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Mutational analysis of U1 function in *Schizosaccharomyces pombe*: Pre-mRNAs differ in the extent and nature of their requirements for this snRNA in vivo

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ABSTRACT

The U1 snRNP is known to play a critical role in spliceosome assembly, at least in part through base pairing of its RNA moiety to the substrate, but many details remain to be elucidated. To further dissect U1 snRNA function, we have analyzed 14 single point mutations in the six nucleotides complementary to the 5′ splice site for their effects on growth and splicing in the fission yeast *Schizosaccharomyces pombe*. Three of the four alleles previously found to support growth of *Saccharomyces cerevisiae* are lethal in *S. pombe*, implying a more critical role for the 5′ end of U1 in fission yeast. Furthermore, a comparison of phenotypes for individual nucleotide substitutions suggests that the two yeasts use different strategies to modulate the extent of pairing between U1 and the 5′ splice site. The importance of U1 function in *S. pombe* is further underscored by the lethality of several single point mutants not examined previously in *S. cerevisiae*. In total, only three alleles complement the U1 gene disruption, and these strains are temperature-sensitive for growth. Each viable mutant was tested for impaired splicing of three different *S. pombe* introns. Among these, only the second intron of the *cdc*2 gene (*cdc*2-I2) showed dramatic accumulation of linear precursor. Notably, *cdc*2-I2 is spliced inefficiently even in cells containing wild-type U1, at least in part due to the presence of a stable hairpin encompassing its 5′ splice site. Although point mutations at the 5′ end of U1 have no discernible effect on splicing of pre-U6, significant accumulation of unspliced RNA is observed in a metabolic depletion experiment. Taken together, these observations indicate that the repertoire of U1 activities is used to varying extents for splicing of different pre-mRNAs in fission yeast.

Keywords: base pairing; secondary structure; splice site selection; U6 snRNA

INTRODUCTION

In spite of its status as the first snRNP known to directly contact the splicing substrate, both historically and kinetically (Mount et al., 1983; Black et al., 1985; Bindereif & Green, 1986; Pikielny et al., 1986), the role of U1 remains enigmatic. Our current view dates to a breakthrough series of experiments conducted in *Saccharomyces cerevisiae* in the late 1980s, which led to the concept of "commitment" (Legrain et al., 1988; Ruby & Abelson, 1988; Séraphin & Rosbash, 1989a). The idea that binding of U1 represents a key regulatory step in splicing rests principally on the observation that complexes containing only this snRNP and a small number of proteins are stable to challenge by an excess of competitor pre-mRNA, i.e., they are committed to the splicing pathway. Subsequent experiments revealed the presence of U1 in early pre-splicing complexes formed in mammalian cell extracts (e.g., Barabino et al., 1990; Michaud & Reed, 1991). Assembly of commitment complexes requires an intact branch point sequence in *S. cerevisiae* (Ruby & Abelson, 1988; Séraphin

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& Rosbash, 1989a) and a strong polypyrimidine tract in mammalian cell extracts (Champion-Arnaud et al., 1995), suggesting that the 5′ and 3′ ends of the intron may be juxtaposed even at this early stage. A plausible mechanism for how this might occur emerged from recent studies of protein–protein interactions among mammalian splicing factors, which suggest the existence of an intron-spanning bridge extending from the 70K protein of the U1 snRNP through one or more SR proteins to the 35-kDa subunit of the U2AF heterodimer (Wu & Maniatis, 1993; Kohtz et al., 1994; reviewed in Fu, 1995).

The role of U1-70K in promoting spliceosome assembly has not yet been integrated with the more traditional role ascribed to the U1 snRNP, base pairing between its RNA moiety and the pre-mRNA. The absolute conservation of an 8-nt sequence near the 5′ end of U1 in species ranging from fungi to plants to mammals (reviewed by Guthrie & Patterson, 1988) suggested early on that recognition of the 5′ junction by this snRNA is integral to the mechanism of splicing. Clearly, pairing of U1 to the substrate can and does facilitate splicing in vivo, because compensatory changes in the snRNA ameliorate the splicing defects conferred by 5′ splice site mutations in a broad spectrum of organisms, in some cases reducing precursor accumulation to near wild-type levels (Zhuang & Weiner, 1986; Zhuang et al., 1987; Séraphin et al., 1988; Siliciano & Guthrie, 1988; Cohen et al., 1993; Lo et al., 1994). Base pairing of U1 to the pre-mRNA could not be demonstrated in every instance, however, presumably because these same nucleotides are targeted by other spliceosomal components. For example, splicing is minimally improved upon restoration of pairing between U1 snRNA and position +5 mutants, and this nucleotide is known to pair subsequently with U6 snRNA (reviewed by Madhani & Guthrie, 1994).

The majority of the point mutations examined to date in the 5′ splice site interaction region of budding yeast U1 support growth at a reduced rate, although a few lethal alleles have been identified (Séraphin et al., 1988; Siliciano & Guthrie, 1988; Séraphin & Rosbash, 1989b). Early studies revealed that, surprisingly, precursors to several pre-mRNAs did not accumulate in cells harboring mutant U1 genes as their sole source of the snRNA (Séraphin et al., 1988; Siliciano & Guthrie, 1988; Séraphin & Rosbash, 1989b). The ensuing proposal that the 5′ end of U1 is conserved in *S. cerevisiae* because it is important for splicing of some as yet unidentified intron(s) (Siliciano et al., 1991) has now been confirmed (Kao & Siliciano, 1996). In budding yeast, even introns whose removal was unaffected by point mutations at the 5′ end of U1 may require other functions of this snRNP, because large deletions in the snRNA blocked their splicing at a step prior to the first transesterification (Goguel et al., 1991; Siliciano et al., 1991). Possibly related to these in vivo observations is the demonstration that mammalian snRNPs in which the 5′ end of U1 RNA is blocked or cleaved to prevent base pairing with the 5′ splice site can still contribute to splicing in vitro, as evidenced by stabilization of complexes containing U2 snRNPs (Barabino et al., 1990) and promotion of splicing in substrates containing spliced leader sequences in place of the normal 5′ junction (Seiwert & Steitz, 1993). At least one function of U1 beyond base pairing to the 5′ splice site requires the structural integrity of stem I (Yuo & Weiner, 1989; Seiwert & Steitz, 1993).

Once the U1 snRNP has located a potential exon/intron boundary, presumably at least in part via base pairing interactions, the remaining snRNPs and an extensive array of other factors bind in a defined sequence to give rise ultimately to a mature spliceosome. Clearly, a key but as yet poorly understood transition is the displacement of the U1-5′ splice site helix in favor of a pairing interaction with U6 snRNA, a likely candidate to play an instructive role via base pairing during the transesterification reactions (reviewed by Wise, 1993; Nilsen, 1994; Ares & Weiser, 1995). Pairing of the 5′ splice site to U1 and U6 involves common nucleotides, and the exclusive nature of these interactions is supported by recent experiments with a model mammalian in vitro system, which demonstrate that the presence of the U1 snRNP at the 5′ splice site poses a barrier to binding of the U4/U6·U5 snRNP (Konforti et al., 1993). Indeed, other recent data provide evidence that U1 might be entirely dispensable for splicing, because high concentrations of SR proteins, a family of factors that also bind to the substrate early in spliceosome assembly and influence splice site selection, can promote a complete reaction in the absence of detectable levels of functional U1 snRNP (Crispino et al., 1994; Tarn & Steitz, 1994). Nonetheless, it should be emphasized that the fidelity of splicing is compromised in reactions lacking U1 (Tarn & Steitz, 1994) and the ability to undergo splicing in the absence of this snRNP is substrate-specific (Crispino & Sharp, 1995). These observations may help to explain why disruption of the U1 snRNA gene is lethal both in *S. cerevisiae* and *Schizosaccharomyces pombe* (Séraphin et al., 1988; Siliciano & Guthrie, 1988; Porter et al., 1990).

To further delineate the role of U1 in premessenger RNA splicing, we have conducted the most extensive mutational analysis to date of the region complementary to the 5′ splice site. A significantly higher fraction of U1 point mutants are lethal in *S. pombe* than in *S. cerevisiae*, possibly reflecting differences between the two yeasts in intron distribution and architecture. The isolation of three viable, temperature-sensitive U1 alleles provided an opportunity to examine the dependence of different fission yeast precursors on the integrity of the 5′ end of the snRNA. Of three introns tested in these strains, only one showed a dramatic splicing defect. This pre-mRNA is spliced inefficiently even in

wild-type cells, at least in part because its 5′ junction is sequestered within a stable hairpin. Notably, metabolic depletion of U1 caused a significant reduction in splicing of a precursor that was unaffected by single base substitutions at the 5′ end of the snRNA. Thus, our data indicate that *S. pombe* introns differ in the extent and nature of their requirements for U1 snRNA in vivo, as do vertebrate introns analyzed in vitro.

RESULTS

Most mutations at the 5′ end of *S. pombe* U1 snRNA are lethal

To identify additional nucleotides at the 5′ end of U1 that play critical roles in splicing, as well as to allow comparisons between the effects of analogous mutations in *S. cerevisiae*, we undertook a systematic mutational analysis of nt 1–6 of the fission yeast snRNA. Fourteen single point mutants (see Fig. 1) were constructed and tested for the ability to function as the sole U1 gene using the experimental strategy described earlier (Porter et al., 1990; Reich et al., 1992). The results of complementation and growth analyses for these mutants are presented in Table 1. Seven of the alleles that we examined in *S. pombe* had been studied previously in *S. cerevisiae*, the only other organism in which a comparable approach is feasible; the phenotypes of these are also shown in Table 1. Beginning from the 5′ end, we found that a C substitution at A1 supports growth of fission yeast, similar to the phenotype of the analogous substitution in budding yeast (Table 1, line 3). The reduced growth rate of our U1 mutant may be attributable to a transcription defect (see below). On the other hand, substitution of a U for the C at nt 2 of *S. pombe* U1 is lethal, whereas the analogous mutation supported growth of *S. cerevisiae* (Table 1, line 4). Transversions at C2 are lethal in both yeasts (Table 1, lines 5 and 6). Complementation data for purine substitutions at the third nucleotide of *S. pombe* U1 were difficult to obtain, but our data are most consistent with lethality (see below). In contrast, a U1-U3A mutant supported growth of *S. cerevisiae* (Table 1, line 8). At position 6, which is a C in the wild-type RNA, a U but not an A is tolerated in budding yeast, whereas neither a transition nor a transversion can support growth of fission yeast (Table 1, lines 14 and 15). In summary, four of the seven mutations examined in both yeasts were found to have similar phenotypes. Significantly, in all cases where a difference was observed, the allele in question supported growth of *S. cerevisiae*, but not *S. pombe*.

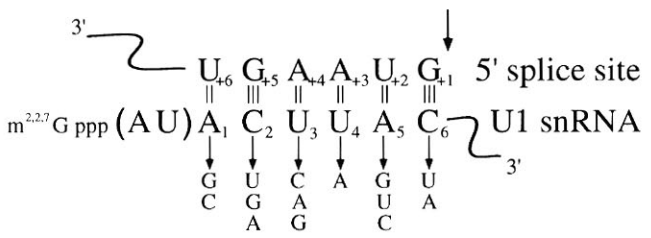

FIGURE 1. Mutations constructed at the 5′ end of *S. pombe* U1 shown in the context of pairing between the snRNA and the fission yeast 5′ splice site consensus sequence. Top: Sequence of the conserved hexanucleotide found at the 5′ end of *S. pombe* introns, shown in inverted orientation; an arrow indicates the 5′ cleavage site. Bottom: Sequence of the complementary region of *S. pombe* U1, with arrows extending from each nucleotide to indicate the single point mutations analyzed in the present study (14 total). In addition, we tested the effect of adding two nucleotides to the 5′ end of U1 (shown in parentheses).

We also analyzed the effects of seven single base changes not examined previously in *S. cerevisiae*. Again proceeding from the 5′ end of the RNA, we found that an A to G substitution at the first nucleotide of *S. pombe* U1 is viable (Table 1, line 2). An allele in which U3 was replaced with a C also complements the fission yeast U1 gene disruption (Table 1, line 7), whereas a G substitution, like U3A, appears to be lethal (see below). The U1-U4A mutant, as well as all three substitutions at A5, are also inviable (Table 1, lines 10–13). In summary, five of the seven point mutants that had not been examined previously in *S. cerevisiae* failed to support growth as the sole U1 gene in *S. pombe*. It is therefore not surprising that all four alleles containing double point mutations are lethal (Table 1, lines 16–19). Finally, because fission yeast U1 lacks two nucleotides that precede the region complementary to the 5′ splice site in virtually every other homologue sequenced, including examples from plants, fungi, and animals (reviewed by Guthrie & Patterson, 1988), we tested whether this truncation is important for optimal U1 function in *S. pombe*. An allele in which an AU dinucleotide is inserted at the beginning of the coding sequence in a gene otherwise identical to wild-type has no discernible impact on growth or viability (Table 1, line 20), indicating that the absence of a 5′ extension in fission yeast U1 (see Fig. 1) is unlikely to be significant.

To confirm that the alleles scored as viable truly are able to support growth as the sole source of U1 in the cell, we performed primer extension sequence analyses with a U1-specific primer on RNA from haploid cells. Results for the three substitution mutants are shown in Figure 2A. Interestingly, in the A1C mutant, there is a band present in all four lanes of the sequencing gel at position 5, suggesting a secondary transcription initiation site. Primer extension analysis in the absence of dideoxynucleotides indicates that approximately 30% of the products migrate at this position (data not shown), which corresponds to the first A downstream from the normal initiating nucleotide. For A1G, the sequence looks identical to that of the wild-type RNA shown in the adjacent lanes, as expected, because a substitution at the first nucleotide cannot be detected by primer extension sequencing. Finally, this

TABLE 1. Phenotypes conferred by mutations at positions 1–6 of U1 snRNA.

Line #	Allele	U1 snRNA 5′ end sequence	Phenotype of *S. pombe* mutant[a]	Phenotype of *S. cerevisiae* mutant[b]
1	Wild-type	ACUUAC		
2	A1G	GCUUAC	Slow (1.6×), TS[c]	ND
3	A1C	CCUUAC	Slow (1.2×), TS[c]	Slow (1.8×[d,e])[f]
4	C2U	AUUUAC	Lethal	Slow (2×[e]; 1.6×[g])[f]
5	C2G	AGUUAC	Lethal	Lethal[d,e]
6	C2A	AAUUAC	Lethal	Lethal[e,g]
7	U3C	ACCUAC	Slow (2.2×), TS[a]	ND
8	U3A	ACAUAC	Lethal?[h]	Slow (1.5×[e,g])[f]
9	U3G	ACGUAC	Lethal?[h]	ND
10	U4A	ACUAAC	Lethal	ND
11	A5G	ACUUGC	Lethal	ND
12	A5U	ACUUUC	Lethal	ND
13	A5C	ACUUCC	Lethal	ND
14	C6U	ACUUAU	Lethal	Slow (2.3×[e])[f]
15	C6A	ACUUAA	Lethal	Lethal[e]
16	U4G,C6U	ACUGAU	Lethal	ND
17	A5C,C6G	ACUUCG	Lethal	ND
18	U3C,C6A	ACCUAA	Lethal	ND
19	U3G,C6G	ACGUAG	Lethal	ND
20	5′ extension	AUACUUAC	Wild-type	NA

[a] The ability of U1 mutant alleles to support growth of *S. pombe* was assessed by genetic complementation as described previously (Porter et al., 1990; Reich et al., 1992). Briefly, the diploid strain *snu1*Δura41, heterozygous for U1 gene disruption, was transformed with a wild-type or mutant U1 plasmid and transformants selected by their ability to grow on media lacking leucine. Following sporulation, ca. 100 haploids were tested for leucine and/or uracil prototrophy. Strains harboring functional U1 alleles give rise to both Leu^+Ura^+ and Leu^+Ura^- spores, whereas diploids transformed with lethal U1 snRNA mutants yield no Ura^+ haploids. ND, not determined; NA, not applicable.

[b] For comparison, complementation and growth data, where available, are provided for corresponding *S. cerevisiae* mutants. Note that there is a discrepancy in numbering between fission and budding yeast U1 due to truncation of the *S. pombe* RNA noted in the text.

[c] TS, mutants are both heat- and cold-sensitive for growth. Growth rates of viable mutants relative to an isogenic wild-type strain were determined by monitoring optical density of cultures grown in minimal medium supplemented with adenine at the indicated temperature.

[d] Séraphin et al. (1988).

[e] Siliciano and Guthrie (1988).

[f] Viable *S. cerevisiae* U1 mutants are also temperature-sensitive (unpubl. data cited in Siliciano et al., 1991), but growth rates have not been reported.

[g] Séraphin and Rosbash (1989b).

[h] We were unable to obtain unequivocal phenotypes for the U3A and U3G mutants after several attempts (see text).

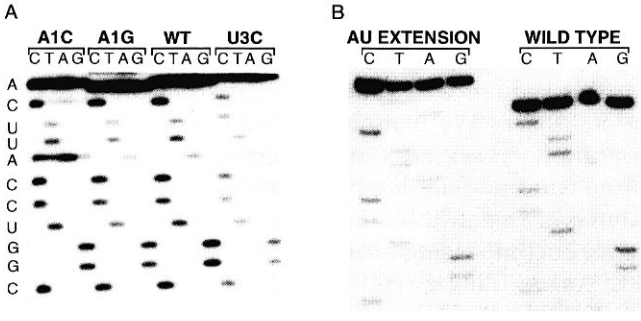

FIGURE 2. Primer extension sequence analysis of U1 from haploid strains harboring viable mutants. Haploid cells carrying a disrupted gene in the chromosome and a plasmid-borne copy of the indicated allele as their sole copy of the U1 gene were propagated at the permissive temperature (30 °C). Total RNA was isolated from cells in mid-log phase and subjected to primer extension sequence analysis with a U1-specific oligonucleotide. **A:** Alleles carrying nucleotide substitutions at or near the 5′ end of U1 snRNA. **B:** Comparison of wild-type U1 and the AU extension mutant.

analysis indicates that the sole RNA sequence present in the U3C mutant contains the change introduced. The sequencing gel shown in Figure 2B demonstrates that U1 RNA from the strain containing an AU insertion at the beginning of the coding sequence is, indeed, extended by two nucleotides.

We also attempted to determine growth rates for the substitution mutants at both the permissive and restrictive temperature (30 and 37 °C, respectively). Even at low temperature, reproducible generation times were not obtained due to the phenotypic instability of these strains; thus, the values given in parentheses in Table 1 represent minimum estimates of their growth defects. The propensity of all three mutants to undergo genetic change was apparent from the observation that they were invariably heat-sensitive when tested immediately after sporulation, but frequently acquired the ability to

grow at 37 °C following propagation (see the Discussion). On solid media, newly sporulated haploids harboring the A1G and A1C mutants formed very small colonies at high temperature, whereas the U3C mutant did not show visible growth, consistent with their relative growth defects under permissive conditions.

Lethal U1 mutants have deleterious effects even in the presence of the wild-type snRNA

Similar studies in *S. cerevisiae* showed a lower proportion of lethality among U1 alleles carrying mutations in the region complementary to the 5′ splice site. One possible explanation for the inability of some mutant RNAs to support growth of *S. pombe* is that they are unstable, for example, due to defects in snRNP assembly. To assess the level to which lethal U1 snRNAs accumulate in the heterozygous state, we performed primer extension sequence analysis; results for six mutants are shown in Figure 3. The data indicate that RNA instability is an unlikely explanation for the prevalence of lethality among U1 mutants in fission yeast, because in only one case did we find that the altered RNA is underrepresented in the population. For the U3G, A5C, and A5G alleles, the mutant RNA is present in several-fold excess over the wild-type RNA, whereas for the U4A, C2A, C2G, and C6A alleles, the mutant and wild-type nucleotides are approximately equally represented in the U1 population. An alternative explanation for the reduced yield of mutant RNA observed with the C6U mutant (Fig. 3) and, on several occasions, with other U1 variants (not shown) is gene conversion of the plasmid-borne allele by the wild-type chromosomal copy. Although instability of the transcript and gene sequence are not mutually exclusive possibilities, we believe that the latter is likely to be the major, if not sole, factor leading to the decline in mutant RNA levels for two reasons. First, in several experiments, sporulation of a diploid heterozygous for the gene disruption in the chromosome and carrying a mutant U1 allele on a plasmid yielded anomalous results. Specifically, although the presence of the mutation in the diploid had been confirmed by RNA sequencing (see Fig. 3), upon analyzing U1 from haploid derivatives, we found exclusively the wild-type RNA (data not shown). These observations suggest that the mutant RNA is being eliminated from the population prior to or during sporulation, most likely via gene conversion. This sequence of events was observed in all three analyses of the U3A mutant and in two of the three experiments with the U3G mutant, with the third yielding only a small number of haploid spores. For all of the other alleles reported in Table 1 to be lethal, evidence of gene conversion was occasionally observed, but, in at least one experiment, the 100 spores analyzed included no viable haploids containing the chromosomal gene disruption.

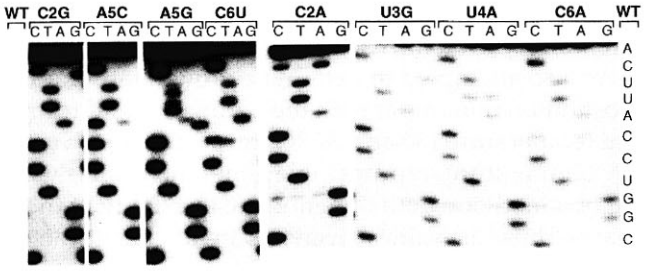

FIGURE 3. Primer extension sequence analysis of U1 from diploid strains harboring lethal mutant alleles. RNA isolated from diploid cells heterozygous for the gene disruption in the chromosome and carrying a plasmid-borne copy of the indicated allele was sequenced with a U1-specific primer as described in the legend to Figure 2.

Although this phenomenon has not been investigated systematically, we note that reduced mutant RNA levels are less prevalent in diploid RNA prepared immediately after transformation (data not shown), consistent with the requirement for a genetic change. Also supporting the existence of selective pressure to eliminate nonfunctional U1 RNAs from the population is the observation that heterozygous diploids invariably grow more slowly than an isogenic strain transformed with a plasmid carrying the wild-type gene. This is in keeping with the semi-dominant phenotypes of U1 5′ splice site interaction region mutants in *S. pombe*. To assess the extent to which the mutant RNAs interfere with wild-type U1 function, we attempted to determine growth rates for strains expressing wild-type U1 from the chromosome and a mutant version from a plasmid-borne gene. Different results were obtained in each of several attempts (data not shown), presumably due to outgrowth of cells in which the mutant gene was converted to wild-type.

Point mutations at the 5′ end of U1 have unequal effects on splicing of different fission yeast introns

The identification of viable point mutations in the region of U1 complementary to the 5′ splice site presented an opportunity to determine the requirements for this sequence in splicing of different pre-mRNAs in *S. pombe*. We examined three different intron-containing precursors in cells containing each of the three conditionally lethal mutant alleles as their sole source of the snRNA. First among these was a transcript encompassing intron 2 of the *cdc*2 gene (*cdc*2-I2) and surrounding exons. The gel in Figure 4 shows that splicing of *cdc*2-I2 is severely compromised in all three viable U1 point mutants (lanes 3–5). No lariat intermediate is detectable in these cells even upon prolonged exposure of the gel, indicating that the block is imposed before step 1, as expected based on the wealth of evidence that U1 promotes events that precede the first catalytic step. However, we were somewhat surprised by the magnitude of the splicing defects, be-

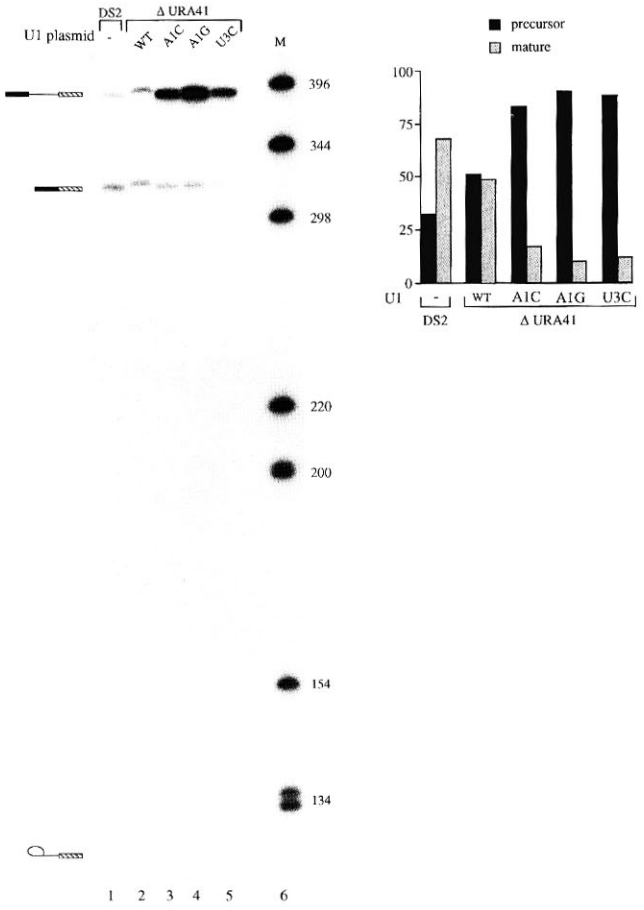

FIGURE 4. Primer extension assay of *cdc*2 intron 2 splicing in viable U1 mutant strains. Left: Primer extension splicing assays on RNA prepared from cells transformed with pREPa-cdc2, which expresses a transcript encompassing intron 2 of the *cdc*2 gene and surrounding exons from the *nmt*1 (no message on thiamine; Maundrell, 1990) promoter. Lane 1, DS2, a haploid strain carrying wild-type U1 in the chromosome; lanes 2–5, *snu1*Δura41 haploids carrying a disrupted U1 gene in the chromosome and the indicated plasmid-borne allele as their sole functional copy. The predicted sizes of the cDNA products derived from *cdc*2 RNA species are: precursor, 388 nt; mature, 317 nt; and lariat intermediate, 121 nt. Right: Quantitation of primer extension data. Levels of precursor and mature message 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 sample, pre-mRNA + mRNA = 100%.

cause the assays were performed on RNA extracted from cells propagated at a temperature (30 °C) where the mutants can grow, albeit more slowly than cells harboring a wild-type U1 plasmid (see Table 1). Careful examination of the data revealed that the increase in linear *cdc*2-I2 precursor had two underlying causes. First, the efficiency with which this intron is spliced appears to depend on the level of U1 in the cell. In RNA from a haploid strain in which U1 is transcribed from a single chromosomal copy of the gene, mature mRNA represents 70% of the total primer extension products (lane 1), whereas in a strain containing the disrupted allele in the chromosome and a multicopy plasmid expressing wild-type U1, the splicing efficiency is only 50% (lane 2). The concentration of the snRNA is approximately twofold elevated in the plasmid-bearing strain as judged by the relative primer extension signals from U1 and U2 snRNAs (data not shown). Although the reduction in *cdc*2-I2 splicing efficiency is highly reproducible, sensitivity to the concentration of U1 in the cell is not observed for another pre-mRNA (see below), and the growth rate of the overexpressing strain is not dramatically reduced (data not shown); these observations suggest that *cdc*2-I2 is unusual among *S. pombe* pre-mRNAs in its response to elevated U1 levels. More importantly, perturbation of the amount of U1 can account for only a fraction of the *cdc*2-I2 splicing defects in cells harboring mutant U1 alleles. For strains that are isogenic except that they carry either the A1G, A1C, or U3C mutant allele rather than wild-type U1 on the extrachromosomal plasmid, mature mRNA represents only 10–17% of the total primer extension products. We attribute the additional ca. 40% reduction in mRNA accumulation to the fact that *cdc*2-I2 splicing is highly dependent on the integrity of the 5′ end of U1 snRNA. The basis for this requirement is addressed by experiments presented below.

To determine the effect of mutating the 5′ end of U1 snRNA on splicing of an endogenous precursor, we analyzed the transcript of the intron-containing U6 snRNA gene (Tani & Ohshima, 1989), which is processed by the pre-mRNA splicing apparatus (Potashkin & Frendewey, 1989). U6 splicing assays performed on the same samples shown in Figure 4 revealed no accumulation of precursor (data not shown), in contrast to the situation with *cdc*2-I2. We therefore proceeded to the experiment shown in Figure 5, in which primer extension analysis was performed on RNA samples prepared from the most severe of our viable mutants, U3C, after growth under standard conditions or at various times after a shift to the nonpermissive temperature; an isogenic wild-type strain was analyzed in parallel as a control. Despite the severe growth defect of the mutant at the restrictive temperature, we observed little, if any, accumulation of unspliced U6 in these cells. Similar experiments with the less deleterious A1C and A1G mutants gave comparable results (data not shown). The lack of an effect is not likely to be due to suppressor outgrowth, because the experiments employed newly sporulated haploids, and optical density measurements indicated that the cells were not actively doubling at 37 °C. However, we did note an intriguing effect of prolonged incubation of the U1-U3C mutant at the nonpermissive temperature: pre-U6 RNA becomes undetectable, whereas the signal from mature U6 (which is presumably quite stable in *S. pombe* as in other organisms) is relatively undiminished (Fig. 5, lane 21; see below).

One possible explanation for the discrepancy in the effect of point mutations at the 5′ end of U1 snRNA is that *cdc*2-I2 is a premessenger RNA and U6 is a splice-

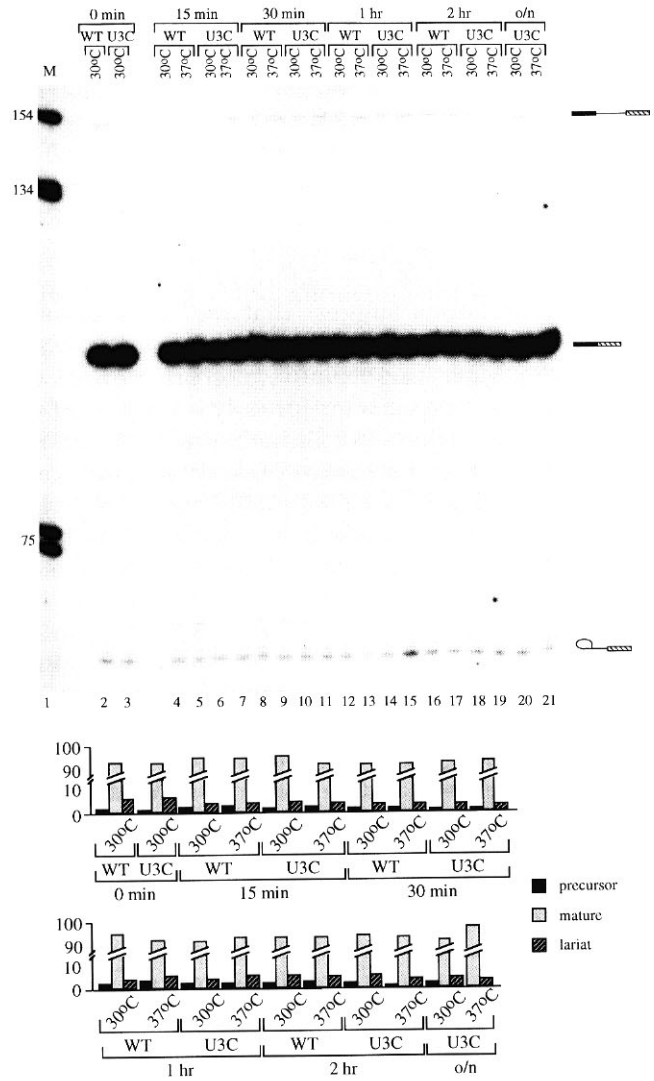

FIGURE 5. Primer extension assay of pre-U6 snRNA splicing in a viable U1 mutant. Top: RNA was prepared from a haploid strain harboring the plasmid-borne U3C allele as the sole functional copy of the gene, either after growth under standard conditions (30 °C) or at various times after a shift to the nonpermissive temperature (37 °C). Primer extension reactions were conducted on total RNA as described earlier (Reich et al., 1992). The predicted sizes of the U6-specific products are: precursor, 149 nt; mature, 99 nt; and lariat intermediate, 66 nt. Bottom: Quantitation of primer extension data. Because U6 is a stable RNA, the amount of precursor is extremely low relative to mature; thus, the middle sections (10–90%) of the bars corresponding to spliced U6 are replaced with a double slash.

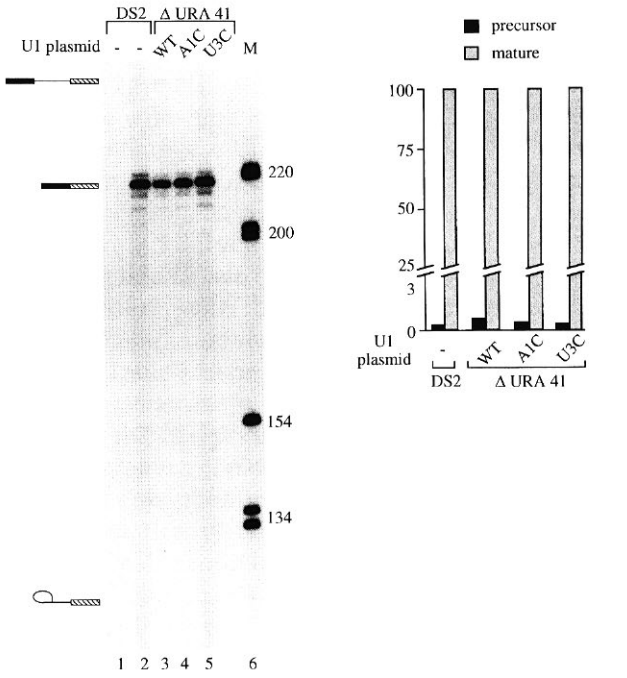

FIGURE 6. Left: Primer extension assay of β-tubulin intron 3 splicing in viable U1 mutants. The experimental procedure was as described in the legend to Figure 4 except that the U1 mutant strains were transformed with pREPa-tub3, which expresses a transcript including intron 3 and surrounding exons of the *nda*3 gene (Hiraoka et al., 1984) from the *nmt*1 promoter. In addition to the control strain harboring a plasmid-borne wild-type U1 gene, we assayed RNA prepared from the untransformed DS2 (parental) strain carrying neither the tub3 nor the U1 plasmid. Predicted sizes of the cDNA products derived from *nda*3 RNA species are: precursor, 261 nt; mature, 220 nt; and lariat intermediate, 116 nt. Right: Quantitation of primer extension data.

osomal snRNA. In *S. pombe*, as in other organisms, the U6 gene is likely to be transcribed by RNA polymerase III (Frendewey et al., 1990; M. Kaiser & D. Brow, pers. comm.), whereas mRNA precursors are synthesized by RNA polymerase II. To determine whether the nature of the transcript accounts for the differential effect of U1 mutations on pre-U6 and *cdc*2-I2 splicing, we analyzed the response of a second pre-mRNA. As illustrated by the data shown in Figure 6, splicing of the third intron from the *nda*3 (β tubulin) gene (*nda*3-I3) is not significantly impaired in the A1C and U3C strains, in contrast to *cdc*2-I2. We attribute the small amount of precursor visible in each lane to background, because its level does not differ dramatically between mutants and, in fact, the highest ratio of precursor:mature mRNA is observed in cells harboring a wild-type U1 plasmid. Importantly, *nda*3-I3 is excised much more efficiently than *cdc*2-I2 and its splicing is insensitive to U1 levels in the cell. Because both intron-containing precursors are transcribed from the same promoter on a plasmid with the same replication origin (see the Materials and methods), these observations argue that the relatively poor splicing of *cdc*2-I2 and its unexpected response to elevated U1 levels are due to properties of the intron itself rather than its mode of expression.

The 5′ splice site of *cdc*2-I2 is sequestered within a stable hairpin

In our initial attempt to discern features that distinguish the *S. pombe* intron that shows a dramatic response to U1 point mutants from the two that do not, we first noted that *S. pombe* introns are in general small (Zhang & Marr, 1994), and *cdc*2-I2 is somewhat larger than the other two intervening sequences (Fig. 7A).

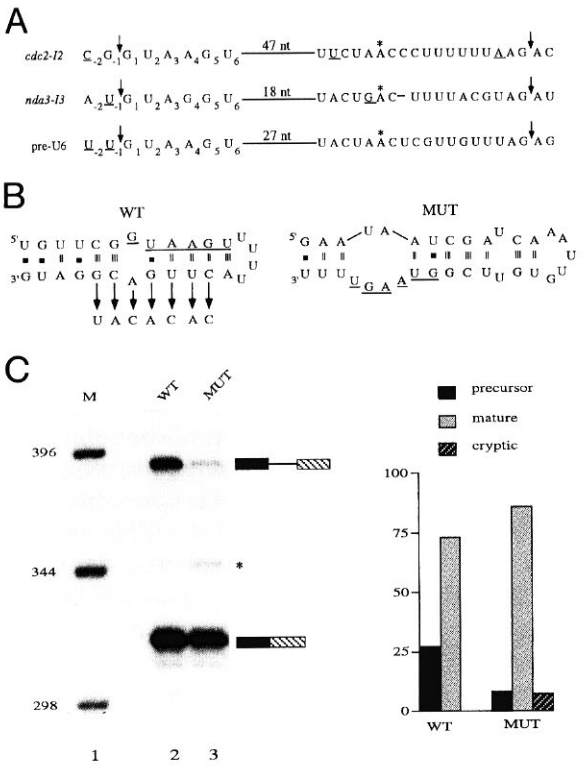

FIGURE 7. A: Schematic representations of the three introns used in splicing assays. Splice junctions are marked by arrows and the site of lariat formation with an asterisk. A dash in the *nda*3-I3 sequence indicates a gap in the alignment, because its branch site-3′ junction distance is one nucleotide shorter than the corresponding regions of *cdc*2-I2 and pre-U6. **B:** Predicted hairpins encompassing the 5′ splice sites of wild-type or mutant *cdc*2-I2. Secondary structure predictions for *cdc*2-I2 transcripts were generated using the MulFold computer program (Jaeger et al., 1989). Diagrammed at the left is the region surrounding the 5′ splice site (underlined) of wild-type *cdc*2-I2. Indicated below the secondary structure is a series of mutations designed to disrupt the intramolecular helix. When this multiple point mutant (MUT) was analyzed using the MulFold program, the 5′ splice site was predicted to reside on the 3′ side of a weak stem rather than the 5′ side of a strong stem (right). Because the predicted secondary structure is different, the nucleotides altered in this mutant do not appear. **C:** Primer extension assays of splicing for wild-type *cdc*2-I2 and the mutant allele containing the sequence changes indicated in B. Left: Autoradiogram of cDNA species. The experimental design was as described in Figure 4. The asterisk indicates a band that arises through use of a cryptic 5′ splice site (see text). Right: Quantitation of data by PhosphorImager analysis.

However, expanding *nda*3-I3 to the size of *cdc*2-I2 had little or no discernible effect on splicing efficiency (C.J. Alvarez & J.A. Wise, unpubl. data). Thus, size per se does not appear to be a critical factor in the differential response of these introns to U1 point mutations. Second, we compared the sequences surrounding the cleavage sites as well as the branch point in *cdc*2-I2, pre-U6, and *nda*3-I3. This was, again, in and of itself uninformative because, as illustrated in Figure 7A, the three introns examined in this study do not differ dramatically in the strength of their splicing signals; in fact, *cdc*2-I2 contains not only a nearly perfect *S. pombe* consensus 5′ splice site, but also the most extensive polypyrimidine tract yet documented in this organism (Hindley & Phear, 1984). A key clue to the underlying cause of the differences is that *cdc*2-I2 is spliced inefficiently even in cells carrying wild-type U1 (Fig. 4). A plausible hypothesis to account for this observation emerged from computer-assisted secondary structure analysis (Jaeger et al., 1989), which revealed the presence of a stable hairpin encompassing the 5′ splice site of *cdc*2-I2 (Fig. 7B, left). Similar analyses of pre-U6 and *nda*3-I3 indicate that their 5′ splice sites are not likely to be involved in stable secondary structures.

To test whether the predicted hairpin contributes to the inefficient splicing of *cdc*2-I2, we incorporated a series of mutations designed to disrupt the stem into an otherwise wild-type allele (Fig. 7B, left). Computer-assisted secondary structure analysis indicates that a different, far less stable helix encompassing the 5′ splice site is likely to be present in the resulting mutant RNA. Most importantly, the 5′ hexanucleotide itself is predicted to adopt a largely single-stranded conformation (Fig. 7B, right). Splicing assays on the mutant allele (Fig. 7C) demonstrate that disrupting the hairpin encompassing the 5′ splice site in *cdc*2-I2 dramatically decreases the precursor:mRNA ratio (cf. lanes 2 and 3). This result strongly implies that the presence of a stable stem that includes the 5′ junction has a major impact on the efficiency of *cdc*2-I2 splicing. Intriguingly, disruption of the stem that encompasses the 5′ splice site in wild-type *cdc*2-I2 has a second effect: activation of a cryptic 5′ junction. This cryptic site is also activated by certain mutations at the *cdc*2-I2 5′ junction, as described in detail elsewhere (C.J. Alvarez & J.A. Wise, in prep.).

Metabolic depletion of U1 snRNA inhibits splicing of pre-U6 snRNA

Given that the role of the U1 snRNP in splicing is not limited to base pairing between its RNA moiety and the substrate (see the Introduction), we wanted to determine whether the complete absence of the snRNA would decrease splicing of an *S. pombe* precursor that is not detectably affected by point mutations. For these experiments, the U1 coding sequence was placed under control of the *nmt*1 promoter, which is regulated by thiamine (see the Materials and methods for details). To examine U1 expression in this strain, we performed primer extension assays using the same U1-specific oligonucleotide employed for sequencing (Figs. 2, 3); the results are shown in Figure 8B. The primer extension analysis demonstrates that the *nmt*1 transcription signals direct accurate initiation, because the 5′ end of U1 snRNA is identical between the strain harboring the repressible allele and the wild-type control (cf. Fig. 8B, lanes 1 and 3). The 3′ end of U1 is likely to be properly formed as well, because the downstream DNA in this construct is derived from the *S. pombe* U1 locus. Most

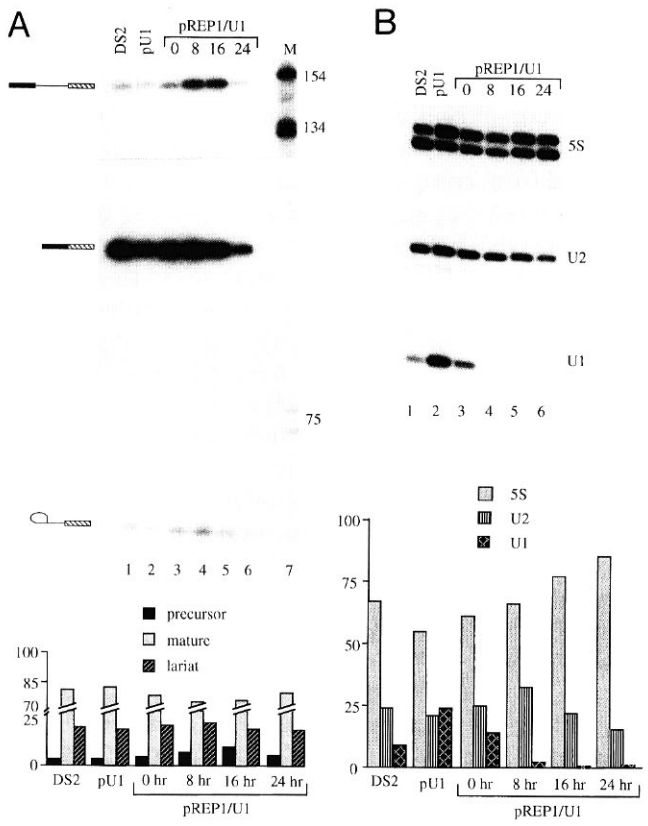

FIGURE 8. Effects of metabolic depletion of U1 snRNA on splicing in fission yeast. The U1 coding sequence, cloned behind the *nmt*1 promoter (see the Materials and methods for a detailed description of the construction of pREP1-U1), was introduced into the *snu1*Δura41 diploid (Porter et al., 1990) and a haploid carrying this allele as its sole functional copy isolated by sporulation. **A:** Time course of pre-U6 snRNA splicing in a strain carrying the U1 gene expressed from a regulated promoter. Top: Total RNA was prepared and assayed by primer extension with a U6-specific oligonucleotide as described in the legend to Figure 5. Lane 1, DS2, a control haploid carrying only the chromosomal copy of U1; lane 2, pCLF3, an *snu1*Δura41 derivative containing U1 under its own promoter on a high-copy plasmid; lanes 3–6, time course of U6 splicing after transfer of the pREP1-U1 strain to medium containing thiamine, which represses transcription from the *nmt*1 promoter (Maundrell, 1990). The upper 25% of the figure shows a 1-h exposure of the gel, whereas the bottom portion shows a 3-min exposure. Bottom: Quantitation of primer extension data. As in Figure 5, the middle sections (25–70%) of the bars corresponding to mature U6 are replaced with a double slash. **B:** Time course of U1 depletion. Primer extension analysis was performed using 5S, U1, and U2-specific primers on the same RNA samples shown in the corresponding lanes of A. The 5S-specific product is a doublet. Bottom right: Quantitation of primer extension data. The sum of the 5S, U2, and U1 bands = 100%.

importantly, U1 RNA expressed from an mRNA promoter is functional, because the regulated allele complements the gene disruption under derepressing conditions (data not shown). As a loading control, we used an oligonucleotide directed against fission yeast 5S ribosomal RNA. U1 levels in a strain transformed with the plasmid-borne allele under *nmt*1 control are comparable to those in a strain lacking extrachromosomal copies of the gene, as judged by the ratio of the U1:5S signals (cf. Fig. 8B, lanes 1 and 3); in contrast, a strain containing both chromosomal and plasmid-borne U1 genes with the natural promoter has approximately threefold elevated levels of U1 (Fig. 8B, lane 2).

Cells harboring the regulated U1 gene were transferred to medium containing thiamine and the efficiency of U6 splicing, as well the level of residual U1, was analyzed by primer extension at various times after the shut-off of transcription. The results of these assays are shown in Figure 8A and B, lanes 3–6. First and most importantly, the data demonstrate that metabolic depletion of U1 snRNA has a significant impact on splicing of the U6 precursor, in contrast to the undetectable consequences of point mutations at the 5′ end. RNA extracted from cells maintained under repressing conditions displays a significant elevation in the ratio of pre-U6:mature U6, whereas the levels of lariat intermediate remain relatively constant (Fig. 8A, lanes 4–6). Thus, the absence of U1 snRNA imposes a block prior to the first step of U6 splicing, as expected. The accumulation of pre-U6 closely parallels the loss of the U1 signal (Fig. 8B, lanes 4–6), which becomes virtually undetectable after 8 h on repressing medium. These results are consistent with a direct cause and effect relationship between the absence of U1 snRNA and diminished splicing of pre-U6 snRNA. Growth data reveal a lag between manifestation of the splicing defect and an increase in the doubling time of the cells (data not shown), as was observed previously upon metabolic depletion of *S. cerevisiae* U5 snRNA (Patterson & Guthrie, 1987).

Intriguingly, after an extended period under repressing conditions (24 h; Fig. 8, lane 6), the accumulation of pre-U6 relative to mature U6 declines. This observation is reminiscent of the undetectable level of this precursor after prolonged incubation of the U1-U3C point mutant at the nonpermissive temperature. In the temperature-shift experiment with the U3C strain, however, there was no discernible decrease in the level of mature U6 snRNA, whereas in the U1 metabolic depletion experiment, a decline in the corresponding primer extension signal is readily apparent (Fig. 8, lane 6). The diminution of mature U6 levels is not likely to reflect loading differences, because the 5S signal remains constant in each of the RNA samples analyzed (Fig. 8B). Moreover, depletion of U1 snRNA results in decreased accumulation of another spliceosomal snRNA, U2, which was analyzed in parallel with 5S and U1 (Fig. 8B, lanes 4–6). Taken together, these observations suggest the existence of a mechanism for co-regulating the levels of spliceosomal snRNAs in fission yeast.

DISCUSSION

Molecular basis of the differential effects of U1 mutations in *S. cerevisiae* and *S. pombe*

The systematic mutational analysis of the first six nucleotides of U1 reported here indicates that the in-

tegrity of this sequence is extremely critical for the function of the snRNA in *S. pombe*. First, haploid strains carrying viable alleles as their sole source of U1 are unhealthy even at the permissive temperature, and frequently acquire the ability to grow at a temperature that was initially restrictive. We are currently investigating whether this is due to the occurrence of spontaneous extragenic suppressor mutations. Second, in diploid strains also carrying a wild-type U1 gene, there appears to be selective pressure to eliminate 5′ end mutants from the RNA population, and these U1 alleles display partial dominance over the wild-type snRNA. Presumably, interference with wild-type U1 function reflects the ability of altered snRNAs to enter presplicing complexes and render them less than fully functional; for example, snRNPs containing the mutant U1's may interact with factors bound at the 3′ end of the intron, but be unable to bind productively at the 5′ splice site. It has been reported previously that certain human U1 variants could not be stably propagated in 293 cells, suggesting that aberrant forms of the snRNA may have dominant negative effects in vertebrates as well (Cohen et al., 1993). Interference with wild-type snRNA function and genetic instability have been reported in budding yeast for mutations in the branch site recognition region of U2 (Parker et al., 1987), but not for mutations at the 5′ end of U1 (Séraphin et al., 1988; Siliciano & Guthrie, 1988). A more critical role for U1 in *S. pombe* versus *S. cerevisiae* is consistent with the higher frequency of intron-containing genes in fission yeast, and may also be due in part to the presence of transcripts containing multiple introns (see below).

In addition to global differences, the analyses described here and in our earlier work (Reich et al., 1992) reveal a surprising number of individual mutations at the 5′ end of U1 that are compatible with growth of *S. cerevisiae*, but not of *S. pombe* (Fig. 9). A likely explanation for discrepancies in the phenotypes of particular U1 mutants between *S. cerevisiae* and *S. pombe* is that the two yeasts have adopted different strategies to fine-tune the strength of pairing between the snRNA and the 5′ exon/intron boundary. Particularly illuminating is the effect of changing the third nucleotide of the snRNA, which sits across from intron position +4 in the standard alignment with the 5′ junction (see Fig. 7A). In *S. cerevisiae*, the consensus nucleotide at the fourth position of the intron is a U, and mutating U1 to allow pairing to the vast majority of 5′ splice sites produces a mild growth defect (Siliciano & Guthrie, 1988; Séraphin & Rosbash, 1989b); this implies that perfect complementarity is detrimental to splicing and, in fact, hyperstabilization of the U1-5′ splice site helix through other manipulations is deleterious to growth (J. Staley & C. Guthrie, pers. comm.). In *S. pombe* and mammals, intron position +4 is most commonly an A, which is complementary to U1, and purine substitutions at the relevant nucleotide in the fission yeast snRNA are lethal. This seeming paradox can be resolved by a model in which splicing in all species is facilitated by base pairing to U1, so long as the complementary regions do not form too strong a helix. Presumably, stable pairing slows down or prevents displacement of U1 snRNA, which is necessary to make way for the entry of U6 (Konforti et al., 1993). In *S. cerevisiae*, destabilization of the helix appears to be accomplished in the same way for virtually all introns, i.e., by a programmed mismatch at position +4, whereas in other organisms, including fission yeast, the position(s) of mispairing is (are) more variable. Consistent with this notion, only 25 of 186 *S. pombe* introns surveyed can form seven or more contiguous base pairs with U1 snRNA (J.A. Wise & C.M. Romfo, unpubl. obs.).

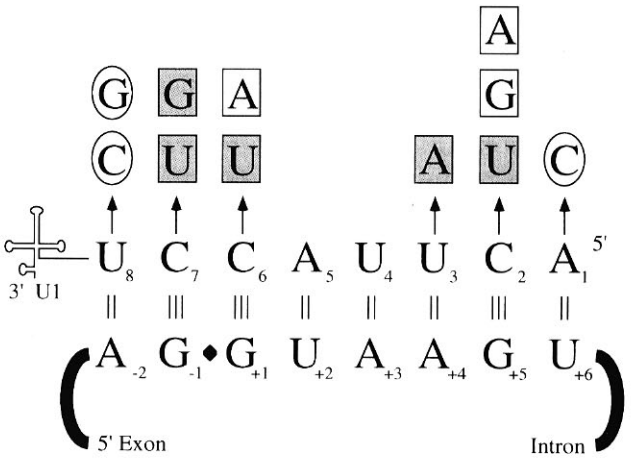

FIGURE 9. Comparison of phenotypes conferred by mutations at the 5′ end of U1 snRNA in budding yeast and fission yeast, shown in the context of potential pairing with the 5′ exon/intron boundary. Substitutions that are viable in both *S. pombe* and *S. cerevisiae* are surrounded by a circle; mutations that are unable to support growth of *S. pombe* are surrounded by a box, with those that are viable in *S. cerevisiae* indicated by shading. See the legend to Table 1 for references to the effects of mutating budding yeast U1 at nt 3–8 (equivalent to nt 1–6 of fission yeast U1); phenotypes for positions 9 and 10 are taken from Séraphin and Kandels-Lewis (1993). The data for fission yeast U1 mutants are taken from the present work for nt 1–6 and from Reich et al. (1992) for nt 7 and 8.

A second difference between the two yeasts is the effect of a C to U change at the second nucleotide of U1, which is complementary to intron position +5; this mutation creates a G·U base pair that is tolerated in budding yeast (Siliciano & Guthrie, 1988; Séraphin & Rosbash, 1989b) but not in fission yeast (Table 1). The discrepancy here may be due to essentially the converse of the situation at position +4. In *S. cerevisiae*, the sequence of the 5′ splice site is nearly invariant (Rymond & Rosbash, 1992), whereas in *S. pombe*, it is quite degenerate (Zhang & Marr, 1994). Moreover, a significantly higher fraction of transcripts contain introns in fission yeast than in budding yeast (Rymond & Rosbash, 1992; Zhang & Marr, 1994). Thus, on a simple statistical basis, disruption of Watson–Crick pairing be-

tween nt 2 of U1 and position +5 of the 5′ splice site is more likely to reduce the strength of the pairing interaction below a critical threshold required for commitment of at least one intron in a pre-mRNA encoding an essential product in *S. pombe*. The need for an uninterrupted region of Watson–Crick pairing between U1 and the 5′ exon/intron boundary may also account for the lethality in *S. pombe* of C to U substitutions at nt 6 and 7, which produce noncanonical G·U pairs, versus their viability in *S. cerevisiae* (Table 1). A C7G mutations is likewise lethal in fission yeast, but not in budding yeast, in keeping with the relative conservation of the terminal exon nucleotide (Fig. 9; Rymond & Rosbash, 1992; Zhang & Marr, 1994). Notably, the three U1 nucleotides at which viable alleles were recovered in both *S. cerevisiae* and *S. pombe* are complementary to poorly conserved nucleotides (−2, +4, and +6) of the consensus 5′ splice site.

In addition to the four U1 nucleotides examined in budding yeast, we determined the phenotypes of mutations at U4 and A5 within the 5′ splice site interaction region of the snRNA. Our observation that any base change at either of these nucleotides is lethal in *S. pombe* provides the first evidence that they are critical for splicing in vivo. A5 of U1 is juxtaposed with the invariant U at the second position of the intron in the standard alignment of the snRNA with the 5′ splice site, whereas U4 sits across from the more variable third intron nucleotide (Fig. 1). In light of the hypothesis advanced above, it will be interesting to learn the effect of mutating the equivalent nucleotides in *S. cerevisiae*. If the budding yeast splicing machinery has indeed evolved toward a stereotypic U1 pairing strategy, mispairing at intron position +3 may be lethal. Notably, the 5′ splice site of the budding yeast *MER2* transcript, whose splicing is regulated by the product of the *MER1* gene, contains a nonconsensus C at this position, and the need for Mer1p can be relieved by U1 mutations that extend its complementarity to the intron beyond the usual pairing region (Nandabalan et al., 1993).

Any discussion of the contribution of U1 base pairing to 5′ splice site selection must take into account the fact that this snRNA is only one of several components that interact with the exon/intron boundary in a sequence-specific manner. Both crosslinking experiments and compensatory base analysis in *S. cerevisiae* demonstrate that nt +4 to +6 of the 5′ splice site pair with an invariant ACA sequence in U6 snRNA (Sawa & Abelson, 1992; Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993). Thus, the mismatch with U1 at position +4 serves a second purpose in this organism by providing for a stronger interaction with U6. Despite the common occurrence of mismatches between U6 snRNA and nt +4 to +6 of mammalian introns, both crosslinking experiments (Sawa & Shimura, 1992; Sontheimer & Steitz, 1993; Konforti & Konarska, 1994) and compensatory base analysis (Sun & Manley, 1995) demonstrate that they interact. Other recent evidence indicates that nt −2 to +4 of the 5′ splice site are recognized in a base-specific fashion by an as yet unidentified component that is neither U1 nor U6 snRNA (Konforti & Konarska, 1994; Crispino & Sharp, 1995). Finally, the terminal region of the exon interacts with U5 snRNA (Newman & Norman, 1991, 1992; Wyatt et al., 1992; Cortes et al., 1993), presumably subsequent to contacting U1.

U1-dependence of *S. pombe* introns correlates with intrinsic splicing efficiency

Our analysis of splicing for three precursors in *S. pombe* revealed that the second intron of the *cdc2* gene is highly dependent on the integrity of the 5′ end of U1, whereas pre-U6 and the third intron of the *nda3* gene are not. What features distinguish the *S. pombe* intron that shows a dramatic response to U1 point mutants from the two that do not? As noted in the Results, the inefficient splicing of *cdc2*-I2 is not due to weak splicing signals, but rather to suboptimal use of its 5′ splice site due to the presence of a stable hairpin. Several naturally occurring RNA secondary structures that modulate splicing efficiency have been documented (e.g., Eng & Warner, 1991), and the product of the *cdc2* gene, $p34^{cdc2}$, functions as a kinase to regulate cell division. An earlier study found no evidence for regulation in *S. pombe* cells of either *cdc2* transcription or splicing of the fourth intron (Durkacz et al., 1986). Does the precursor accumulation we observe consistently for the *cdc2*-I2 transcript reflect a subpopulation of cells in which splicing is completely blocked, or is the effect of the secondary structure on splicing efficiency nonspecific? The magnitude of the defect correlates quite well with the effects on splicing in *S. cerevisiae* of artificial hairpins resembling the natural *cdc2*-I2 structure, in that the 5′ junction is situated on the 5′ side of a stem capped by a four-base loop (Goguel et al., 1993). However, this congruence does not rule out the existence of a specific regulatory mechanism. We attempted to determine whether the structure surrounding the 5′ splice site in *cdc2*-I2 is conserved through evolution, an approach that provided strong evidence for the biological relevance of an imperfect hairpin implicated in regulated splicing of the budding yeast *RPL32* transcript (Eng & Warner, 1991). Unfortunately, the *CDC28* gene of *S. cerevisiae*, which is clearly equivalent to *S. pombe cdc2*, does not contain introns (Lörincz & Reed, 1984), and the proliferation of cyclin-dependent kinases in metazoa makes assigning homology difficult (reviewed by Norbury & Nurse, 1992); only a few genomic sequences have been determined, and none of these resembles *cdc2* in exon–intron organization. Thus, resolution of the role of the *cdc2*-I2 hairpin in regulating splicing must await further developments.

Although the issue of whether *cdc*2-I2 splicing is regulated remains open, our data suggest that a more complex mechanism of 5′ splice site recruitment may pertain for this intron than for the other two fission yeast precursors examined. In particular, its more dramatic response to 5′ end mutations in U1 snRNA may reflect the participation of *trans*-acting factors not required for splicing of *nda*3-I3 and pre-U6, which in turn could help explain the puzzling inhibition of *cdc*2-I2 splicing when U1 levels are moderately elevated. For example, the excess snRNA might sequester a factor that promotes or disrupts pairing with the 5′ splice site such as an annealing protein or helicase, and/or a protein that cooperates with the snRNP to promote juxtaposition of the 5′ and 3′ splice sites (see below). It may also be relevant that, in its natural context, this intron is the second of four in the *S. pombe cdc*2 transcript (Hindley & Phear, 1984). Because pre-mRNAs containing multiple introns are commonplace in *S. pombe* (Zhang & Marr, 1994), it is tempting to speculate that the apparently greater reliance of fission yeast splicing on an invariant sequence at the 5′ end of U1 might be related to the need to match appropriate 5′ and 3′ splice sites in precursors containing many possible combinations.

The role of U1 snRNA in vivo extends beyond base pairing

As noted in the Introduction, U1 snRNP-independent splicing has been documented in vitro for a subset of the pre-mRNAs tested (Crispino & Sharp, 1995). In principle, the efficient splicing of some precursors in *S. pombe* cells carrying point mutations in their sole copy of the U1 gene could reflect a similar situation in vivo. Counter to this possibility, the ratio of precursor:mature U6 increased dramatically over the time course of our metabolic depletion experiment (Fig. 8), indicating that U1 snRNA does, in fact, contribute to splicing of pre-U6 despite its apparent indifference to single base substitutions. Similar to our results for U1, metabolic depletion of U5 snRNA in *S. cerevisiae* significantly reduced the efficiency of premessenger RNA splicing (Patterson & Guthrie, 1987). The effects of decreasing the amount of budding yeast U1 on splicing through regulated expression in vivo have not been reported. However, deleting large portions of this RNA did result in diminished splicing of precursors that were unaffected by point mutations in the snRNA (Goguel et al., 1991; Siliciano et al., 1991). Taken together, these data imply that U1 as a whole, presumably in conjunction with its associated proteins, is likely to be important for splicing of all precursors in both yeasts. A likely possibility is that an intact U1 snRNP is required for splicing in *S. pombe* as a component of an intron-bridging complex such as those proposed recently in extracts from mammalian cells (Wu & Maniatis, 1993; Kohtz et al., 1994; reviewed in Fu, 1995) and *S. cerevisiae* (Abovich et al., 1994). Although there is, as yet, no direct evidence that a similar mechanism is used to juxtapose intron termini in fission yeast, our recent data hint that this may be the case. In particular, we find that the efficiency of *cdc*2-I2 splicing is increased dramatically upon expanding its polypyrimidine tract (C.M. Romfo & J.A. Wise, in prep.), a feature that in mammalian introns anchors the bridging complex to the 3′ end of the intron (Fu, 1995). The dramatic reduction in *cdc*2-I2 splicing efficiency in response to U1 point mutations may also reflect decreased formation of such complexes, because pairing between the substrate and the snRNA is presumably required to stabilize snRNP binding to the 5′ end of the intron. It is quite likely that the stable secondary structure encompassing the *cdc*2-I2 5′ splice site inhibits splicing by decreasing the accessibility of this sequence to *trans*-acting factors including U1 snRNA.

In summary, we have taken advantage of the amenability of fission yeast to genetic manipulation to further dissect the role of U1 snRNA in premessenger RNA splicing. Two major conclusions emerge. First, the markedly different effects of mutating the 5′ end of U1 snRNA in *S. pombe* and *S. cerevisiae* provide additional evidence that the basic mechanism of splicing can become specialized to the needs of a particular organism during the course of evolution. Second, the unequal contribution of fission yeast U1 to splicing of different pre-mRNAs hints that the complex array of U1 functions elucidated in mammalian cells and extracts is relevant to the role of this snRNA even in a simple eukaryote. Thus, our data both extend and shed new light on the results of earlier studies in organisms studied more commonly.

MATERIALS AND METHODS

Plasmid construction and mutagenesis

Construction of the pCLF3 plasmid, which contains the *S. pombe* U1 gene on a 1.8-kb *Eco*R I–*Hin*d III fragment, was described previously (Reich et al., 1992). The following oligonucleotides were used to mutagenize the 5′ splice site interaction region of the snRNA:

U1-ext: 5′CCAGGTAAGT**AT**AAAGAACCGTAA3′;

U1-HB: 5′CTCATGCCA$\mathbf{N_1N_1N_2}$ $\mathbf{N_3N_3N_1N_2}$AAAGAACCG TA3′ ($\mathbf{N_1}$ = 85% G, 5% each C, A, and T; $\mathbf{N_2}$ = 85% T, 5% each A, G, and C; $\mathbf{N_3}$ = 85% A, 5% each T, G, and C);

U1-N2: 5′CCAGGTAA**HT**AAAGAACC3′ (**H** = equal amounts of A, C, and T);

U1-N3: 5′CATGCCAGGTA**Y**GTAAAGAACCGTA3′ (**Y** = 50% each T and C);

U1-N5: 5′ATGCCAGG**V**AAGTAAAGAAC3′ (**V** = equal amounts of G, A, and C);

U1-N6: 5′CATGCCAG**H**TAAGTAAAG3′ (**H** = equal amounts of A, C, and T).

Mutations were confirmed by sequencing with the primer U1-R2: 5′GGGCTCAGT$\mathbf{N_1N_2}$AATGCCAAG3′ ($\mathbf{N_1}$ = 50% G, 16.7% each A, T, and C; $\mathbf{N_2}$ = 50% C, 16.7% each G, A, and T).

To express U1 under control of the regulated *nmt*1 promoter, we first modified the pREP1 vector (Maundrell, 1990) by site-directed mutagenesis with the oligonucleotides LEU2-RV (5′TTGGTGACATCATCTCC3′), which eliminates the *Eco*R V site in the LEU2 gene, and nmt1+RV (5′CTTATTC GATATCTGATATGCCAG3′), which introduces an *Eco*R V site at the predicted start site of transcription. The U1 coding sequence, along with ca. 900 nt of 3′ flanking DNA, was then amplified by PCR from a pTZ19 subclone with the oligonucleotides U1F (5′TTACGGTTCAGTACTTACXYGGCAT G3′; X = 40% C, 20% each of G, T, and A; Y = 40% T, 20% each of G, C, and A; *Sca* I site underlined) and the Universal sequencing primer. The PCR fragment was cleaved with *Sca* I and *Sst* I and used to replace *nmt*1 sequences extending from the *Eco*R V site in the 5′ flanking DNA to the *Sst* I site in the polylinker of the modified pREP1 vector. Thus, the DNA downstream of the U1 coding sequence was derived from the *snu*1 locus (Porter et al., 1990).

Construction of pREP2-cdc2 and pREPa-cdc2, in which a transcript consisting of the second intron of the *S. pombe cdc*2 gene, together with flanking exon sequences, is expressed under control of the *nmt1* promoter, are described elsewhere (C.M. Romfo & J.A. Wise, in prep.; C.J. Alvarez & J.A. Wise, in prep.). A similar construct, designated pREP2-tub3, was generated by using PCR to amplify the third intron of the *nda*3 (β-tubulin) gene (Hiraoka et al., 1984; kindly provided by M. Yanagida), together with its flanking exons, and inserting this product between the *Nde* I and *Bam*H I sites of pREP2 (Maundrell, 1990). The PCR primers were 5′GGAAT TCCATATGGTCTACCATTGCT GATG3′ (*Nde*I site underlined) and 5′GCGGATCCCTCGTTAAAATAAACGTTC3′ (*Bam*HI site underlined). To make a version of the *nda*3-I3 expression construct carrying an *ade*6 selectable marker, designated pREPa-tub3, we inserted a 2.5-kb *Pst* I–*Sst* I fragment carrying both *nda*3 and *nmt*1 sequences into the plasmid pAD3 (Althoff et al., 1994).

To create mutations that disrupt the predicted hairpin encompassing the 5′ splice site of *cdc*2-I2, we used the following oligonucleotide, in which mutant positions are indicated in bold:

2Str: 5′ATGTAAACATTCCCATGCATC**ATGTGATG**
AAAAACTTACCGAACACAATTTG3′.

S. pombe manipulations

Complementation assays for U1 alleles were conducted by transforming the fission yeast strain *snu1Δura41* (Porter et al., 1990), which is heterozygous for disruption of the U1 gene, with plasmids carrying either wild-type or mutant U1 alleles. Sporulation and ascal wall digestion were performed as described previously (Liao et al., 1989). Spores were plated on EMM2 supplemented with uracil and minimal adenine (10 mg/L); haploid colonies, distinguished by their red color, were tested for leucine and/or uracil prototrophy (Althoff et al., 1994). The *S. pombe* strain DS2 (h^+, *ade6-210, leu1-32, ura4-d18*; Reich et al., 1992) was the host for assaying splicing of *cdc*2-I2 and *nda*3-I3. Cells were transformed by the lithium acetate method using approximately 1 μg of purified plasmid; ca. 10^5 transformants were obtained per microgram of DNA.

RNA preparation and analyses

Primer extension RNA sequencing to confirm the presence of U1 mutations was performed as described previously (Porter et al., 1990) using the primer U1-PE2 (Reich et al., 1992), which was also used for standard primer extension analysis.

Primer extension analysis of 5S RNA, which was used as an internal control in the U1 depletion experiments, was performed with the oligonucleotide 5S-PE (5′AGTGATCGGA CGGGA3′), which is complementary to nt 33–47 of the coding region (Mao et al., 1982); due to the higher abundance of the rRNA relative to snRNAs, this oligonucleotide was diluted 25-fold before addition to the reaction mixture. In these experiments, the levels of U2 snRNA were measured using the oligonucleotide L15 (Brennwald et al., 1988).

Primer extension assays of *cdc*2-I2, *nda*3-I3, and pre-U6 splicing were performed as described elsewhere (Reich et al., 1992; C.J. Alvarez & J.A. Wise, in prep.). Quantitation was performed on a Molecular Dynamics PhosphorImager using ImageQuant software (version 3.1).

Computer-assisted secondary structure analysis

A 277-nt sequence encompassing *cdc*2 intron 2 (71 nt) and the adjoining 5′ and 3′ exons (140 and 66 nt, respectively) was analyzed using the MulFold program for RNA secondary structure prediction (Jaeger et al., 1989). The program was run using standard parameters and the three structures predicted to have the highest stabilities were compared in each case. For *nda*3-I3, the 41-nt intron as well as 48 nt of 5′ exon and 61 nt of 3′ exon sequence were analyzed. For U6, 20 nt of the 5′ exon and 30 nt of the intron sequence were included in the secondary structure prediction.

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