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REPORT

Activation of a cryptic 5′ splice site by U1 snRNA

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ABSTRACT

In the course of analyzing 5′ splice site mutations in the second intron of *Schizosaccharomyces pombe cdc*2, we identified a cryptic 5′ junction containing a nonconsensus nucleotide at position +2. An even more unusual feature of this cryptic 5′ junction was its pattern of activation. By analyzing the profile of splicing products for an extensive series of *cdc*2 mutants in the presence and absence of compensatory U1 alleles, we have obtained evidence that the natural 5′ splice site participates in activation of the cryptic 5′ splice site, and that it does so via base pairing to U1 snRNA. Furthermore, the results of follow-up experiments strongly suggest that base pairing between U1 snRNA and the cryptic 5′ junction itself plays a dominant role in its activation. Most remarkably, a mutant U1 can activate the cryptic 5′ splice site even in the presence of a wild-type sequence at the natural 5′ junction, providing unambiguous evidence that this snRNA redirects splicing via base pairing. Although previous work has demonstrated that U5 and U6 snRNAs can activate cryptic 5′ splice sites through base pairing interactions, this is the first example in which U1 snRNA has been implicated in the final selection of a cryptic 5′ junction.

Keywords: *S. cerevisiae*; splice site selection; U5 snRNA; U6 snRNA

INTRODUCTION

It has long been known that mutating the 5′ splice sites of nuclear premessenger RNAs can lead to the activation of cryptic 5′ junctions that are ignored by the splicing machinery under normal circumstances (reviewed in Black, 1995). Although the factors that promote the use of most cryptic 5′ splice sites have not been identified, a few examples have been reported in which activation was clearly due to base pairing with a specific snRNA. The first of these was discovered through a genetic screen in *Saccharomyces cerevisiae* that sought extragenic mutations that allowed removal of an intron containing a G+1A change at the natural 5′ splice site (Newman & Norman, 1991). Splicing occurred not at the mutant junction, but rather at a cryptic 5′ splice site which bore little resemblance to the *S. cerevisiae* consensus. Cloning of the suppressor gene revealed that activation was due to a mutation in U5 snRNA that allowed base pairing with nucleotides just upstream from the cryptic 5′ junction (Newman & Norman, 1991, 1992). Similarly, mutant U5 snRNAs activated nonconsensus 5′ splice sites via base pairing to nucleotides upstream from the scissile bond in mammalian cells (Cortes et al., 1993).

Other cryptic 5′ splice sites were shown to be activated through a distinct mechanism: pairing between nucleotides downstream from the scissile bond and a highly conserved sequence in U6 snRNA. Early studies had shown that mutating position +5 of the 5′ splice site in *S. cerevisiae* led to the accumulation of lariat intermediates resulting from transesterification at an aberrant upstream site (Jacquier et al., 1985; Parker & Guthrie, 1985; Fouser & Friesen, 1986), but the molecular mechanism remained a mystery for several years. In the wake of crosslinking data that placed U6 snRNA in proximity to the 5′ splice site (Sawa & Abelson, 1992; Sawa & Shimura, 1992; Sontheimer & Steitz, 1993), compensatory base analysis was used to demonstrate that these aberrant 5′ splice sites were activated via base pairing to U6 snRNA (Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993). Like the U5/exon interaction, the U6/intron interaction is important for splicing in mammals as well as yeast (e.g., Crispino & Sharp, 1995; Hwang & Cohen, 1996).

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A third snRNA implicated in the selection of natural 5′ splice sites is U1, and its potential role in activation of cryptic 5′ splice sites has also been investigated. These studies revealed, first, that RNA fragments containing three cryptic 5′ junctions found in human β-globin can be coprecipitated with the U1 snRNP (Chabot & Steitz, 1987). Second, improving the complementarity of one of these to the 5′ end of U1 snRNA increased its use (Nelson & Green, 1990). Third, mutating the first G of the large rabbit β-globin intron shifted the site of the first transesterification 1 nt upstream, and the displacement was proposed to result from a change in the register of optimal U1 pairing (Aebi et al., 1987). However, in none of these cases has compensatory base analysis of the sort used to unambiguously demonstrate roles for U5 and U6 snRNAs in cryptic splice site activation been performed.

Here, we report the identification of a cryptic 5′ splice site in *Schizosaccharomyces pombe cdc*2 and present direct evidence that it is activated via base pairing to U1 snRNA. These findings have important implications for the mechanism by which natural 5′ splice sites are selected.

RESULTS

A subset of 5′ splice site mutations in the second intron of *cdc*2 activate an unusual cryptic 5′ junction

Mammalian 5′ splice sites display a great deal of variation about the consensus (Burge et al., 1999) whereas in the budding yeast *Saccharomyces cerevisiae*, 5′ junction sequences are highly constrained (Spingola et al., 1999). Consistent with the relative degrees of conservation, cryptic splice 5′ site activation upon mutation of natural 5′ junctions is much more common in mammals (Krawczak et al., 1992). In the fission yeast *S. pombe*, 5′ splice sites display an intermediate degree of degeneracy (Zhang & Marr, 1994; M. Lyne, K. Rutherford, and V. Wood, pers. comm.) and in one study, it was found that mutating the 5′ splice site of an artificial intron resulted in activation of a cryptic 5′ junction (Gatermann et al., 1989). To evaluate the frequency of cryptic 5′ splice site activation in a natural fission yeast pre-mRNA that contains a consensus 5′ hexanucleotide, we selected the second intron in the *cdc*2 gene (hereafter designated *cdc*2-Int2). Splicing was assayed by primer extension analyses on total RNA from the mutants; a representative set is shown in Figure 1. The profiles of products from each of the mutants allowed them to be divided into two distinct classes, which are summarized in Figure 1B.

The most striking result of mutating the 5′ splice site in *S. pombe cdc*2-Int2 was the appearance of an additional band migrating approximately midway between the two major products in position +6 mutants and in longer exposures of position +4 mutants (Fig. 1, lane 9; see also Figs. 3 and 5). Based on the degeneracy of *S. pombe* splicing signals, we suspected that the novel species might arise via use of a cryptic 5′ junction. PCR sequence analysis, the results of which are displayed schematically in Figure 1B, confirmed this hypothesis. Consistent with other evidence that splice site pairing in *S. pombe* occurs via intron definition (Romfo et al., 2000), the cryptic junction is located within the intron; in contrast, cryptic 5′ splice sites are located almost exclusively within exons in larger eukaryotes, which employ exon definition during the initial recognition of pre-mRNAs (reviewed in Berget, 1995). The most unusual feature of the cryptic 5′ splice site in *cdc*2, however, is its sequence, which differs from those of previously identified cryptic junctions (including the one from *S. pombe*) by the presence of a noncanonical A at position +2 rather than the standard U. In addition, the cryptic 5′ junction in *cdc*2-Int2 differs from the natural 5′ splice site (and from the *S. pombe* consensus) in containing a U at position +4, which is complementary to U6 snRNA but not to U1.

Based on the potential base pairing between U6 and nucleotides +4 through +6 of the unusual cryptic 5′ junction in *S. pombe cdc*2-Int2, together with the fact that this snRNA had been implicated in cryptic splice site activation in other organisms (see Introduction), we tested the effect of extending the U6 complementarity by 2 nt. Consistent with the results of similar experiments in both *S. cerevisiae* and mammals (Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993; Crispino & Sharp, 1995), augmenting the pairing between U6 snRNA and the cryptic 5′ splice site in *S. pombe cdc*2 did increase splicing there. However, the effect was quite small (data not shown), suggesting that U6 participates in splicing at the cryptic 5′ junction but is not a key factor in its activation.

In seeking an alternative route by which the cryptic 5′ splice site in *cdc*2 might be activated, we noted that it also differs from the natural 5′ splice site at position −2 (see Fig. 1B). Because studies in other organisms had demonstrated that cryptic 5′ splice sites can be activated via pairing between nucleotides −1 and −2 and the uridine-rich loop of U5 snRNA (Newman & Norman, 1991, 1992), we used mutagenesis to test whether the presence of two purines at positions −1 and −2 of the cryptic 5′ junction in *cdc*2 versus a pyrimidine/purine combination at the natural 5′ junction was significant. Although replacing both purines preceding the cryptic 5′ junction with pyrimidines eliminated its use, introducing a second pyrimidine just upstream from the natural 5′ splice site reduced splicing there but did not lead to significant activation of the cryptic 5′ junction (data not shown). Together, these results suggest that U5 snRNA pairing is necessary but not sufficient for activation of the cryptic 5′ splice site.

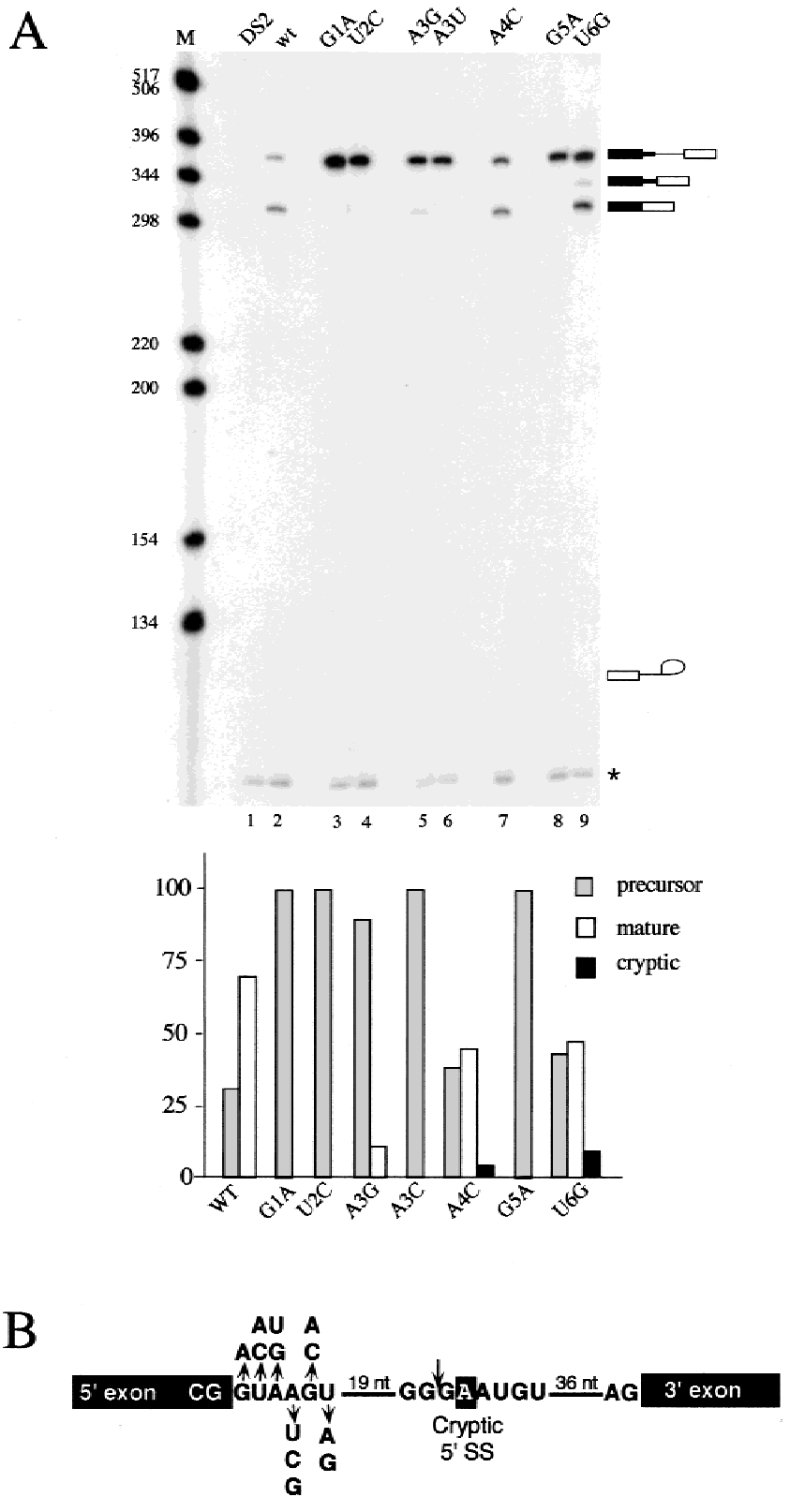

FIGURE 1. **A**: Primer extension splicing assays on a subset of *cdc*2-Int2 5′ splice site mutants. The indicated alleles were introduced into fission yeast cells by transformation and the profiles of splicing products analyzed by primer extension on total RNA as previously described (Alvarez et al., 1996). Splicing assays for additional mutants, as well as duplicate assays for those shown here, are shown in Figures 3 and 5. Because the primer (nmt1-poly(A); Alvarez et al., 1996) was complementary to sequences within the expression vector, there is no signal from endogenous *cdc*2. Upper panel: Autoradiogram of primer extension products. M: molecular weight markers (1 kb ladder). Lane 1: untransformed recipient strain (DS2); the band marked with an asterisk arises from chromosomally expressed *nmt*1 mRNA and thus serves as a loading control. Lane 2: wild-type *cdc*2-Int2. Lanes 3–9: 5′ splice site mutants. Because the signal from endogenous *nmt*1 in lane 2 is comparable to that in the other samples, it is not clear why there appears to be less *cdc*2-specific RNA present. The band migrating between linear precursor and mature mRNA in lane 9 arises via splicing at a cryptic 5′ junction (see text). In no case was a signal detected at the position of lariat intermediate, in contrast to observations in other organisms (e.g., Jacquier et al., 1985; Newman et al., 1985; Vijayraghavan et al., 1986; Aebi et al., 1987). The various parts of the *cdc*2 pre-mRNA are indicated by symbols alongside the gel as follows: 5′ exon: black box; 3′ exon: open box; intron: thick line upstream from the cryptic 5′ splice site and thin line downstream from it. The predicted sizes of the primer extension products are: precursor; 388 nt; mature mRNA: 317 nt; lariat intermediate: 121 nt. Lower panel: Quantitation of primer extension products. The levels of precursor, mature message, and cryptic product were determined by PhosphorImager analysis and are displayed as a bar graph in which the y axis shows the percentage of each species. For each mutant, the three species combined equals 100%. **B**: Schematic representation of the *cdc*2-Int2 pre-mRNA depicting the location of the cryptic 5′ splice site and the effects of mutations at the natural 5′ splice site. The figure summarizes data from Figures 1, 3, and 5, which allow the 5′ splice site mutants to be divided into two classes. In the first, which includes substitutions at intron positions +1, +2, +3, and +5, linear precursor is the sole species detectable (this figure, lanes 3, 4, 6, and 8) except for a small amount of mature mRNA produced in the position +3 transition mutant (this figure, lane 5). In the second, which encompasses substitutions at position +4 and +6, a mixture of linear precursor, correctly spliced mRNA, and mRNA spliced at the cryptic 5′ junction is observed (this figure, lanes 7 and 9). Mutations that activate the cryptic splice site are indicated by arrows pointing downwards and those that block splicing are indicated by arrows pointing upwards. The unusual second nucleotide in the cryptic junction is shown in white type against a black background.

A subset of compensatory U1 snRNA mutants promotes splicing at the cryptic 5′ junction

Because neither U5 nor U6 snRNA appeared to play a decisive role in the choice between the natural and cryptic 5′ splice sites in *cdc*2, another obvious candidate was U1. The first question that we wanted to address was whether introducing mutant U1 snRNAs that were perfectly complementary to mutations at the natural 5′ splice site would repress splicing at the cryptic

5′ junction in favor of the normal exon/intron boundary. Figure 2A illustrates the effect of introducing either a compensatory or a noncompensatory U1 allele in combination with each possible mutation at position +4 of the *cdc*2-Int2 5′ splice site. For all three mutants, the ratio of precursor to mature mRNA, a commonly accepted measure of in vivo splicing efficiency (Pikielny & Rosbash, 1985; Fouser & Friesen, 1986), was found to decrease (Fig. 2A, compare lanes 3 and 4, 6 and 7, and 9 and 10). This result was not unexpected, because suppression of a 5′ splice site position +4 mutant via U1 pairing had previously been observed in mammals (Zhuang & Weiner, 1986). In our experiment, the most dramatic change caused by the introduction of a compensatory U1 allele was observed with the 5′SS-A+4G mutant: whereas precursor was by far the predominant species in the absence of a complementary U1 mutant, restoration of pairing to this snRNA returned the pre-mRNA:mRNA ratio nearly to the wild-type level. Notably, the substantial increase in correctly spliced mRNA did not lead to a concomitant decrease in splicing at the cryptic 5′ junction, because the fraction of product corresponding to this species increased slightly in two of the mutants and remained constant in the third.

As expected, noncompensatory U1 mutants failed to rescue splicing and, in fact, actually increased precursor accumulation relative to the level observed with the 5′ splice site mutations alone (compare lanes 3 and 5, 6 and 8, and 9 and 11). These observations are consistent with our previous report that U1 mutants interfere with splicing of wild-type pre-mRNAs in *S. pombe* (Alvarez et al., 1996).

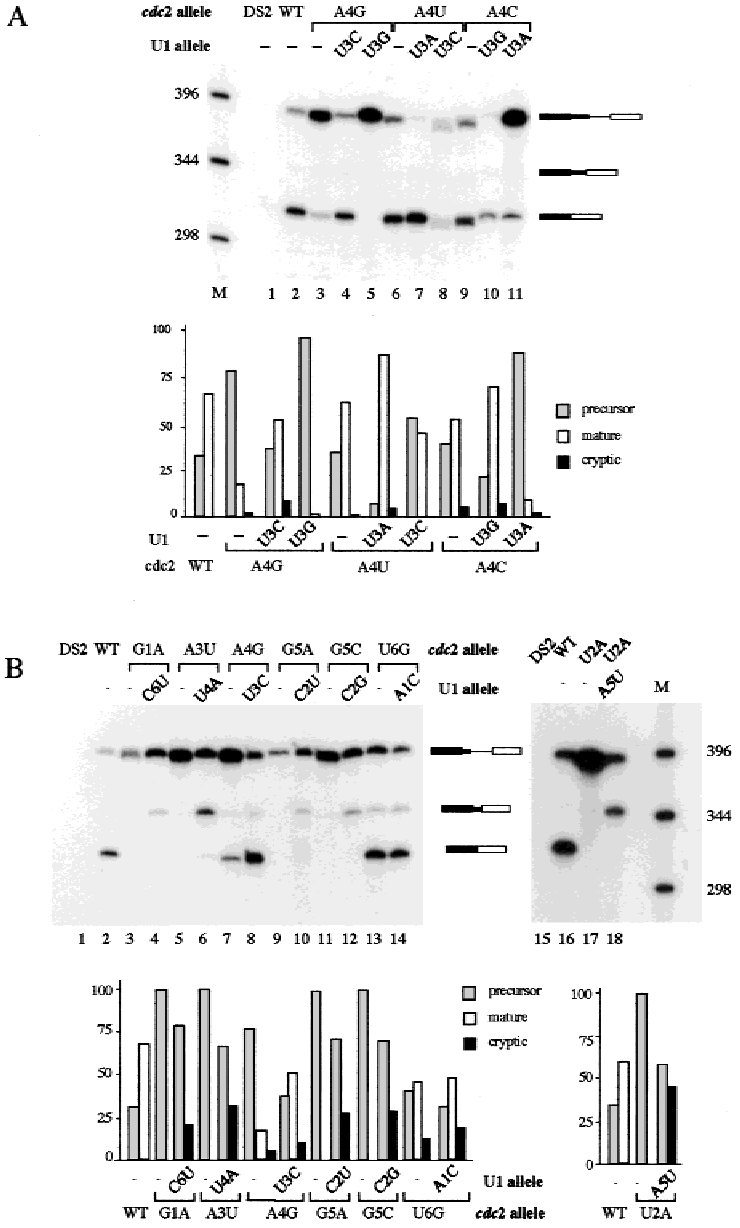

FIGURE 2. Effects of U1 mutants on splicing of *cdc*2-Int2 5′ splice site mutants. Splicing was assayed as described in the legend to Figure 1. **A**: Analysis of position +4 mutants. Lanes 1 and 2: negative and positive controls as in Figure 1; lanes 3, 6, and 9: no U1 plasmid; lanes 4, 7, and 10: compensatory U1 alleles; lanes 5, 8, and 11: noncompensatory U1 alleles. The fuzziness of the bands in lanes 8 and 9 is due to a flaw in the gel and was not observed in other analyses of the same mutants (see, e.g., Fig. 1, lane 7). **B**: Effects of compensatory changes in U1 snRNA on 5′ splice sites mutants carrying changes at all positions. Because only 3 of the 12 U1 mutants employed here could support growth (Alvarez et al., 1996), their effects on splicing of *cdc*2-Int2 5′ splice site mutants were assessed in cells that also retained a wild-type copy of the U1 gene.

The effects of compensatory U1 mutants on the profiles of products from a panel of *cdc*2-Int2 alleles including at least one change at each position of the 5′ splice site are shown in Figure 2B. At position +6, the other nucleotide where mutations activated the cryptic 5′ splice site in *cdc*2-Int2, restoration of splicing by compensatory U1 mutants has also been observed previously in other organisms (Séraphin & Rosbash, 1989; Lo et al., 1994). In the *S. pombe cdc*2-Int2 5′ SS U+6G mutant, a complementary U1 allele produced a very modest increase in mRNA spliced at the natural exon/intron boundary (Fig. 2B, compare lanes 13 and 14). As at position +4, the increase in correctly spliced mRNA occurred at the expense of precursor, because the fraction of cryptic product was again slightly higher than in the absence of the suppressor U1.

Because the introduction of U1 alleles that could pair with mutations at positions +4 and +6 of the natural 5′ splice site did not reduce use of the cryptic 5′ junction, we wondered whether and how compensatory U1 alleles would affect the profiles of products from 5′ splice site mutants that do not, on their own, lead to splicing at the cryptic site. The introduction of complementary U1 mutations in combination with 5′ splice sites containing changes at positions +1, +2, +3, and +5 (Fig. 2B) produced results that were strikingly different from those observed at positions +4 and +6. Although we did observe a decrease in precursor accumulation in these samples, splicing occurred principally at the unusual cryptic 5′ splice site rather than at the authentic (but mutant) 5′ junction (Fig. 2B, compare lanes 3 and 4, 5 and 6, 9 and 10, 11 and 12, and 17 and 18).

The data presented in Figure 2 are summarized in Figure 3 to illustrate an intriguing reciprocity in the pattern of cryptic 5′ splice site activation: that is, the effect of mutating the natural 5′ splice site alone was the mirror image of the effect of compensatory U1 alleles. Specifically, mutations at 5′ splice site positions +4 and +6 activated the cryptic junction on their own, whereas mutations at positions +1, +2, +3, and +5 did not lead to significant use of the cryptic site; conversely, U1 mutants designed to pair with mutations at positions +4 and +6 of the standard 5′ splice site promoted splicing principally at the natural exon/intron boundary, whereas U1 alleles designed to pair with mutations at the other four positions promoted splicing principally at the cryptic 5′ junction.

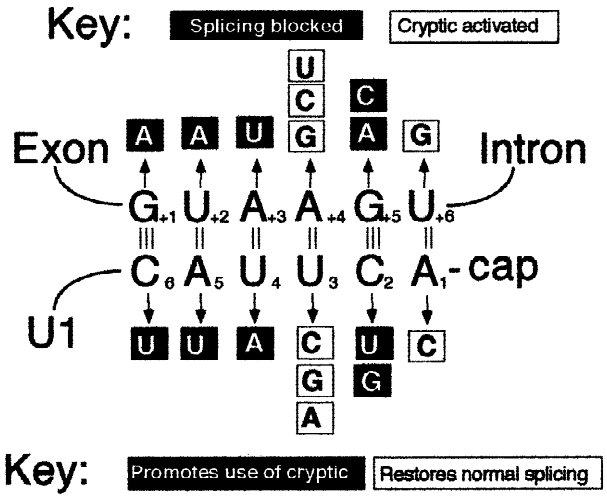

FIGURE 3. Schematic representation of the mirror-image relationship between the effects of *cdc*2-Int2 5′ splice site mutations on their own and in the presence of compensatory alleles of *S. pombe* U1 snRNA. Potential base pairing between the wild-type 5′ splice site and wild-type U1 snRNA is indicated at the center of the figure. Shown above and below this are the mutants employed in compensatory base analysis. The effects of the *cdc*2-Int2 mutations alone on splicing and of the U1 mutations on splicing of the complementary 5′ splice site mutants (Fig. 2) are indicated as described in the key.

A U1 allele with extensive complementarity to the cryptic 5′ splice site activates it even when the wild-type sequence is present at the standard 5′ junction

To provide an initial assessment of whether the mutant U1 snRNAs might function directly to activate splicing at the cryptic site, we examined their ability to base pair with the normally silent 5′ junction in *cdc*2. As illustrated in Figure 4A, a strong correlation was observed between the extent of complementarity to this snRNA and the magnitude of cryptic 5′ junction activation, consistent with a role for U1 pairing at the cryptic site itself. The U1-A5U allele, which can form four contiguous Watson–Crick pairs, was the most potent activator of the cryptic 5′ splice site (44% of the total primer extension products), whereas the other extreme was represented by the C6U mutant, which cannot form a canonical pair with position +1 of the cryptic 5′ junction. The other three U1 alleles examined can form at least two Watson–Crick pairs that span the scissile bond at the cryptic 5′ junction and activated it to similar intermediate extents.

The correlation between the ability of U1 snRNA mutants to activate the cryptic 5′ junction and their potential to pair there prompted us to perform the *cis*-competition experiment depicted in Figure 4B, in which two U1 alleles that pair to different extents with the cryptic 5′ junction were tested in combination with a panel of *cdc*2 alleles. The most striking result is shown in Figure 4B, lane 7, which demonstrates that a U1 mutant capable of extensive pairing with the cryptic 5′ junction, specifically U1-A5U, promoted splicing at the downstream site even when a wild-type sequence was present at the standard exon/intron boundary. In fact, the major primer extension product resulted from splicing at the unusual cryptic 5′ junction (40% of the total versus 25% normally spliced mRNA). Notably, the fraction of *cdc*2 RNA that remained as precursor in this sample was similar to wild type (30%; Fig. 4B, lane 1).

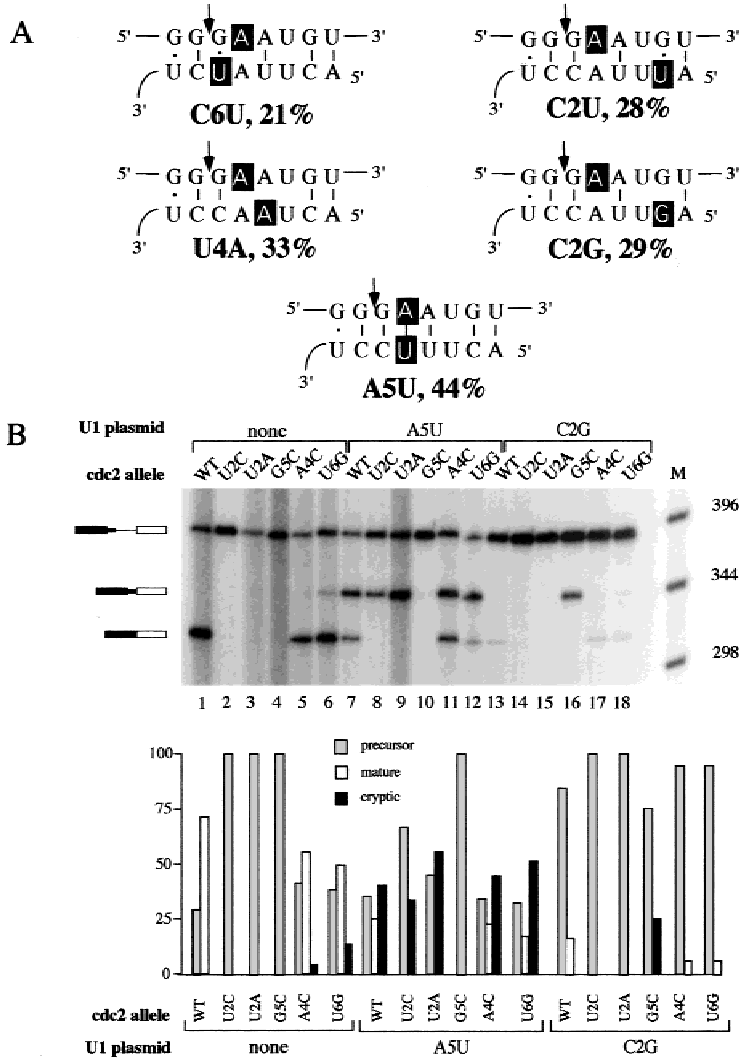

FIGURE 4. **A**: Potential base pairing of mutant U1 snRNAs to the cryptic 5′ splice site. The aberrant nucleotide within the cryptic junction and the nucleotide mutated in each U1 allele are shown in white against a black background. The percentage of product corresponding to mRNA spliced at the cryptic junction is taken from the experiment shown in Figure 3B. **B**: Effects of two U1 alleles that pair to different extents with the cryptic 5′ splice site on the profile of products from *cdc*2-Int2 mutants. The experimental design was as in Figure 3.

Thus, cryptic 5′ junction activation due to extensive pairing with a mutant U1 occurred at the expense of the natural 5′ splice site.

The conclusion that pairing to U1 snRNA determines which of the two competing 5′ splice sites is selected in *cdc*2-Int2 was reinforced by analyzing the effect of the U1-A5U mutant on the remaining 5′ junction mutants. Except when a G+5C mutation was present at the standard 5′ splice site, use of the cryptic site in *cdc*2-Int2 was increased in all strains harboring the U1-A5U allele (Fig. 4B, compare lanes 2 and 8, 3 and 9, 4 and 10, 5 and 11, and 6 and 12). The U1-C2G mutant, which cannot form an extensive pairing interaction with the cryptic 5′ splice site (Fig. 4B, lanes 13–18), serves as a negative control. With this U1 allele, the aberrant cryptic 5′ junction was utilized only in the presence of a G+5C mutation at the standard 5′ splice site, to which it is perfectly complementary (Fig. 4B, lane 16). This result strongly suggests that pairing of the mutant U1 to the normal 5′ junction also contributes to splicing at the downstream site.

DISCUSSION

In this report, we present evidence that U1 snRNA is a key *trans*-acting factor in the activation of an unusual cryptic 5′ splice site in the second intron of the *cdc*2 gene from *S. pombe*. In experiments described elsewhere, we have altered the distance between the cryptic 5′ junction and the other splicing signals within this intron (Romfo et al., 2000). The results demonstrate that moving the cryptic 5′ splice site farther away from the 3′ splicing signals dramatically diminished its use, indicating that proximity to the branchpoint is critical for its activation. Conversely, decreasing the size of the intron allowed the natural 5′ splice site to compete much more effectively with the cryptic 5′ junction. These findings are consistent with the prevalence of extremely small introns in *S. pombe* (Zhang & Marr, 1994; Romfo et al., 2000). An important goal of future work will be to uncover the factor(s) that collaborate with U1 to impose the distance constraints on splice site pairing in *S. pombe*.

Because the vast majority of 5′ splice sites from diverse species, whether natural or cryptic, contain a U as the second nucleotide (Newman & Norman, 1991; Krawczak et al., 1992; Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993; Crispino & Sharp, 1995; Burge et al., 1999; Spingola et al., 1999), the presence of an A at position +2 of the cryptic 5′ junction in *S. pombe cdc*2 is unusual. However, it is not unprecedented, as both a developmentally regulated alternative 5′ splice site in the terminal intron of *Drosophila melanogaster Antp* (Bermingham & Scott, 1988) and a cryptic 5′ splice site in mammalian *ras* (Cohen et al., 1994) share this anomaly. How do these 5′ junctions circumvent the normal sequence requirements for splicing? We speculate that in all three examples, spliceosome assembly is initiated at a nearby site via pairing to U1 snRNA but is diverted to the aberrant 5′ junction prior to catalytic activation. In *Antp*, a consensus 5′ splice site that is used for splicing at later times in development is located just upstream of the noncanonical site in several species of *Drosophila* (Bermingham & Scott, 1988; Hooper et al., 1992), whereas a natural (but mutant) 5′ junction most likely facilitates the recruitment of splicing factors in human *ras* (Cohen et al., 1994) and fission yeast *cdc*2 (this work).

The *trans*-acting factors that mediate the switch in splice site usage in *Antp* have not been investigated but may well include one or more snRNAs. In human *ras* and fission yeast *cdc*2, the data point to different snRNAs as playing the dominant role in cryptic 5′ splice site activation. Although the designs of the two sets of experiments were not precisely parallel, the similarities were sufficient that we believe a comparison is meaningful. A useful framework for discussing the differences is to consider 5′ splice site selection as occurring in two sequential stages: regional, which involves the identification of a domain of the pre-mRNA within which the 5′ splice site will be chosen; and final, in which the specific phosphodiester bond to be attacked in the first transesterification reaction is selected. In the case of human *ras*, the data clearly pointed to a role for U1 snRNA in regional 5′ splice site selection, because the cryptic 5′ junction was activated by several distinct "shift U1s" in which the entire 5′ end of the snRNA was modified to pair with different nearby sequences, some of which were unrelated to the 5′ splice site consensus (Cohen et al., 1994). These results were reminiscent of earlier examples of U1 "action at a distance" in mammalian cells, in which mutant snRNAs promoted splicing at nearby sites to which they were not complementary (Yuo & Weiner, 1989). Our data (Fig. 2) similarly demonstrate that U1 snRNA can act from a distance (i.e., the natural 5′ splice site that has been mutated at highly conserved positions) to promote the use of a nearby 5′ splice site (the unusual cryptic junction) in *S. pombe cdc*2.

In contrast to the earlier work on human *ras*, however, our experiments point to a role for U1 snRNA not only in regional selection of the 5′ splice site, but also in determining the final site of the first transesterification reaction. The most compelling evidence supporting this conclusion is provided by our observation that a mutant U1 snRNA with extensive complementarity to the cryptic 5′ splice site in *cdc*2 promotes splicing there even when a wild-type sequence is present at the natural 5′ junction (Fig. 4). In human *ras*, on the other hand, the evidence pointed to U6 as the key snRNA in final 5′ splice site selection (Hwang & Cohen, 1996). These findings were, in turn, reminiscent of experiments in budding yeast in which U1 alleles complementary to a natural 5′ splice site mutated at position +5 did not restore splicing there, but rather enhanced the use of an aberrant upstream 5′ junction that was activated to a lesser degree by the 5′ splice site mutation alone (Séraphin et al., 1988; Siliciano & Guthrie, 1988). As in *ras*, subsequent work indicated an instructive role for U6 snRNA in diverting splicing to the cryptic 5′ splice site in *S. cerevisiae* rp51 (Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993).

In light of our evidence that U1 is the dominant snRNA in redirecting splicing of *S. pombe cdc*2, it is interesting to note that the 5′ splice site consensus sequences differ slightly between budding and fission yeast. In *S. cerevisiae*, U is the most common nucleotide at position +4 and is complementary to U6, whereas in fission yeast, this nucleotide is most frequently an A, which is complementary to U1 (Zhang & Marr, 1994; Spingola et al., 1999; M. Lyne, K. Rutherford, and V. Wood, pers. comm.). Thus, the contributions of the two snRNAs to activation of cryptic 5′ splice sites may reflect their relative importance in selection of natural 5′ junctions. Do these observations reflect fundamental differences between *S. cerevisiae* and *S. pombe*? We believe not, as they can be reconciled by a model in which U1 and U6 snRNAs collaborate to select the final site of the first transesterification reaction. Consistent with this idea, recent evidence from both yeast and metazoa revealed the existence of a spliceosomal assembly intermediate that contains both the U1 snRNP and the U4/U6·U5 tri-snRNP (Staley & Guthrie, 1999; Maroney et al., 2000). Within this pre-spliceosome, it appears that the departure of U1 snRNA from the 5′ splice site and the entrance of U6 are coupled (Staley & Guthrie, 1999). Our evidence that mutant U1 snRNAs function in the final selection of a cryptic 5′ junction provides another indication that this snRNA continues to influence 5′ splice site selection at a later stage than imagined until recently.

MATERIALS AND METHODS

Construction of pREP2-cdc2, which contains the second intron of the *S. pombe cdc*2 gene and its flanking exons, was described elsewhere (Romfo & Wise, 1997). To alter the sequence of the *cdc*2-Int2 5′ splice site, we used standard site-directed mutagenesis procedures as previously described

(Alvarez et al., 1996) with the following mutagenic oligonucleotides (mutations in bold):

G_1U_2: 5′-AAAAACTT**DH**CGAACACAATTTG-3′ (**D** = 50% A, 25% T, and G; **H** = 50% C, 25% each A and T)
A_3A_4: 5′-GTAAAAAC**BH**ACCGAACACAATTTG-3′ (**B** = 50% T, 25% each C and G; **H** = 50% T, 25% each A and C)
G_5U_6: 5′-AAGTAAAA**HB**TTACCGAACACAA-3′ (**H** = 50% A, 25% each T and C; **B** = 50% C, 25% each G and T)
U_2: 5′-GTAAAAACTT**T**CCGAACACAATTTG-3′
A_4: 5′-GTAAAAAC**M**TACCGAACACAATTTG-3′ (**M** = 50% C, 50% A)
U_6: 5′-CAAGTAAAA**B**CTTACCGAACACA-3′ (**B** = $33\frac{1}{3}$% each T, C, and G)

The profiles of products from *cdc*2 5′ splice site mutants in the presence and absence of U1 mutants were analyzed via primer extension as described previously (Alvarez et al., 1996). The phenotypes of the U1 mutants used in the present study were published elsewhere (Alvarez et al., 1996). Because only 3 of the 12 alleles employed in the studies reported here could support growth (Alvarez et al., 1996), their effects on splicing of the *cdc*2-Int2 5′ splice site mutants were assessed in cells that also retained a wild-type copy of the U1 gene.

The location of the cryptic 5′ splice site in *cdc*2-Int2 was determined by PCR sequence analysis, which is described in detail elsewhere (Alvarez, 1996).

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REFERENCES

Aebi M, Hornig H, Weissman C. 1987. 5′ cleavage site in eukaryotic pre-mRNA splicing is determined by the overall 5′ splice site region, not by the conserved 5′ GU. *Cell 50*:237–246.

Alvarez CJ. 1996. Sequences and factors required for 5′ splice site selection in *S. pombe*. Ph.D. Thesis, University of Illinois at Urbana–Champaign.

Alvarez CJ, Romfo CM, VanHoy RW, Porter GL, Wise JA. 1996. Mutational analysis of U1 function in *S. pombe*: pre-mRNAs differ in the extent and nature of their requirements for this snRNA in vivo. *RNA 2*:404–418.

Berget SM. 1995. Exon recognition in vertebrate splicing. *J Biol Chem 270*:2411–2414.

Bermingham JR, Scott MP. 1988. Developmentally regulated alternative splicing of transcripts from the *Drosophila* homeobox gene *Antennapedia* can produce four different proteins. *EMBO J 7*: 3211–3222.

Black DL. 1995. Finding splice sites within a wilderness of RNA. *RNA 1*:764–771.

Burge CB, Tuschl T, Sharp PA. 1999. Splicing of precursors to messenger RNAs by the spliceosomes. In: Gesteland RF, Atkins JF, eds. *The RNA world*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 525–560.

Chabot B, Steitz JA. 1987. Recognition of mutant and cryptic 5′ splice sites by the U1 small ribonucleoprotein in vitro. *Mol Cell Biol 7*:698–707.

Cohen JB, Snow JE, Spencer SD, Levinson AD. 1994. Suppression of mammalian 5′ splice site defects by U1 small nuclear RNAs from a distance. *Proc Natl Acad Sci USA 91*:10470–10474.

Cortes JJ, Sontheimer EJ, Seiwert SD, Steitz JA. 1993. Mutations in the conserved loop of human U5 snRNA generate the use of novel cryptic 5′ splice sites in vivo. *EMBO J 12*:5191–5200.

Crispino JD, Sharp PA. 1995. A U6 snRNA:pre-mRNA interaction can be rate-limiting for U1 independent splicing. *Genes & Dev 9*: 2314–2323.

Fouser LA, Friesen JD. 1986. Mutations in a yeast intron demonstrate the importance of specific conserved nucleotides for the two stages of nuclear mRNA splicing. *Cell 45*:81–93.

Gatermann KB, Hoffman A, Rosenberg GH, Käufer NF. 1989. Introduction of functional artificial introns into the naturally intronless *ura*4 gene of *Schizosaccharomyces pombe*. *Mol Cell Biol 9*: 1526–1535.

Hooper JE, Pérez-Alonso M, Bermingham JR, Prout M, Rocklein BA, Wagenbach M, Edstrom J-E, De Fritos R, Scott MP. 1992. Comparative studies of *Drosophila Antennapedia* genes. *Genetics 132*:453–469.

Hwang D-Y, Cohen JB. 1996. U1 snRNA promotes the use of nearby 5′ splice sites by U6 snRNA in mammalian cells. *Genes & Dev 10*:338–350.

Jacquier A, Rodriguez JR, Rosbash M. 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.

Kandels-Lewis S, Séraphin B. 1993. Involvement of U6 snRNA in 5′ splice site selection. *Science 262*:2035–2039.

Krawczak M, Reiss J, Cooper DN. 1992. The mutational spectrum of single-pair substitutions in mRNA splice junctions of human genes: Causes and consequences. *Hum Genet 90*:41–54.

Lesser CF, Guthrie C. 1993. Mutations in U6 snRNA that alter splice site specificity: Implications for the active site. *Science 262*: 1982–1988.

Lo PCH, Debjani R, Mount SM. 1994. Suppressor U1 snRNAs in *Drosophila*. *Genetics 138*:365–378.

Maroney PM, Romfo CM, Nilsen TW. 2000. Functional recognition of the 5′ splice site by U4/U6.U5 tri-snRNP defines a novel ATP-dependent step in early spliceosome assembly. *Mol Cell 6*: 317–328.

Nelson KK, Green MR. 1990. Mechanism for cryptic splice site activation during pre-mRNA splicing. *Proc Natl Acad Sci USA 87*: 6253–6257.

Newman A, Norman C. 1991. Mutations in yeast U5 snRNA alter the specificity of 5′ splice-site cleavage. *Cell 65*:115–123.

Newman A, Norman C. 1992. U5 snRNA interacts with exon sequences at 5′ and 3′ splice sites. *Cell 68*:743–754.

Newman AJ, Lin R-J, Cheng S-C, Abelson J. 1985. Molecular consequences of specific intron mutations on yeast mRNA splicing in vivo and in vitro. *Cell 42*:335–344.

Parker R, Guthrie C. 1985. A point mutation in the conserved hexanucleotide at a yeast 5′ splice site uncouples recognition, cleavage and ligation. *Cell 41*:107–118.

Pikielny CW, Rosbash M. 1985. mRNA splicing efficiency in yeast and the contribution of non-conserved sequences. *Cell 41*:119–126.

Romfo CM, Alvarez CJ, van Heeckeren WJ, Webb CJ, Wise JA. 2000. Evidence for splice site pairing via intron definition in *Schizosaccharomyces pombe*. *Mol Cell Biol 20*:7955–7970.

Romfo CM, Wise JA. 1997. Both the polypyrimidine tract and the 3′ splice site function prior to the first step of splicing in fission yeast. *Nucleic Acids Res 25*:4658–4665.

Sawa H, Abelson J. 1992. Evidence for a base-pairing interaction between U6 small nuclear RNA and the 5′ splice site during the splicing reaction in yeast. *Proc Natl Acad Sci USA 89*:11269–11273.

Sawa H, Shimura Y. 1992. Association of U6 snRNA with the 5′ splice site region of pre-mRNA in the spliceosome. *Genes & Dev 6*: 244–254.

Séraphin B, Kretzner L, Rosbash M. 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.
Séraphin B, Rosbash M. 1989. Mutational analysis of the interactions between U1 small nuclear RNA and pre-mRNA of yeast. *Gene 82*:145–151.
Siliciano PG, Guthrie C. 1988. 5′ splice site selection in yeast: Genetic alterations in base-pairing with U1 reveal additional requirements. *Genes & Dev 2*:1258–1267.
Sontheimer EJ, Steitz JA. 1993. The U5 and U6 small nuclear RNAs as active site components of the spliceosome. *Science 262*: 1989–1996.
Spingola M, Grate L, Haussler D, Ares M Jr. 1999. Genome-wide bioinformatic and molecular analysis of introns in *Saccharomyces cerevisiae*. *RNA 5*:221–234.
Staley JP, Guthrie C. 1999. An RNA switch at the 5′ splice site requires ATP and the DEAD box protein Prp28p. *Mol Cell 3*: 55–64.
Vijayraghavan U, Parker R, Tamm J, Iimura Y, Rossi J, Abelson J, Guthrie, C. 1986 Mutations in conserved intron sequences affect multiple steps in the yeast splicing pathway, particularly assembly of the spliceosome. *EMBO J 5*:1683–1695.
Yuo C-Y, Weiner AM. 1989. A U1 small ribonucleoprotein particle with altered specificity induces alternative splicing of an adenovirus E1A mRNA precursor. *Mol Cell Biol 9*:697–707.
Zhang MQ, Marr TG. 1994. Fission yeast gene structure and recognition. *Nucleic Acids Res 22*:1750–1759.
Zhuang Y, Weiner AM. 1986. A compensatory base change in U1 snRNA suppresses a 5′ splice site mutation. *Cell 46*:827–835.