-U2 interactions) that are absent or have been replaced by RNA-protein or protein-protein interactions in vertebrates.

How can the selection frequency of the "weaker" downstream site be improved by a mutation in a *trans*-acting factor that should weaken the splicing machinery further? Our preferred interpretation assumes that splicing complex formation takes place rapidly during transcription, thereby favoring the upstream 3'-splice site region and accounting for the proximity effects. Consistent with this assumption is the fact that no proximity effect is manifest during *in vitro* splicing of a comparable substrate (data not shown). If the U1 snRNP mutants interact with the 3'-splice site region less well or less rapidly, a larger fraction of the nascent transcripts will remain uncommitted until the second 3'-splice site region is transcribed. Emerging from these considerations is a model that features splice site order, perhaps due to sequential transcription ("first come-first served"; Aebi and Weissmann 1987), modulated by splice site competition whenever the rate of stable complex formation is relatively slow.

It is important to compare these results with what is known about order and splice site selection in metazoan organisms. In some systems spliceosome assembly and splicing clearly take place on nascent transcripts (Beyer and Osheim 1988; LeMaire and Thummel 1990), but the generality of these observations and their contribution to splice site selection are not yet known. Also, mammalian experiments designed to search for a relationship between order and splice site selection have given rise to contradictory conclusions (Kühne et al. 1983; Lang and Spritz 1983). It is tempting to attribute this situation to the fact that mammalian splice sites are less conserved and therefore perhaps less strong than their yeast counterparts. The existence of weaker splice sites probably enhances the interplay between splice site order and splice site competition and may contribute to the regulation of alternative splicing in metazoa. This point of view suggests that the influence of polarity on splice site selection might be easily visible only if the competing splice sites are very strong and of equivalent strengths, a circumstance that may be the rule only in yeast where the 5'-splice site and the branchpoint sequences are unusually highly conserved.

Materials and methods

Plasmid constructions

Plasmids were constructed according to standard protocols (Maniatis et al. 1982) and propagated in *Escherichia coli* strain TG1. All yeast vectors are derived from vector HZ18 containing the

rp51A intron inserted in a reporter gene (Teem and Rosbash 1983). 5'-Splice site duplications were made by the insertion of a *XhoI*-*SstI* fragment encompassing the 5'-splice site from HZ18 between the *SaII* and the *SstI* sites of the HZ18 vector. The resulting construction, VG18-18, contains two wild-type 5'-splice sites separated by 75 bp. Mutations were generated by using the vector HZ12 containing the 5'II mutation GTATGT → GTATaT (Jacquier et al. 1985). The resulting constructs have this mutation at the upstream site (VG12-18), the downstream site (VG18-12), or both sites (VG12-12). To construct the 3'-splice site duplication, a 95-bp PCR fragment encompassing the TACTAAC box and the 3' exon/intron junction was cloned into HZ18 digested with *SaII*. Resulting vectors are HZ1-18 with an additional upstream 3' site from HZ18 and HZ2-18, with an additional upstream 3' site from HZ10, which contains the 3'III mutation, TACTAAC → TACTAcC (Jacquier and Rosbash 1986).

β-Galactosidase assays

DNAs were introduced into yeast by the LiCl method of Ito et al. (1983). Strains used are derived from the wild-type strain XL8 [*MATa*, *leu2-3*, *leu2-112*, *ura3-52*, *trp1-289*, *arg4*, *ade2*, *snr19::LEU2*, pXL8 (Liao et al. 1990)]. After induction of the reporter gene for 3 hr in 2% galactose, the *β*-galactosidase assays were performed as described (Legrain and Rosbash 1989).

Primer extension analyses

RNA extraction and primer extension were carried out according to published procedures (Pikielny and Rosbash 1985). The primer used, RB1, is complementary to the beginning of the second exon (Teem and Rosbash 1983). Extension products were analyzed on 6% polyacrylamide denaturing gels.

Acknowledgments

We thank members of the Rosbash lab and B. Séraphin for suggestions and comments on the manuscript. Helpful comments on the manuscript were also made by D. Rio, D. Herschlag, M. Green, P. Zamore, A. Jacquier, and P. Legrain. A. Jacquier contributed to preliminary experiments, and one of the U1 snRNA deletion genes was constructed by L. Kretzner and B. Séraphin. T. Tishman provided expert secretarial assistance. Support was from the National Institutes of Health (GM-23549), and V. Goguel was supported by the ARC (Association pour la Recherche sur le Cancer).

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References

- Aebi, M. and C. Weissmann. 1987. Precision and orderliness in splicing. *Trends Genet.* **3**: 102–107.
- Barabino, S.M.L., B.J. Blencowe, U. Ryder, B.S. Sproat, and A.I. Lamond. 1990. Targeted snRNP depletion reveals an additional role for mammalian U1 snRNP in spliceosome assembly. *Cell* **63**: 293–302.
- Beyer, A.L. and Y.N. Osheim. 1988. Splice site selection, rate of splicing, and alternative splicing on nascent transcripts. *Genes & Dev.* **2**: 754–765.
- Brody, E. and J. Abelson. 1985. The "spliceosome": Yeast pre-messenger RNA associates with a 40S complex in a splicing-dependent reaction. *Science* **288**: 963–967.
- Cheng, S.C. and J. Abelson. 1987. Spliceosome assembly in yeast. *Genes & Dev.* **1**: 1014–1027.
- Eperon, L.P., J.P. Estibeiro, and I.C. Eperon. 1986. The role of nucleotide sequences in splice site selection in eukaryotic pre-messenger RNA. *Nature* **324**: 280–282.
- Faye, G., D.W. Leung, K. Tatchell, B.D. Hall, and M. Smith. 1981. Deletion mapping of sequences essential for *in vivo* transcription of the iso-1-cytochrome *c* gene. *Proc. Natl. Acad. Sci.* **78**: 2258–2262.
- Freier, S.M., R. Kierzek, J.A. Jaeger, N. Sugimoto, M.H. Caruthers, T. Neilson, and D.H. Turner. 1986. Improved free-energy parameters for predictions of RNA duplex stability. *Proc. Natl. Acad. Sci.* **83**: 9373–9377.
- Friendewey, D. and W. Keller. 1985. Stepwise assembly of a pre-mRNA splicing complex requires U-snRNPs and specific intron sequences. *Cell* **42**: 355–367.
- Furdon, P.J. and R. Kole. 1988. The length of the downstream exon and the substitution of specific sequences affect pre-mRNA splicing *in vitro*. *Mol. Cell. Biol.* **8**: 860–866.
- Ge, H. and J.L. Manley. 1990. A protein factor, ASF, controls cell-specific alternative splicing of SV40 early pre-mRNA *in vitro*. *Cell* **62**: 25–34.
- Grabowski, P.J., S.R. Seiler, and P.A. Sharp. 1985. A multicomponent complex is involved in the splicing of messenger RNA precursors. *Cell* **42**: 345–353.
- Green, M.R. 1986. Pre-mRNA splicing. *Annu. Rev. Genet.* **20**: 671–708.
- Guthrie, C. and B. Patterson. 1988. Spliceosomal RNAs. *Annu. Rev. Genet.* **22**: 387–419.
- Heinrichs, V., M. Bach, G. Winkelmann, and R. Lührmann. 1990. U1-specific protein C needed for efficient complex formation of U1 snRNP with a 5' splice site. *Science* **247**: 69–72.
- Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **53**: 163–168.
- Jacquier, A. and M. Rosbash. 1986. RNA splicing and intron turnover are greatly diminished by a mutant yeast branch point. *Proc. Natl. Acad. Sci.* **83**: 5835–5839.
- Jacquier, A., J.R. Rodriguez, and M. Rosbash. 1985. A quantitative analysis of the effects of 5' junction and TACTAAC box mutants and mutant combinations on yeast mRNA splicing. *Cell* **43**: 423–430.
- Konarska, M.M. and P.A. Sharp. 1986. Electrophoretic separation of complexes involved in the splicing of precursors to mRNAs. *Cell* **46**: 845–855.
- . 1987. Interactions between small nuclear ribonucleoprotein particles in formation of spliceosomes. *Cell* **49**: 763–774.
- Krainer, A.R., G.C. Conway, and D. Kozak. 1990. The essential pre-mRNA splicing factor SF2 influences 5' splice site selection by activating proximal sites. *Cell* **62**: 35–42.
- Kühne, T., B. Wieringa, J. Reiser, and C. Weissmann. 1983. Evidence against a scanning model of RNA splicing. *EMBO J.* **2**: 727–733.
- Lamond, A.I., M.M. Konarska, P.J. Grabowski, and P.A. Sharp. 1988. Spliceosome assembly involves the binding and release of U4 small nuclear ribonucleoprotein. *Proc. Natl. Acad. Sci.* **85**: 411–415.
- Lang, K.M. and R.A. Spritz. 1983. RNA splice site selection: Evidence for a 5' → 3' scanning model. *Science* **220**: 1351–1355.
- Lear, A.L., L.P. Eperon, I.M. Wheatley, and I.C. Eperon. 1990.

- Hierarchy for 5' splice site preference determined in vivo. *J. Mol. Biol.* **211**: 103–115.
- Legrain, P. and M. Rosbash. 1989. Some *cis*- and *trans*-acting mutants for splicing target pre-mRNA to the cytoplasm. *Cell* **57**: 573–583.
- Legrain, P., B. Séraphin, and M. Rosbash. 1988. Early commitment of yeast pre-mRNA to the spliceosome pathway. *Mol. Cell. Biol.* **8**: 3755–3760.
- LeMaire, M.F. and C.S. Thummel. 1990. Splicing precedes polyadenylation during *Drosophila* E74A transcription. *Mol. Cell. Biol.* **10**: 6059–6063.
- Liao, X., L. Kretzner, B. Séraphin, and M. Rosbash. 1990. Universally conserved and yeast-specific U1 snRNA sequences are important but not essential for U1 snRNP function. *Genes & Dev.* **4**: 1766–1774.
- Maniatis, T. and R. Reed. 1987. The role of small nuclear ribonucleoprotein particles in pre-mRNA splicing. *Nature* **325**: 673–678.
- Maniatis, T., E.T. Fritsch, and J. Sambrook. 1982. *Molecular cloning. A laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Nelson, K.K. and M.R. Green. 1988. Splice site selection and ribonucleoprotein complex assembly during in vitro pre-mRNA splicing. *Genes & Dev.* **2**: 319–329.
- . 1990. Mechanism for cryptic splice site activation during pre-mRNA splicing. *Proc. Natl. Acad. Sci.* **7**: 6253–6257.
- Padgett, R.A., P.J. Grabowski, M.M. Konarska, S. Seiler, and P.A. Sharp. 1986. Splicing of messenger RNA precursors. *Annu. Rev. Biochem.* **55**: 119–150.
- Parker, R., P.G. Siliciano, and C. Guthrie. 1987. Recognition of the TACTAAC box during mRNA splicing in yeast involves base pairing to the U2-like snRNA. *Cell* **49**: 229–239.
- Peebles, C.L., P.S. Perlman, K.L. Mecklenburg, M.L. Petrillo, J.H. Tabor, K.A. Jarell, and H.L. Cheng. 1986. A self-splicing RNA excises an intron lariat. *Cell* **44**: 213–223.
- Pikielny, C.W. and M. Rosbash. 1985. mRNA splicing efficiency in yeast and the contribution of nonconserved sequences. *Cell* **41**: 119–126.
- . 1986. Specific small nuclear RNAs are associated with yeast spliceosomes. *Cell* **45**: 869–877.
- Pikielny, C.W., J.L. Teem, and M. Rosbash. 1983. Evidence for the biochemical role of an internal sequence in yeast nuclear mRNA introns: Implications of U1 RNA and metazoan mRNA splicing. *Cell* **34**: 395–403.
- Pikielny, C.W., B.C. Rymond, and M. Rosbash. 1986. Electrophoresis of ribonucleoproteins reveals an ordered pathway of yeast splicing complexes. *Nature* **324**: 341–345.
- Reed, R. 1989. The organization of 3' splice-site sequences in mammalian introns. *Genes & Dev.* **3**: 2113–2123.
- Reed, R. and T. Maniatis. 1986. A role for exon sequences and splice-site proximity in splice-site selection. *Cell* **46**: 681–690.
- Ruby, S.W. and J. Abelson. 1988. An early hierarchic role of U1 small nuclear ribonucleoprotein in spliceosome assembly. *Science* **242**: 1028–1035.
- Ruskin, B., P.D. Zamore, and M.R. Green. 1988. A factor, U2AF, is required for U2 snRNP binding and splicing complex assembly. *Cell* **52**: 207–219.
- Rymond, B.C. and M. Rosbash. 1986. Differential nuclease sensitivity identifies tight contacts between yeast pre-mRNA and spliceosomes. *EMBO J.* **5**: 3517–3523.
- . 1988. A chemical modification/interference study of yeast pre-mRNA spliceosome assembly and splicing. *Genes & Dev.* **2**: 428–439.
- Rymond, B.C., D.D. Torrey, and M. Rosbash. 1987. A novel role for the 3' region of introns in pre-mRNA splicing of *Saccharomyces cerevisiae*. *Genes & Dev.* **1**: 238–246.
- Séraphin, B. and M. Rosbash. 1989. Identification of functional U1 snRNA-pre-mRNA complexes committed to spliceosome assembly and splicing. *Cell* **59**: 349–358.
- . 1990. Exon mutations uncouple 5' splice site selection from U1 snRNA pairing. *Cell* **63**: 619–629.
- . 1991. The yeast branchpoint sequence is not required for the formation of a stable U1 snRNP-pre-mRNA complex and is recognized in the absence of U2 snRNA. *EMBO J.* **10**: 1209–1216.
- Séraphin, B., L. Kretzner, and M. Rosbash. 1988. A U1 snRNA: pre-mRNA base pairing interaction is required early in yeast spliceosome assembly but does not uniquely define the 5' cleavage site. *EMBO J.* **7**: 2533–2538.
- Siebel, C.W. and D.C. Rio. 1990. Regulated splicing of the *Drosophila* P transposable element third intron in vitro: Somatic repression. *Science* **248**: 1200–1208.
- Siliciano, P.G. and C. Guthrie. 1988. 5' splice site selection in yeast: Genetic alterations in base-pairing with U1 reveal additional requirements. *Genes & Dev.* **2**: 1258–1267.
- Smith, C.W.J., E.B. Porro, J.G. Patton, and B. Nadal-Ginard. 1989. Scanning from an independently specified branch point defines the 3' splice site of mammalian introns. *Nature* **342**: 243–247.
- Steitz, J.A., D.L. Black, V. Gerke, K.A. Parker, A. Kramer, D. Frendewey, and W. Keller. 1988. Functions of the abundant U-snRNPs. In *Structure and function of major and minor small nuclear ribonucleoprotein particles* (ed. M.L. Birnstiel), pp. 115–154. Springer Verlag, Berlin.
- Teem, J.L. and M. Rosbash. 1983. Expression of a β -galactosidase gene containing the ribosomal protein 51 intron is sensitive to the *rna2* mutation of yeast. *Proc. Natl. Acad. Sci.* **80**: 4403–4407.
- Van der Veen, R., A.C. Arnberg, G. van der Horst, L. Bonen, H.F. Tabak, and L.A. Grivell. 1986. Excised group II introns in yeast mitochondria are lariats and can be formed by self-splicing in vitro. *Cell* **44**: 225–234.
- Woolford, J.L., Jr. 1989. Nuclear pre-mRNA splicing in yeast. *Yeast* **5**: 439–457.
- Wu, J. and J.L. Manley. 1989. Mammalian pre-mRNA branch site selection by U2 snRNP involves base pairing. *Genes & Dev.* **3**: 1542–1554.
- Zamore, P.D. and M.R. Green. 1989. Identification, purification, and biochemical characterization of U2 small nuclear ribonucleoprotein auxiliary factor. *Proc. Natl. Acad. Sci.* **86**: 9243–9247.
- . 1991. Biochemical characterization of U2 snRNP auxiliary factor: An essential pre-mRNA splicing factor with a novel intranuclear distribution. *EMBO J.* **10**: 207–214.
- Zhuang, Y. and A.M. Weiner. 1986. A compensatory base change in U1 snRNA suppresses a 5' splice site mutation. *Cell* **46**: 827–835.
- . 1989. A compensatory base change in human U2 snRNA can suppress a branch site mutation. *Genes & Dev.* **3**: 1555–1561.
- Zhuang, Y., H. Leung, and A.M. Green. 1987. The natural 5' splice site of simian virus 40 large T antigen can be improved by increasing the base complementarity to U1 RNA. *Mol. Cell. Biol.* **7**: 3018–3020.
- Zillmann, M., S.D. Rose, and S.M. Berget. 1987. U1 small nuclear ribonucleoproteins are required early during spliceosome assembly. *Mol. Cell. Biol.* **7**: 2877–2883.

U1 snRNP can influence 3'-splice site selection as well as 5'-splice site selection.

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Genes Dev. 1991, **5**:

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