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Molecular genetic analysis of U2AF^{59} in *Schizosaccharomyces pombe*: Differential sensitivity of introns to mutational inactivation

CHARLES M. ROMFO, SUJATA LAKHE-REDDY, and JO ANN WISE

Case Western Reserve University, School of Medicine, Department of Molecular Biology and Microbiology, Cleveland, Ohio 44106-4960, USA

ABSTRACT

The large subunit of the mammalian U2AF heterodimer (U2AF^{65}) is essential for splicing in vitro. To expand our understanding of how this protein functions in vivo, we have created a null allele of the gene encoding the *Schizosaccharomyces pombe* ortholog, U2AF^{59}, and employed it in a variety of genetic complementation assays. First, analysis of an extensive series of double amino acid substitutions indicates that this splicing factor is surprisingly refractory to mutations. Second, despite extensive structural conservation, we find that metazoan large subunit orthologs cannot substitute in vivo for fission yeast U2AF^{59}. Third, because the activity of U2AF^{65} in vitro involves binding to the 3′ polypyrimidine tract, we examined the splicing of introns containing or lacking this feature in a U2AF^{59} mutant described here as well as a previously isolated temperature-sensitive mutant (Potashkin et al., 1993, *Science 262*:573–575). Our data indicate that all four introns tested, including two that lack extensive runs of pyrimidines between the branchpoint and 3′ splice site, show splicing defects upon shifting to the nonpermissive condition. In all cases, splicing is blocked prior to the first transesterification reaction in the mutants, consistent with the role inferred for human U2AF^{65} based on in vitro experiments.

Keywords: spliceosome assembly; temperature-sensitive mutant; U2AF^{35}; U2AF^{65}; UAP56

INTRODUCTION

U2 auxiliary factor (U2AF), a heterodimer composed of 65- and 35-kDa subunits (Zamore & Green, 1989), was originally identified by biochemical complementation as a component of mammalian splicing extracts that promotes stable association of the U2 snRNP with the branchpoint (Ruskin et al., 1988). The large subunit is composed of five distinct domains: an amino-terminal segment rich in Arg-Ser dipeptides, followed by a short hinge region responsible for dimerization with the small subunit, and a large carboxy terminus consisting of two standard RNA recognition motifs (RRMs) and a third RRM-like module sometimes referred to as a pseudo-RRM (Zamore et al., 1992; Birney et al., 1993). Orthologs of U2AF^{65} have now been identified in a variety of organisms, including budding yeast (Abovich et al., 1994), fission yeast (Potashkin et al., 1993), *Drosophila* (Kanaar et al., 1993), and *Caenorhabditis elegans* (Zorio et al., 1997). U2AF large-subunit function has been extensively analyzed in vitro (e.g., Ruskin et al., 1988; Zamore & Green, 1991; Zamore et al., 1992; Valcarcel et al., 1993, 1996; Gaur et al., 1995; Singh et al., 1995; Zuo & Maniatis, 1996; reviewed in Krämer, 1996), while only limited information is as yet available regarding its function in vivo (Kanaar et al., 1993; Potashkin et al., 1993; Rudner et al., 1998a, 1998b). Splicing activity can be restored to depleted human extracts by the addition of either human U2AF^{65} or *Drosophila* U2AF^{50}, indicating that the function as well as the structure of this factor is conserved among metazoa (Ruskin et al., 1988; Zamore & Green, 1991). Consistent with the in vitro data, chromosomal deletions encompassing the gene encoding *Drosophila* U2AF^{50} result in embryonic lethality (Kanaar et al., 1993), and RNA interference experiments indicate that blocking production of the *C. elegans* large subunit is also lethal (T. Blumenthal, pers. comm.). However, it has not been possible to show directly that loss of large subunit function in either flies or worms leads to a splicing defect (D. Rudner and D. Rio, unpubl. observations cited in Rudner et al., 1998a, 1998b; T. Blumenthal, pers. comm.). A gene encoding the *S. pombe* ortholog of the large subunit, designated U2AF^{59}, was cloned via genetic comple-

Reprint requests to: Jo Ann Wise, Case Western Reserve University, School of Medicine, Department of Molecular Biology and Microbiology, 10900 Euclid Avenue, Cleveland, Ohio 44106-4960, USA; e-mail: jaw17@po.cwru.edu.

mentation of the *prp*2.1 mutant, which displays a splicing defect at the nonpermissive temperature (Potashkin & Frendewey, 1989; Potashkin et al., 1989, 1993). A genetic screen similar to the one that yielded *prp*2.1 produced a second conditional allele, designated *prp*2.2 (Urushiyama et al., 1996), whereas the *prp*2.3/*mis*11 allele emerged as a chromosome segregation mutant (Takahashi et al., 1994); the implications of the latter observation remain to be explored.

A key aspect of mammalian large-subunit function in vitro is its ability to bind RNAs containing extensive runs of pyrimidines. Gel mobility shift experiments with recombinant human U2AF^{65} demonstrated that the affinity of this protein for oligoribonucleotides varied as a function of both their length and pyrimidine content (Zamore et al., 1992), and the preference of U2AF^{65} for pyrimidine-rich RNAs was underscored by the results of SELEX experiments (Singh et al., 1995). Moreover, there is evidence that the observed binding affinities are functionally relevant, since the extent of cross-linking between U2AF^{65} and the polypyrimidine tract located between the branchpoint and 3′ splice site correlated with splicing efficiency for several mammalian introns (e.g., Hoffman & Grabowski, 1992; Gaur et al., 1995). While the importance of U2AF/pyrimidine-tract binding to splicing in vitro has been extensively documented, a role for this interaction in vivo has not yet been demonstrated. In contrast to the presence of an extensive run of pyrimidines near the 3′ end of virtually every mammalian intron, *Saccharomyces cerevisiae* pre-mRNAs generally lack this feature, and association of the large subunit ortholog, Mud2p, with the intron depends instead on an intact branchpoint recognition sequence (Abovich et al., 1994; Abovich & Rosbash, 1997). In *S. pombe*, the sequence between the branchpoint and 3′ splice site varies widely in base composition and can even be purine-rich (Zhang & Marr, 1994; Romfo & Wise, 1997). Nonetheless, our data indicate that a 3′ polypyrimidine tract, when present, functions prior to the first transesterification reaction to ensure efficient splicing in fission yeast cells (Romfo & Wise, 1997), as in mammalian extracts. These observations, in combination with the ability to detect splicing defects in U2AF^{59} mutants, make *S. pombe* an attractive target for addressing the relationship between polypyrimidine tracts and large-subunit function in vivo.

In addition to binding the polypyrimidine tract, U2AF^{65} has been shown to contact a variety of other splicing factors, and the list continues to grow (Zhang et al., 1992; McKinney et al., 1996; Wentz-Hunter & Potashkin, 1996; Abovich & Rosbash, 1997; Fleckner et al., 1997; Gozani et al., 1998; Rain et al., 1998; Rudner et al., 1998b). For several of these factors, a binding site on the large subunit has been mapped. First, the small subunits of human, *Drosophila,* and *S. pombe* U2AF interact with the hinge regions of the corresponding large subunits (Zhang et al., 1992; Wentz-Hunter & Potashkin, 1996; Rudner et al., 1998b). Second, a member of the DEAD box helicase family of proteins designated UAP56 (56-kDa U2AF-associated protein) contacts U2AF^{65} in a region spanning the boundary between the hinge and the first RRM (Fleckner et al., 1997). Finally, the C-terminal pseudo-RRM binds to a component of the 17S U2 snRNP designated SAP155 and also to a non-snRNP splicing factor known as SF1 or BBP (branchpoint bridging protein; Abovich & Rosbash, 1997; Gozani et al., 1998; Rain et al., 1998). Specific large-subunit amino acids that participate in contacts with these splicing factors have not yet been mapped. Moreover, most of the interactions have been documented only via two-hybrid or other pair-wise protein–protein interaction assays. Thus, much remains to be learned about the details and dynamics of U2AF assembly into early splicing complexes.

For the past several years, we have been investigating the mechanism and regulation of pre-mRNA splicing in the fission yeast *Schizosaccharomyces pombe*, a genetically tractable unicellular eukaryote that shares key features of exon/intron structure as well as splicing signals and *trans*-acting factors with mammals. Here, we report the results of a series of experiments to address several outstanding issues regarding the role of the large subunit of U2AF in vivo. Our data indicate that 3′ polypyrimidine tracts are relevant to, but not the sole determinant of, U2AF^{59} requirements for splicing in vivo. As in mammalian cell extracts, loss of large-subunit function blocks splicing prior to the first transesterification reaction in *S. pombe* cells. However, despite these commonalities, the fission yeast and human large subunits are not interchangeable.

RESULTS

Human U2AF^{65} cannot replace fission yeast U2AF^{59} in vivo

As noted above and illustrated in Figure 1A, the large subunit of U2AF can be divided into five distinct domains. All five of these are conserved among metazoan orthologs, as well as *S. pombe* U2AF^{59}, whereas the *S. cerevisiae* Mud2 protein displays similarity mainly in the C-terminal RRM-like module (Abovich et al., 1994). Figure 1B shows a sequence alignment of the fission yeast and human large subunits; in addition to having the same overall architecture, U2AF^{59} shares moderate conservation throughout (28% identity and 50% similarity; Potashkin et al., 1993). Given the structural similarities, it would be expected that critical amino acids identified via mutational analysis of *S. pombe* U2AF^{59} will also be functionally important in other orthologs. To make possible the use of genetic complementation as an assay for mutant protein function, we constructed a disruption allele and used it to replace one chromo-

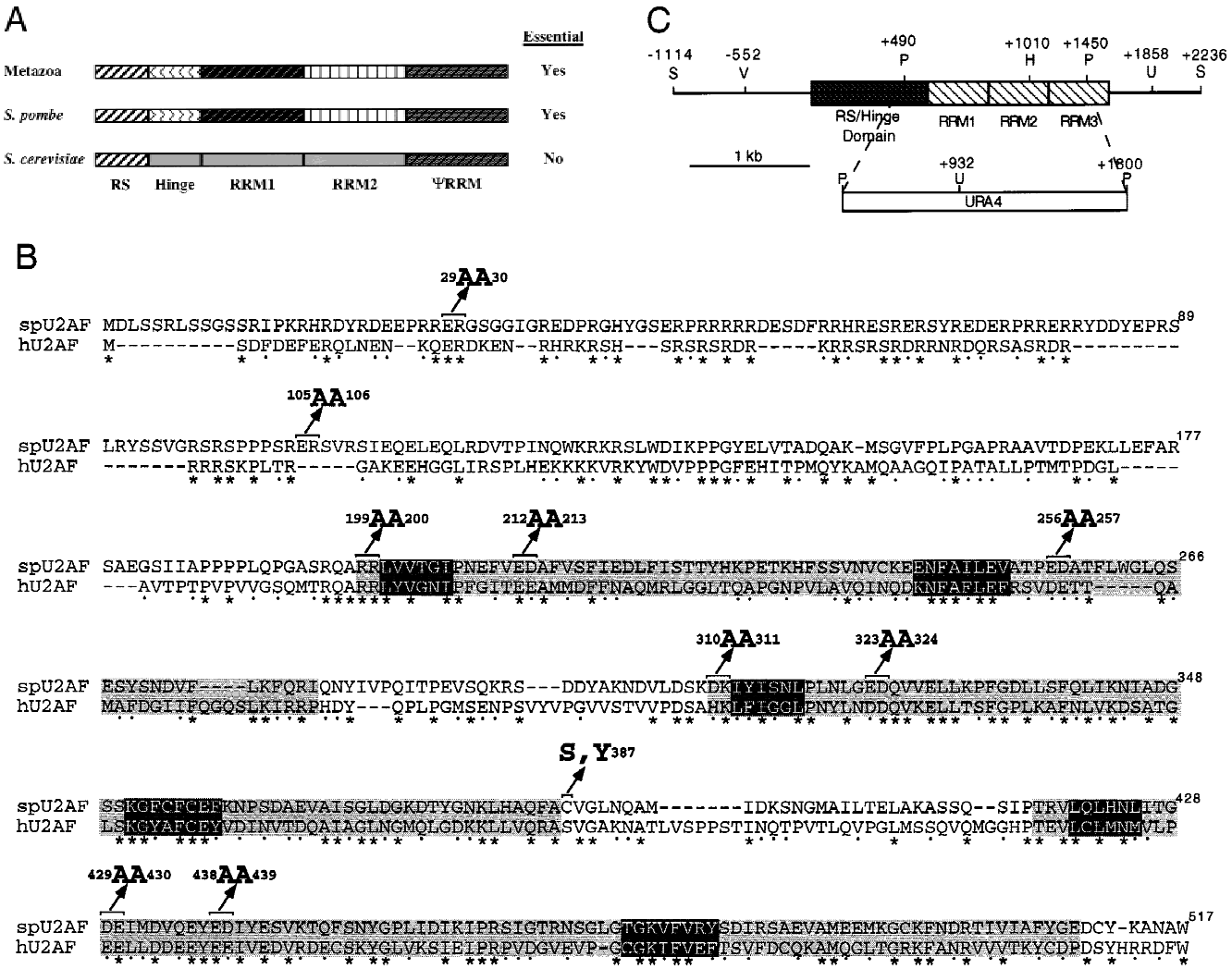

FIGURE 1. A: Domain structure and phenotypes conferred by biochemical or genetic depletion of the large subunit of U2AF. The different shading of the *S. cerevisiae* protein indicates that, while Mud2p is approximately the same length as the other homologs, it shows no significant similarity in the regions corresponding to the hinge, RRM1, and RRM2. References: Human, Ruskin et al. (1988); *Drosophila*, Kanaar et al. (1993); *C. elegans*, T. Blumenthal (pers. comm.); *S. cerevisiae*, Abovich et al. (1994). **B**: Sequence alignment of fission yeast U2AF[59] and human U2AF[65]. The alignment was produced using the ClustalW program with minor manual refinement; *: amino acid identity, ·: amino acid similarity. Numbers at the end of each line refer to the *S. pombe* sequence. The three RNA recognition motifs are shaded in gray with the RNP-1 and RNP-2 submotifs highlighted in black. The following amino acids were considered to be similar: T and S; V, L, and I; D and E; K and R; N and Q; G and A; F and Y. Also illustrated in the figure are the locations of the U2AF[59] mutations described in Table 2. **C**: Restriction map of the wild-type and disruption alleles of *prp*2. H: *Hin*dIII; S: *Sac*I; R: *Eco*RV; V: *Pvu*II; P: *Pst*I; N: *Nhe*I; E: *Eco*RI; B: *Bam*HI; U: *Stu*I. Numbering of restriction sites is relative to the ATG start codon.

somal copy in a diploid strain (see Materials and Methods for details). As illustrated in Figure 1C, the fragment replaced by a *ura*4 selectable marker includes most of the coding sequence. Following verification of correct integration by Southern blot analysis (data not shown), we performed random spore analysis to assess the haploid phenotype conferred by the deletion/replacement allele. Table 1, line 1 shows data from one of four such experiments conducted with SpCR1, a diploid heterozygous for the gene disruption at the *prp*2 locus. The lack of any surviving Ura$^+$ spores indicates that U2AF^{59} is essential for vegetative growth in *S. pombe*, as expected based on the earlier isolation of a temperature-sensitive mutation in the *prp*2 gene (Potashkin et al., 1989, 1993). Both biochemical and genetic experiments indicate that the large subunit has an essential role in metazoa, while a one-step gene disruption in *S. cerevisiae* demonstrates that Mud2p is dispensable (Fig. 1A, right column).

In addition to paving the way for genetic complementation assays of U2AF^{59} mutants, the availability of a *prp*2 null allele allows orthologs from other organisms to be tested for their ability to function in *S. pombe*. As controls for these experiments, we first conducted complementation assays with two plasmids carrying the wild-type U2AF^{59} coding sequence. Table 1, line 2 dem-

TABLE 1. Results of random spore and plasmid complementation analyses in a strain heterozygous for the *prp*2 gene disruption.

Diploid strain sporulated	Number of haploids analyzed[a]	Number of colonies on EMM2+AL[b]	Number of colonies on EMM2+AU[c]	Number of colonies on EMM2+A[d]
SpCR-1	90	0	0	0
SpCR-1/PRP2-pIRT3	90	58	90	58
SpCR-1/PRP2-pREP1	91	41	91	41
SpCR-1/pIRT3	90	0	90	0
SpCR-1/pREP1	90	0	90	0

The diploid strain SpCR1 (genotype *prp2::ura4/prp2*$^+$, *ade6-M210/ade6-M216, ura4d18/ura4d18, leu1-32/leu1-32*) was transformed with plasmids containing the wild-type *prp*2 gene (pIRT3-Prp2 or pREP1-Prp2) or the empty vectors (pIRT3 or pREP1). The untransformed recipient, as well as the four plasmid-bearing strains, were sporulated and tested for growth on defined media as described in Materials and Methods.

[a] Spores were plated initially on rich medium (YEA; Moreno et al., 1991) to allow growth of all viable haploids, which are distinguished from residual diploids by their red or pink color; these cells were then tested for growth on defined media.

[b] Minimal medium (Moreno et al., 1991) supplemented with adenine and leucine, each at 100 mg/L. Cells harboring the disrupted allele (which carries a functional *ura*4 gene) will grow on these plates only if the gene product is not essential.

[c] Minimal medium supplemented with adenine and uracil, each at 100 mg/L. Cells harboring the plasmid (which carries a functional *leu*2 gene) will grow on these plates.

[d] Minimal medium supplemented with adenine. Cells must carry both the disrupted allele and the plasmid to grow.

onstrates that a plasmid carrying the *prp*2 gene with natural flanking sequences allows growth of spores containing the gene disruption, as does a construct in which U2AF^{59} is expressed using the regulatable *nmt*1 (no message on thiamine; Maundrell, 1990) promoter and polyadenylation signal (Table 1, line 3; see Materials and Methods); neither vector on its own can complement (Table 1, lines 4 and 5). To test whether human U2AF^{65} can support growth of haploid cells lacking an intact *prp*2 gene, we expressed the protein using the same *nmt*1 vector used for the complementation with wild-type U2AF^{59} (see Materials and Methods). Unexpectedly, upon transformation of SpCR1 with this plasmid in the absence of thiamine (derepressing conditions; Maundrell, 1990), we recovered very few colonies, less than one percent of the number observed with the vector only control; similar results were obtained with an analogous construct containing *Drosophila* U2AF^{50} (data not shown). Thus, the metazoan large-subunit cDNAs not only fail to complement, but show evidence of dominant lethality in *S. pombe*. To confirm that the human protein prevents growth of *S. pombe*, we introduced the *nmt*1 construct into a wild-type haploid strain under repressing conditions. In this case, the transformation efficiency was normal, but the vast majority of the colonies recovered were unable to grow under derepressing conditions (data not shown). Thus, large-subunit orthologs from metazoa are apparently toxic to *S. pombe*.

Because the *nmt*1 promoter is transcribed at a very high level (Basi et al., 1993; Forsburg, 1993), we were concerned that the dominant lethality might be due to overexpression. To determine whether a lower concentration of the human splicing factor would allow complementation, we reduced its expression via two well-characterized *nmt*1 TATA box mutations (Basi et al., 1993). Dominant negative effects on growth were still observed even with the most defective version of the promoter (data not shown). Thus, we were unable to find conditions under which human U2AF^{65} could functionally substitute for fission yeast U2AF^{59}.

Having demonstrated that overexpression of human U2AF^{65} is lethal in fission yeast, we wanted to determine whether this was due to a block in splicing. As illustrated in Figure 2, cell division ceases ~10–12 h (~3–4 generations) after the shift to medium lacking thiamine (Fig. 2A), with the growth curve paralleling the expression level of the human protein (Fig. 2B). Primer-extension splicing assays were then performed on two different introns in strains overexpressing either the human or *Drosophila* large subunit (Fig. 2C; data not shown). As illustrated in Figure 2C for the endogenous *S. pombe* U6 snRNA transcript, splicing is blocked prior to the first transesterification reaction by human U2AF^{65}, as indicated by the appearance of a precursor band 12 h after the shift to derepressing conditions (Fig. 2C, lane 3). This band persists throughout the time course, at approximately the same level relative to mature U6. As in earlier assays of U6 splicing (Reich et al., 1992; Alvarez et al., 1996), approximately equal amounts of lariat intermediate are observed at all time points.

In an effort to shed light on the molecular basis of the dominant interference, we first tested the effect of co-expressing the small subunit of human U2AF. This protein did not reverse the lethality of the large subunit but was not inhibitory to growth on its own (data not shown).

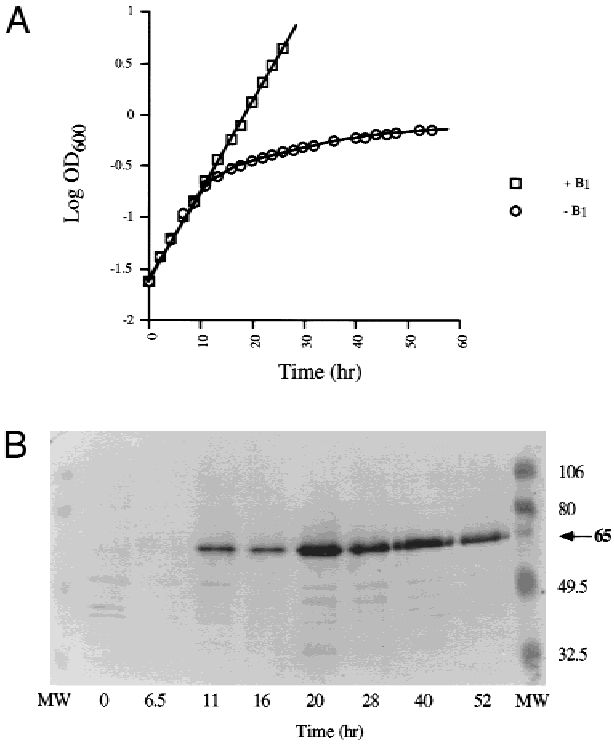

FIGURE 2. Effect of expressing the large subunit of human U2AF in *S. pombe*. **A:** Growth curves in the presence and absence of thiamine (B_1) in strains harboring plasmids in which human U2AF[65] is expressed from the fission yeast *nmt*1 promoter (see Materials and Methods). Thiamine was added to the appropriate cultures at time 0 and growth was monitored by measuring the OD_{600}. **B**: Western blot analysis to monitor human U2AF[65] levels. Extracts were prepared from cells harvested at the indicated times and the proteins resolved by SDS-PAGE, transferred to nitrocellulose, and probed as described previously (Althoff et al., 1994) using antibodies directed against the large subunit of the human protein. **C**: Time course of splicing inhibition in a strain expressing human U2AF[65]. A primer-extension assay (Reich et al., 1992) was used to monitor splicing of the endogenous U6 snRNA transcript. **D**: Effect of deleting and exchanging the RS/Hinge region of human U2AF[65]. The haploid strain DS2 was transformed with the indicated constructs and plated on minimal medium supplemented with thiamine. Transformants were then tested for growth in the absence of thiamine (derepressing conditions).

Second, we attempted to overcome the effect of the human protein by overexpressing fission yeast U2AF[59] using the *nmt*1 promoter. Given its ability to complement the gene disruption (see above), it was somewhat surprising that this construct was unable to relieve the dominant lethality of human U2AF[65]. However, control experiments revealed that the excess of U2AF[59] produced by the combination of *nmt*1-driven expres-

sion from a plasmid and an intact chromosomal copy in a haploid strain produces colonies that are noticeably smaller than those from the untransformed control (data not shown); thus, overexpression of the native protein is also somewhat detrimental to the growth of *S. pombe*. To determine which portion of human U2AF^{65} is responsible for its dominant lethality, we analyzed a series of deletion and domain-swap alleles, with the results shown in Figure 2D. The data indicate that it is the C-terminal portion of the protein containing the RRMs rather than the N-terminal RS/Hinge region that interferes with growth and splicing in fission yeast. In light of the domain swap data, it seems likely that the dominant lethal effect of overexpressing human U2AF^{65} and the detrimental effect of overexpressing the native protein may have different underlying causes.

Most charged-to-alanine substitutions in U2AF^{59} have no phenotypic consequences

As noted above, we created the *prp*2 null allele primarily to take advantage of the facile genetic manipulations available in *S. pombe* to perform an in vivo structure/function analysis on U2AF^{59}. The strategy that we adopted for this purpose involves the replacement of adjacent pairs of charged amino acids with alanine. Because this approach specifically targets amino acids that are likely to be located on the surface of a protein and thus accessible for interactions with other macromolecules (Wertman et al., 1992, and references therein), we hoped to identify particular residues involved in the protein–protein interactions described in the Introduction. Moreover, in previous studies, charged-to-alanine scanning mutagenesis has been found to produce a high frequency of conditional lethal alleles (e.g., Wertman et al., 1992; Diamond & Kirkegaard, 1994; Reijo et al., 1994). In an effort to avoid generating a large number of mutations that produce no phenotypic consequences, we used conservation between the human and fission yeast proteins as an added criterion for selecting mutagenesis targets. The locations and phenotypes of the mutations analyzed are listed in Table 2 and indicated on the sequence alignment shown in Figure 1B; growth defects were assessed as described in the footnotes to Tables 1 and 2. To test our assay system, we first re-created the original *prp*2.1 mutation, C387Y. As expected, this allele displays a temperature-sensitive phenotype (Table 2, line 1). In addition, we tested the effect of mutating cysteine 387 to serine, the amino acid found at the corresponding position in human U2AF^{65} (Fig. 1B). This substitution produced no phenotypic effects (*prp*2.4, Table 2, line 2).

Among the new mutants, the two charged-to-alanine substitutions located within the RS domain (*prp*2.5 and *prp*2.6) displayed wild-type growth (Table 2, lines 3 and 4), which is perhaps not surprising given the generally poor sequence conservation in this region (Fig. 1B). On the other hand, the fact that six of the seven remaining alleles (*prp*2.7–13) also had no discernible impact on growth (Table 2, lines 5–11) was quite unexpected considering that the RRMs display extensive sequence conservation, and we specifically targeted amino acids that are similar or identical between the human and fission yeast proteins (Fig. 1B). As a potential resolution for this paradox, we con-

TABLE 2. Phenotypic analysis of *prp*2 mutants.

Line number	Allele	Mutation(s)	Phenotype	Location
1	*prp*2.1	C387Y	TS lethal	RRM2/RRM3 linker
2	*prp*2.4[a]	C387S	Wild-type	RRM2/RRM3 linker
3	*prp*2.5	E29A/R30A	Wild-type	RS domain
4	*prp*2.6	R105A/E106A	Wild-type	Hinge region
5	*prp*2.7	R199A/R200A	Wild-type	N-terminus of RRM1
6	*prp*2.8	E212A/D213A	Wild-type	RRM1, near RNP2
7	*prp*2.9	E256A/D257A	TS lethal	RRM1, near RNP1
8	*prp*2.10	D310A/K311A	Wild-type	N-terminus of RRM2
9	*prp*2.11	E323A/D324A	Wild-type	RRM2, near RNP2
10	*prp*2.12	D429A/E430A	Wild-type	ΨRRM, near RNP2
11	*prp*2.13	E438A/D439A	Wild-type	ΨRRM, first one-third
12	*prp*2.14	^{195}SRQARR/VALAEE^{200}	Lethal	Hinge/RRM1 junction

Complementation assays with the indicated mutant alleles were performed as described in the footnotes to Table 1. Alleles that supported growth at the standard growth temperature (30 °C) were also assayed for growth at high (37 °C) and low (18 °C) temperature by replica plating.

[a]The *prp*2.2 and *prp*2.3 point mutants, described in the Introduction, were not analyzed here; the amino-acid substitutions in these alleles have only recently been determined (J. Potashkin, pers. comm.).

sidered the possibility that some or all of the mutant alleles are actually dominant lethal, and consequently cells are under selective pressure to eliminate them. Although transformation efficiencies with the charged-to-alanine mutants were not markedly low, this could be because of facile gene conversion of point mutants, as observed for several *prp*2 alleles to be described elsewhere (S. Lakhe-Reddy, C.M. Romfo, T. Tao, & J.A. Wise, unpubl. data). To determine whether the charged-to-alanine mutants are genetically stable, we recovered plasmid from each strain and performed DNA sequence analysis. In no case did we find evidence of gene conversion (data not shown). Thus, we conclude that the large subunit of fission yeast U2AF is quite insensitive to charged-to-alanine substitutions.

Particularly surprising was our finding that the R199A/R200A mutant (*prp*2.7 allele; Table 2, line 8) had no discernible impact on growth, since the altered amino acids lie within a highly conserved region (Fig. 1B) corresponding to the site of UAP56 interaction in human U2AF^{65} (Fleckner et al., 1997). This observation demanded further investigation, particularly in light of the fact that a protein closely related to human UAP56 has been identified through the fission yeast genome sequencing project (www.sanger.ac.uk/Projects/S_pombe/; S. Lakhe-Reddy & J.A. Wise, unpubl. observations). To explain the apparent indifference of U2AF^{59} to the R199A/R200A substitutions, we hypothesized that the relatively subtle changes introduced do not perturb UAP56 binding to an extent sufficient to affect growth. To test this possibility, we constructed the *prp*2.14 allele, in which two uncharged amino acids (S195 and Q197) are replaced with residues of similar size but different chemical characteristics, a basic amino acid (R196) is replaced with alanine, and the two basic amino acids altered in the original mutant (R199 and R200) are replaced with acidic residues. Consistent with our hypothesis, this multiple point mutant is unable to support growth of *S. pombe* (Table 2, line 12). Thus, the charged-to-alanine mutagenesis did in fact target a functionally important region yet failed to produce a phenotype.

The *prp*2.9 mutant is defective in mRNA accumulation as well as pre-mRNA splicing

The single new temperature-sensitive allele that we isolated by charged-to-alanine scanning mutagenesis, *prp*2.9 (Table 2, line 7), affects a contiguous pair of acidic residues located just downstream of the conserved RNP1 octapeptide in the first RNA recognition motif (Fig. 1B). As the first step in characterizing this mutant, we compared its growth under permissive and restrictive conditions to that of an isogenic strain harboring the *prp*2.1 allele. As illustrated in Figure 3, the growth patterns displayed by these mutants following a shift to high temperature are dramatically different. As previously reported (Potashkin et al., 1989), the C387Y mutation causes a very rapid cessation of growth; no further increase in optical density occurs after ap-

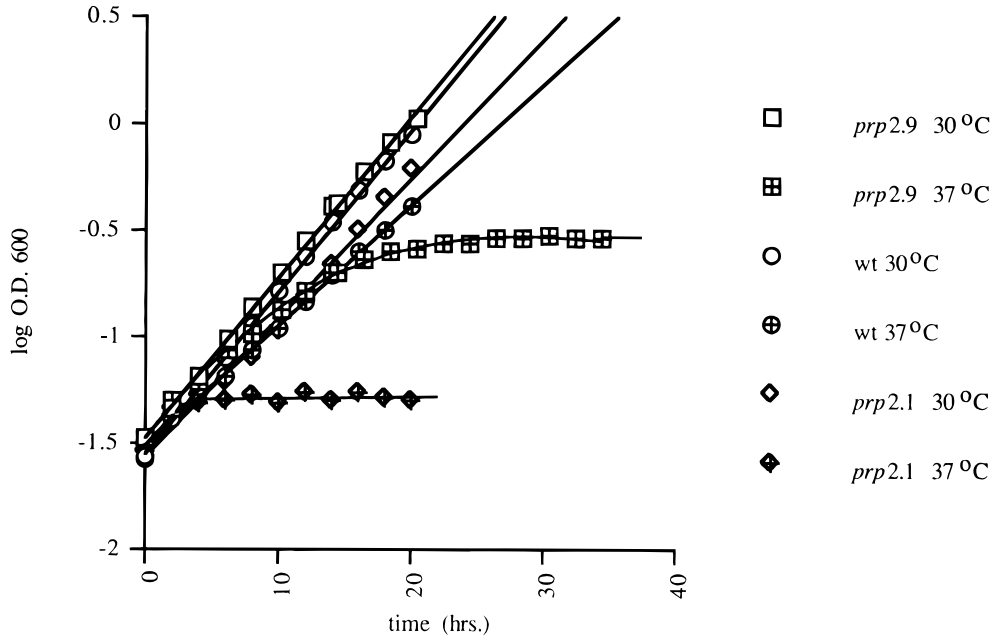

FIGURE 3. Growth curves for the two temperature-sensitive *prp*2 mutants. Growth of isogenic strains harboring either a wild-type (*prp*2^{+}), C387Y (*prp*2.1), or E256A/D257A (*prp*2.9) allele was monitored by measuring the OD_{600} of cultures propagated at the standard growth temperature (30 °C) or at high temperature (37 °C).

proximately half a generation (~2 h) at the elevated temperature. In contrast, the E256A/D257A mutant displays a protracted lag before the optical density plateaus at the higher temperature. In this strain, growth ceases only after nearly five generations under restrictive conditions. At 30 °C, the *prp*2.9 strain grows at a rate indistinguishable from an isogenic wild-type strain, while *prp*2.1 shows a mild growth defect. The distinctive growth properties conferred by these mutations suggest that they affect the function of U2AF[59] in different ways (see Discussion).

To further characterize the *prp*2.9 mutant, we performed primer-extension assays to determine the splicing profiles of three different pre-mRNAs, using the growth curve (Fig. 3) as a guide to determine time points at which to harvest cells for RNA isolation following the shift to the nonpermissive temperature. The pre-mRNAs tested are designated *cdc*2-Int2, *nda*3-Int3 and *cgs*2-Int1 and contain, respectively, the second of four introns from the cell division cycle gene *cdc*2 (Hindley & Phear, 1984), the third of five introns from the β-tubulin gene (Hiraoka et al., 1984), and the first of three introns from the gene encoding cyclic AMP phosphodiesterase (DeVoti et al., 1991), together with their flanking exons (see Materials and Methods). The results of primer extension analyses, shown in Figure 4, indicate that the *prp*2.9 mutation has two distinct effects on mRNA metabolism. First, throughout the time course but more dramatically at early time points (6 and 12 h post-shift; Fig. 4, lanes 2 & 3, 7 & 8, and 12 & 13), we observe a splicing defect for each of the pre-mRNAs. Importantly, however, while all three pre-mRNAs tested show splicing defects, the ratio of precursor to mature mRNA, which is generally taken as a measure of splicing efficiency (Pikielny & Rosbash, 1985), is quite different in each case. The most dramatic accumulation of linear pre-mRNA at the expense of mature message is observed for *cdc*2-Int2 and *nda*3-Int3, which have pyrimidine-rich 3′ ends (Hindley & Phear, 1984; Hiraoka et al., 1984), while *cgs*2-Int1, which has a purine-rich 3′ end (DeVoti et al., 1991), shows only a slight effect. In light of these results, we have attempted to determine whether the fission yeast splicing factor displays a preference for binding to pyrimidine-rich RNAs in vitro, similar to its mammalian counterpart; these efforts have thus far been unsuccessful for technical reasons. Although the analyses presented here were performed on mini-genes cloned under a heterologous promoter, primer-extension analysis on U6 snRNA, which contains an mRNA-like intron in *S. pombe*, demonstrates that splicing of endogenous transcripts is also affected in the *prp*2.9 mutant (data not shown). Moreover, others have previously reported that splicing of chromosomally expressed α- and β-tubulin, as well as U6 snRNA, is impaired in the *prp*2.1 mutant (Potashkin et al., 1989, 1993). We elected to employ primer extension rather than Northern blotting or RNAse protection to assay splicing to determine which step is affected. Notably, the only species that accumulates after incubating the mutant strain under nonpermissive conditions is linear precursor; the position where lariat is predicted to migrate shows no signal (data not shown). This result is consistent with a block in spliceosome assembly, which would be predicted based on the in vitro activities of U2AF[65] (Ruskin et al., 1988; Michaud & Reed, 1991).

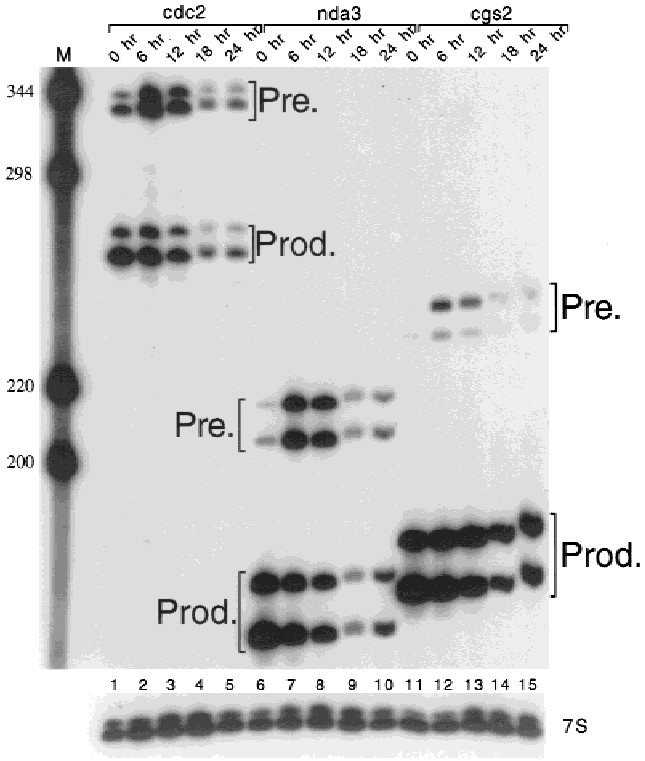

FIGURE 4. Primer-extension assays of splicing in the *prp*2.9 strain. Total RNA was isolated from *prp*2.9 cells transformed with either pAD4-cdc2, pAD4-nda3 or pAD4-cgs2 (see Materials and Methods) at 0, 6, 12, 18, or 24 h following a shift to the nonpermissive temperature. The relative amounts of linear pre-mRNA and mature RNA were determined using primer-extension analysis as previously described (Alvarez et al., 1996; Romfo & Wise, 1997). Lanes 1–5: time course of *cdc*2-Int2 splicing at the nonpermissive temperature; lanes 6–10: time course of *nda*3-Int3 splicing; lanes 11–15: time course of *cgs*2-Int1 splicing. Both the pre-mRNA and mature mRNA extension products appear as doublets due to a pair of transcription initiation sites in the *adh* promoter. The sizes of the lower sets of cDNA products derived from pAD4-cdc2 are: precursor, 334 nt and mature, 262 nt; from pAD4-nda3: precursor, 205 nt and mature, 165 nt; and from pAD4-cgs2: precursor, 235 nt and mature, 174 nt. The region where lariats are predicted to migrate was devoid of signal and is therefore not shown.

The second and more unexpected outcome of the *prp*2.9 splicing assays is the significant decrease in the yield of all three RNAs analyzed at the later time points (18 and 24 h) following the shift to nonpermissive conditions (Fig. 4, lanes 4 & 5, 9 & 10, and 14 & 15). The decline does not appear to be due to general RNA degradation, since the signal from the loading control,

7S RNA of signal recognition particle (Brennwald et al., 1988), remains nearly constant throughout the time course. Moreover, the reduced message levels appear to be unique to the *prp*2.9 mutant, because we have performed similar experiments with the *prp*2.1 strain and have seen no decline in primer-extension signals from intron-containing transcripts following a 6-h incubation at 37 °C (data not shown), which is comparable to the 18-h time point with the *prp*2.9 strain (see Fig. 3). One possible explanation for the reduced mRNA yields in the *prp*2.9 strain is that the mutations render U2AF^{59} susceptible to proteolysis in vivo. If this were the case, then the effects on RNA metabolism might be indirect, that is, the absence of the protein prevents early spliceosome formation, thereby exposing pre-mRNAs to nucleases. Counter to this notion, Western blot analysis demonstrates that U2AF^{59} levels remain quite constant at high temperature in the *prp*2.9 mutant, as well as in *prp*2.1 (data not shown). Moreover, the levels of precursor decline more rapidly in the former strain than the levels of mature mRNA, consistent with a defect at the earliest stage of spliceosome assembly. Further investigation will be required to determine whether the E256A/D257A mutations directly affect commitment complex formation.

Splicing of all introns tested is also impaired in a different U2AF^{59} mutant

As noted in the Introduction, the relevance of 3′ polypyrimidine-tract recognition to U2AF large-subunit function in vivo had not previously been examined for a variety of reasons. Because our results with the *prp*2.9 mutant suggested a correlation between the 3′ pyrimidine content of an intron and its U2AF^{59} dependence, we wanted to explore this issue more systematically. First, in order to determine whether the sensitivity of introns with pyrimidine-rich 3′ ends is specific to the *prp*2.9 mutant, we conducted splicing assays in the original *prp*2.1 strain. Second, we analyzed a more diverse set of pre-mRNAs including *nda*3-Int3 and *cgs*2-Int1, which were also examined in the *prp*2.9 mutant, and *cam*1-Int1, which contains the lone intron from the calmodulin gene together with its flanking sequences (Takeda & Yamamoto, 1987; Moser et al., 1995). As depicted schematically in Figure 5A, these pre-mRNAs differ not only with respect to the branchpoint to 3′ splice site distance and base composition in this interval, but also span a fairly wide range of overall lengths; *S. pombe* introns are generally extremely small, with an average size of 44 nt (Zhang & Marr, 1994). The

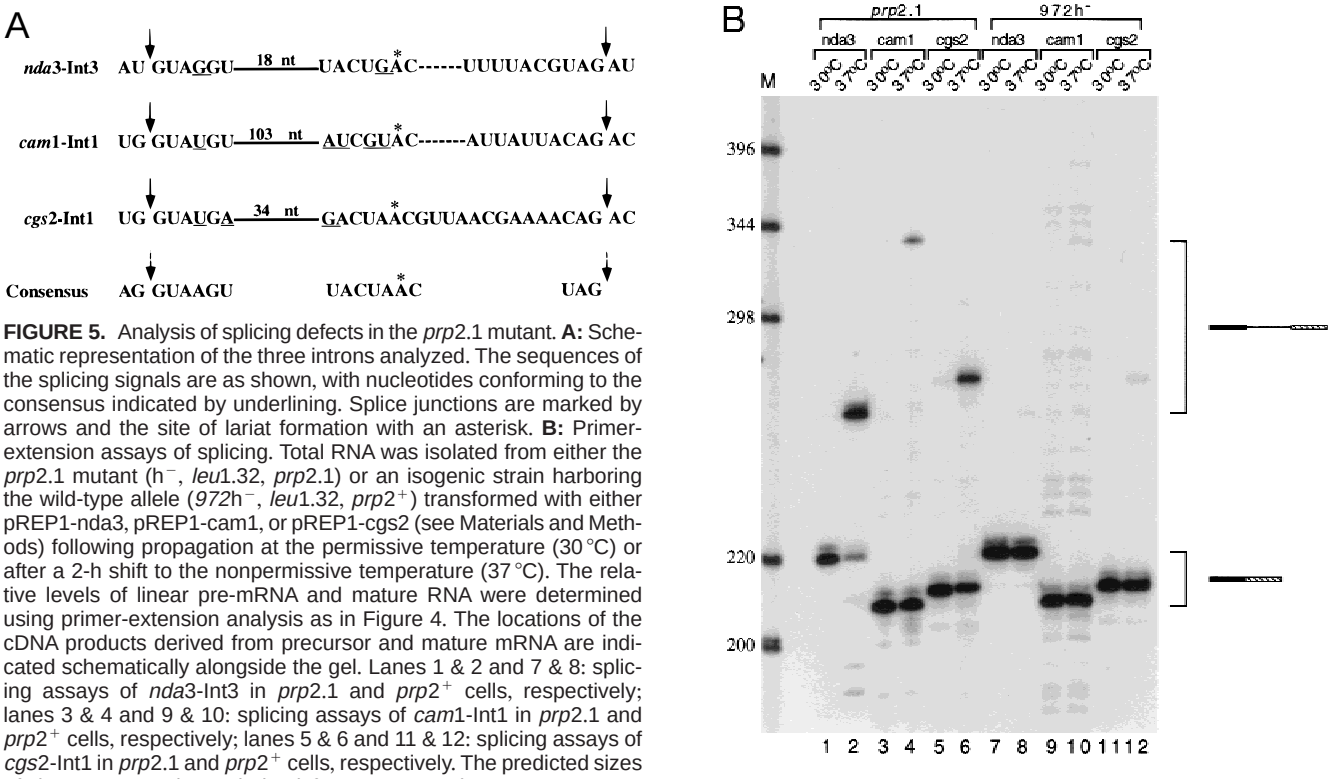

FIGURE 5. Analysis of splicing defects in the *prp*2.1 mutant. **A:** Schematic representation of the three introns analyzed. The sequences of the splicing signals are as shown, with nucleotides conforming to the consensus indicated by underlining. Splice junctions are marked by arrows and the site of lariat formation with an asterisk. **B:** Primer-extension assays of splicing. Total RNA was isolated from either the *prp*2.1 mutant (h$^-$, *leu*1.32, *prp*2.1) or an isogenic strain harboring the wild-type allele (*972*h$^-$, *leu*1.32, *prp*2$^+$) transformed with either pREP1-nda3, pREP1-cam1, or pREP1-cgs2 (see Materials and Methods) following propagation at the permissive temperature (30 °C) or after a 2-h shift to the nonpermissive temperature (37 °C). The relative levels of linear pre-mRNA and mature RNA were determined using primer-extension analysis as in Figure 4. The locations of the cDNA products derived from precursor and mature mRNA are indicated schematically alongside the gel. Lanes 1 & 2 and 7 & 8: splicing assays of *nda*3-Int3 in *prp*2.1 and *prp*2$^+$ cells, respectively; lanes 3 & 4 and 9 & 10: splicing assays of *cam*1-Int1 in *prp*2.1 and *prp*2$^+$ cells, respectively; lanes 5 & 6 and 11 & 12: splicing assays of *cgs*2-Int1 in *prp*2.1 and *prp*2$^+$ cells, respectively. The predicted sizes of the cDNA products derived from pREP1-nda3 are: precursor, 261 nt; mature, 220 nt; and lariat intermediate, 116 nt; from pREP1-cam1: precursor, 333 nt; mature, 206 nt; and lariat intermediate, 139 nt; and and from pREP1-cgs2: precursor, 270 nt; mature, 209 nt; and lariat intermediate, 60 nt.

results of primer-extension splicing assays performed on RNA isolated from the *prp*2.1 mutant propagated at either the permissive or the nonpermissive temperature (Fig. 5B) indicate that splicing of all three introns tested is inhibited following the shift to high temperature, as evidenced by the accumulation of precursor at the expense of mature mRNA seen in Figure 5B, lanes 2, 4, and 6. Thus, in this mutant, as in *prp*2.9, the participation of U2AF^{59} in splicing in vivo is not restricted to introns with pyrimidine-rich 3′ ends. Of the three introns examined here, splicing of *nda*3-Int3, which contains the most pyrimidine-rich 3′ end (five uridines and one cytosine in the 8 nt between the branchpoint consensus and 3′ splice site; Fig. 5A), is the most dramatically impaired (Fig. 5B, lane 2), similar to what was observed in the *prp*2.9 mutant. One possible interpretation of this result is that a 3′ polypyrimidine tract, when present, is recognized by the fission yeast U2AF large subunit in vivo, as in mammalian extracts in vitro. On the other hand, for the other two introns analyzed in this experiment, the magnitude of the splicing defect observed following the temperature shift is not strictly proportional to 3′ pyrimidine content (Fig. 5B, compare lanes 4 and 6). In the case of *cgs*2-Int1, which contains a purine-rich 3′ end (8 of 11 nt between the branchpoint consensus and 3′ splice site are G or A; Fig. 5A), precursor accumulation is more significant than for *cam*1-Int1, which contains a balanced 3′ end (three purines and four pyrimidines between the branchpoint consensus and 3′ splice site; Fig. 5A). The sensitivity of *cgs*2 and *cam*1 splicing to *prp*2 mutations suggests that U2AF^{59}, and not a distinct factor with different binding specificity, participates in excision of *S. pombe* introns lacking strong 3′ pyrimidine tracts. This point is underscored and extended by the data described in the next section.

As noted above, Western blot analysis indicates that U2AF^{59} levels remain quite constant in the *prp*2.1 strain as well as in *prp*2.9. Nevertheless, we were concerned that the splicing defects observed might still be indirect, for example, arising as a consequence of heat shock or some other response to elevating the growth temperature. To exclude this possibility, we tested the effect of shifting an isogenic *prp*2^{+} (972h^{-}) culture to 37 °C. In contrast to the effect of the temperature shift on the *prp*2.1 mutant, no significant precursor accumulation was observed in the wild-type strain for any intron tested (Fig. 5B, compare lanes 7, 9, and 11 with 8, 10, and 12). Moreover, all three introns are spliced efficiently when the mutant strain is propagated at a temperature permissive for growth (Fig. 5B, lanes 1, 3, and 5). Finally, because the *prp*2.1 cells were still growing exponentially at the time they were harvested from the 37 °C cultures (Fig. 3), it is unlikely that the inhibition of splicing is attributable to cell death. In summary, the data presented in this and the preceding section indicate that, while introns with pyrimidine-rich 3′ ends show the most severe splicing defects upon shifting to nonpermissive conditions, splicing of introns that lack strong polypyrimidine tracts is also impaired in the mutants.

Splicing of *cdc*2-Int2 is abolished upon U2AF^{59} inactivation irrespective of its 3′ pyrimidine content

To further explore the relationship between 3′ pyrimidine content and U2AF^{59} dependence in vivo, we took advantage of a series of mutant alleles previously constructed in the second intron of the *cdc*2 gene (Romfo & Wise, 1997); the 3′ sequences of the polypyrimidine tract variants analyzed here are shown in Figure 6A. This intron had been selected for our earlier studies because it contains the longest uninterrupted run of pyrimidines between the branchpoint and 3′ splice site of any intron sequenced to date in fission yeast (Zhang

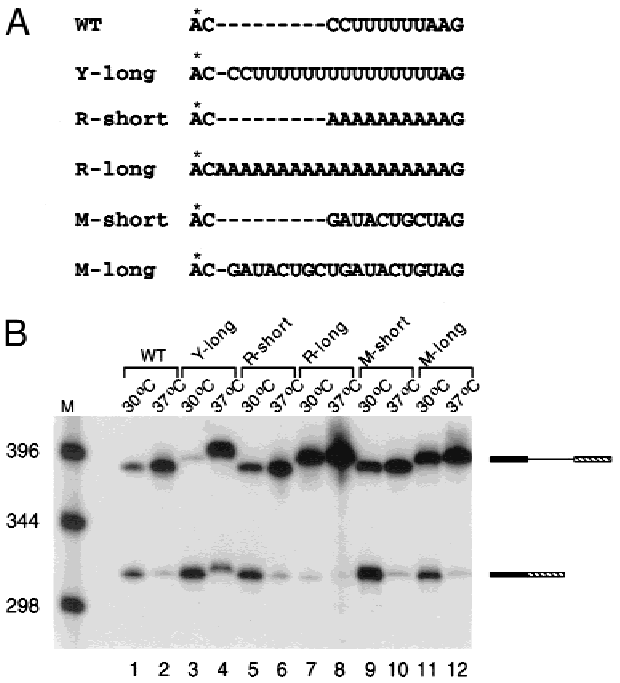

FIGURE 6. Primer-extension assays of splicing for *cdc*2-Int2 polypyrimidine tract variants in the *prp*2.1 strain. **A**: Splicing was assayed for the indicated *cdc*2-I2 alleles (Romfo & Wise, 1997) as described in the legend to Fig. 5. **B**: Lanes 1 and 2: splicing assays of the wild-type (WT) second intron of *cdc*2. Lanes 3 and 4: splicing assays of a mutant with a long branchpoint to 3′ junction distance containing exclusively pyrimidines (Y-long). Lanes 5 and 6: splicing assays of a mutant in which the polypyrimidine tract was replaced with purines (R-short). Lanes 7 and 8: splicing assays of a mutant with a long branchpoint to 3′ junction distance containing exclusively purines (R-long). Lanes 9 and 10: splicing assays of a mutant in which the polypyrimidine tract was replaced with an unbiased mixture of nucleotides (M-short). Lanes 11 and 12: splicing assays of a mutant in which the branchpoint to 3′ junction was doubled and the interval filled with an unbiased mixture of nucleotides (M-long). The sizes of the cDNA products corresponding to precursor for the "long" variants is 397 nt.

& Marr, 1994; J.A. Wise & C.M. Romfo, unpubl. observations). We reported earlier (Romfo & Wise, 1997) that both the branchpoint-to-3′ splice site distance and the pyrimidine content in this interval are critical in determining the splicing efficiencies of *cdc*2-Int2 variants in a wild-type background; these results are recapitulated for the *prp*2.1 mutant propagated at the permissive temperature in the odd-numbered lanes of Fig. 6B. Upon shifting this strain to the nonpermissive temperature, splicing of *cdc*2-Int2 containing its natural 3′ end sequence is dramatically impaired, (Fig. 6B, compare lanes 1 and 2). Thus, as in *prp*2.9, this intron is highly sensitive to mutational inactivation of U2AF^{59} in vivo.

Among the *cdc*2-Int2 pyrimidine tract variants, we were particularly interested in the response of the R-short allele, in which the natural cdc2-Int2 polypyrimidine tract has been replaced with a run of adenosines. A priori, it seemed possible that the *prp*2.1 mutation would not affect splicing in this case because of the absence of a strong U2AF^{59} binding site. Counter to this notion, while a signficant amount of mature mRNA is produced by R-short at the permissive temperature, its splicing is almost completely abolished following the temperature shift, similar to wild-type *cdc*2-Int2. This observation reinforces the conclusion that a different splicing factor is unlikely to replace U2AF^{59} during splicing of introns with purine-rich 3′ ends. Another very interesting allele is Y-long, in which the length of the polypyrimidine tract has been doubled. In this case, it might be anticipated that an extensive polypyrimdine tract, by providing a stronger binding site for the splicing factor, could at least partially compensate for the impaired function of the mutant protein. However, splicing of the Y-long variant is also nearly eliminated at 37 °C (Fig. 6B, lane 4). The remaining polypyrimidine tract variants, which produce varying amounts of mature mRNA at the permissive temperature, also remain unspliced following a shift of the *prp*2.1 mutant to nonpermissive conditions (Fig. 6B, compare lanes 6, 8, 10, and 12 with 5, 7, 9, and 11). In aggregate, our results with *cdc*2-Int2 suggest that the dependence of a particular intron on the *prp*2 gene product is unaffected by changes in its pyrimidine content.

DISCUSSION

In the work presented here, we have exploited "reverse" *S. pombe* genetics to investigate the relevance in vivo of insights gained through biochemical analysis of mammalian U2AF^{65} in vitro, and to initiate a structure/function analysis of the fission yeast protein. Although U2AF^{59} is remarkably resistant to subtle point mutations, we did identify one new conditional allele with growth and RNA accumulation properties distinct from those of the original mutant. While our results indicate that the essential role played by fission yeast U2AF^{59} in vivo cannot be fulfilled by its human counterpart, we find that, like U2AF^{65}, the *S. pombe* U2AF large subunit affects splicing prior to the first transesterification reaction.

U2AF^{59} is refractory to mutational perturbation

As noted in Results, we anticipated that charged-to-alanine scanning mutagenesis of U2AF^{59} would be fruitful, since its mammalian counterpart has a large number of interacting partners, and *S. pombe* homologs of many of these factors have already been identified either through two-hybrid analyses (McKinney et al., 1996; Wentz-Hunter & Potashkin, 1996) and/or the genome sequencing project (www.sanger.ac.uk/Projects/S_pombe/). Clearly, this prediction was not borne out by our results. In the case of the R199A/R200A (*prp*2.7) mutant, which affects the putative binding site of fission yeast UAP56, we have shown that the region targeted is in fact functionally important, since a multiple point mutant that more dramatically alters these and surrounding amino acids, is lethal. It is possible that the more subtle charged-to-alanine mutant has no phenotypic consequences because it does not affect UAP56 binding. A more likely possibility, however, is that the changes produce only a partial loss of function that is compensated by interactions between UAP56 and macromolecules other than U2AF^{59} within the prespliceosome. The existence of redundant interactions would have the advantage of ensuring efficient formation of early splicing complexes on pre-mRNAs that lack the recognition sites for one or more components of such complexes, as discussed further below.

One U2AF^{59} binding partner that was not targeted by our charged-to-alanine mutagenesis is the small subunit of fission yeast U2AF, due simply to the lack of adjacent charged residues in the hinge region, its site of contact with the large subunit (Wentz-Hunter & Potashkin, 1996). We (S. Lakhe-Reddy, C.M. Romfo, & J.A. Wise, unpubl. observations) and others (Rudner et al., 1998b) have now identified several critical amino acids within this region. The sites of interaction for other proteins that bind the large subunit such as SAP155 and SF1/BBP have not yet been precisely mapped but lie in the C-terminal pseudo-RRM (Abovich & Rosbash, 1997; Gozani et al., 1998; Rain et al., 1998). Two of our phenotypically inert charged-to-alanine mutants (D429A/R430A and E438A/D439A) are located in this domain (see Fig. 1B), and it will be interesting to determine whether, as for R199A/R200A, more dramatic changes in the conserved peptides that surround the sites targeted confer conditional or absolute lethality.

Molecular basis of the *prp*2.1 and *prp*2.9 phenotypes

As noted in Results, the amino acid substitutions in the two temperature-sensitive U2AF^{59} mutants analyzed

here do not appear to destabilize the proteins at high temperature, and thus it is likely that their effects on RNA metabolism reflect alterations in RNA binding or protein–protein interactions. The contrasting growth and RNA accumulation profiles of the mutant strains implies different underlying molecular defects. First, the rapid cessation of growth in *prp*2.1 suggests that preexisting protein containing the C387Y mutation is inactivated upon elevating the temperature, whereas the long lag before growth stops in *prp*2.9 may indicate that only newly synthesized protein containing the E256A/D257A substitutions is inactive at 37 °C. These phenotypic differences may reflect distinct effects on the structure of U2AF^{59}. The original *prp*2.1 mutation, C387Y, is located just beyond the C-terminus of the second RRM in fission yeast U2AF^{59} (Fig. 1B); the corresponding amino acid in *C. elegans* U2AF^{65} is also cysteine, whereas the human and *Drosophila* proteins contain a conservative change to serine at this position (Zamore et al., 1992; Kanaar et al., 1993; Zorio et al., 1997). Because, in RRM proteins known to contact RNA, this region lies near the substrate binding face (Oubridge et al., 1994, and references therein), it is possible that introduction of a bulky tyrosine might alter the specificity or affinity of U2AF^{59} for RNA. Again assuming that the U2AF^{59} RRMs adopt structures similar to members of this superfamily for which three-dimensional models are available (Nagai et al., 1990; Wittekind et al., 1992; Oubridge et al., 1994), the mutations in the newly isolated conditional allele, E256A/D257A, are predicted to lie on the side of the protein opposite from the RNA-binding surface (reviewed in Burd & Dreyfuss, 1994). The conservation from fission yeast to man (Fig. 1B) of a pair of adjacent acidic residues within a generally quite variable region of the RRM (reviewed in Birney et al., 1993) implies that they play a critical role in either the structure or function of the protein. One possibility is that these negatively charged amino acids participate in an interaction between the first RRM and another segment of the protein, an appealing notion in light of the biochemical phenotype of the E256A/D257A strain. Specifically, a misfolded protein might be completely unable to form stable complexes with the pre-mRNA, which could explain the defect in RNA accumulation observed in this mutant. Determining whether these mutations disrupt an interdomain interaction will require information about the orientations of the RRMs relative to each other and to the RS/Hinge region, which is as yet unavailable.

Regardless of their structural basis, it is likely that mutations in the *prp*2 gene cause temperature-sensitive growth via direct effects on splicing and not as an indirect consequence of an impairment in some other cellular process(es). Concern about this issue arises principally because, as noted in the Introduction, a conditional mutant that maps to the *prp*2 gene was isolated in a screen for cells with chromosome segregation defects (Takahashi et al., 1994). We consider it unlikely that the splicing defects in the *prp*2.1 mutant are a secondary effect of impaired DNA metabolism primarily based on the fact that they can be observed after a relatively brief incubation under nonpermissive conditions. In this strain, dramatic pre-mRNA accumulation is observed less than half a generation after the temperature shift (Figs. 5 and 6; Potashkin et al., 1989, 1993); thus, it seems more likely that in *prp*2.3, which also shows a splicing defect (J. Potashkin, pers. comm.), disrupting this process perturbs nuclear architecture, which in turn causes altered chromosome segregation. A second possible concern, that mutations in the *prp*2 gene exert primary effects only on splicing of U6 snRNA, which contains an mRNA-like intron in *S. pombe* (Tani & Ohshima, 1989), seems unlikely because the onset of U6 and pre-mRNA splicing defects after shifting the *prp*2.1 strain to high temperature are coincident (Potashkin & Frendewey, 1989; Potashkin et al., 1989).

Fission yeast and human U2AF large subunits are not interchangeable

In light of the structural similarities between fission yeast U2AF^{59} and human U2AF^{65}, and the fact that the *Drosophila* and human large subunits appear to be interchangeable, at least in vitro (see Introduction), it was of obvious interest to determine whether the human protein could functionally substitute for the *S. pombe* ortholog in vivo. It was previously reported that transformation of the *prp*2.1 strain with a human U2AF^{65} cDNA weakly rescued its ability to grow at high temperature (Potashkin et al., 1993); however, because this point mutation is potentially suppressible and also may not produce a complete loss of U2AF^{59} activity, complementation of a null allele clearly provides a more rigorous test of functional conservation. Despite considerable effort, we found no conditions under which human U2AF^{65} could rescue growth of fission yeast cells lacking an endogenous source of U2AF^{59}. Although we cannot rule out differences in experimental protocol to explain the discrepancy with the earlier work, our observation that expression of even low levels of the human protein in *S. pombe* interferes with growth strongly suggests that there may be another explanation for the apparent complementation previously reported. One possibility is that, during growth of the *prp*2.1 strain at the permissive temperature, spontaneous second-site mutations may have emerged that allowed subsequent growth at high temperature.

While we do not yet know the precise molecular basis of the dominant negative effects of human U2AF^{65} in *S. pombe*, our data do suggest that the toxicity is meaningful, since a modest growth defect is also observed when the native fission yeast protein is present in excess. At a minimum, these results imply that the relative stoichiometries of splicing factors is quite crit-

ical in fission yeast (see also Alvarez et al., 1996). Based on the fact that the segment responsible for dominant interference lies in the C-terminal rather than the N-terminal portion of the protein, we can envision two general underlying mechanisms: (1) human U2AF^{65} may titrate one or more fission yeast splicing factors that interact with the C-terminal RRMs (see Introduction), or (2) U2AF^{65} may block access of native U2AF^{59} to the polypyrimidine tract of one or more introns located in essential genes. Because runs of pyrimidines of sufficient length to support high affinity binding of U2AF^{65} (Zamore et al., 1992) are as yet unknown in *S. pombe* (Zhang & Marr, 1994; Romfo & Wise, 1997), we favor the former alternative.

Diverse introns require U2AF^{59} for splicing in vivo

At the outset of our work on U2AF^{59}, we considered it entirely possible that splicing of the many fission yeast introns lacking an extended run of pyrimidines between the branchpoint and 3′ splice site might not require this splicing factor. Precedent for such a scenario is set by the finding that two *S. cerevisiae* splicing factors, Slu7p and Prp18p, are required for splicing only of introns with long branchpoint to 3′ splice site distances (Jones et al., 1995; Zhang & Schwer, 1997). Counter to this notion, we find that splicing of a diverse array (and thus, more than likely, all) introns in this organism is inhibited upon mutational inactivation of U2AF^{59}. Moreover, the requirement for functional U2AF^{59} to splice *cdc*2-Int2 persisted even after replacement of its pyrimidine tract with purines. Taken together, these observations render unlikely the possibility that a distinct factor with a different binding specificity replaces U2AF^{59} during splicing of *S. pombe* pre-mRNAs lacking strong polypyrimidine tracts. Rather, features other than, or in addition to, the 3′ polypyrimidine tract must facilitate recruitment of this protein to the substrate. One possibility is that fission yeast U2AF^{59} also recognizes the 3′ AG dinucleotide that, like the polypyrimidine tract, is required prior to the first transesterification reaction in this organism (Romfo & Wise, 1997). Consistent with this idea, an early study with partially purified human U2AF indicated a preference for substrates containing an intact 3′ splice site (Ruskin et al., 1988). In addition, there is evidence to suggest that the short polypyrimidine tract and 3′ splice site are recognized as a unit by U2AF^{65} in *C. elegans* (Zorio et al., 1997). An alternative, but not exclusive, scenario is that U2AF^{59} does not directly contact the 3′ ends of introns lacking a strong polypyrimidine tract, but rather is tethered to the pre-spliceosome by interactions with other splicing factors such as SF1/BBP (see Introduction). This notion is also appealing for *S. cerevisiae* Mud2p, which displays conservation of the pseudo-RRM that is involved in protein–protein interactions, but not of the two classical RRMs that resemble proteins known to directly contact RNA (Abovich et al., 1994). The lack of a role for sequences downstream from the branchpoint prior to the first transesterification reaction in *S. cerevisiae* (Patterson & Guthrie, 1991; Rymond & Rosbash, 1992) is in keeping with this model.

While all splicing events examined here appear to have at least some requirement for functional U2AF^{59}, our data also suggest that a polypyrimidine tract is relevant to the participation of this protein in splicing of pre-mRNAs in vivo. In particular, the second intron of the *cdc*2 gene, which contains exclusively pyrimidines between the branchpoint and 3′ splice site, displayed the most dramatic splicing defect of any intron tested in both temperature-sensitive mutants analyzed (Figs. 4, 5, and 6). We have shown elsewhere that the relatively inefficient splicing of wild-type *cdc*2-Int2 is a consequence of several factors including its relatively large size for this organism (C.M. Romfo, C.J. Alvarez, & J.A. Wise, in prep.), the presence of a stem-loop structure encompassing the 5′ splice site (Alvarez et al., 1996), and a nonconsensus nucleotide at position −3 relative to the 3′ exon/intron boundary (Romfo & Wise, 1997). To compensate for these deficiencies, *cdc*2-Int2 may be under selective pressure to maintain a strong polypyrimidine tract to ensure efficient recruitment of U2AF^{59} and thereby guarantee its entry into the spliceosome assembly pathway. At the other extreme of U2AF^{59} dependence is the calmodulin pre-mRNA. Although splicing of *cam*1-Int1 has not been as extensively investigated as that of *cdc*2-Int2, it may be relevant that this intron is also quite impervious to 5′-splice-site mutations that completely abolish splicing of other pre-mRNAs in *S. pombe* (C.J. Alvarez & J.A. Wise, in prep.). Because 5′- and 3′-splice-site recognition by the U1 snRNP and U2AF are believed to occur within the same pre-splicing complex (Abovich & Rosbash, 1997, and references therein), the *cam*1 pre-mRNA must possess additional features that ensure efficient assembly of this complex; these are not obvious by inspection.

In conclusion, the data presented in this report extend our understanding of the role played by the large subunit of U2AF in splicing. We anticipate that further analysis of the mutants identified here, as well as others, will bring the picture into sharper focus.

MATERIALS AND METHODS

Plasmid construction and mutagenesis

To generate a null allele of *prp*2 in which most of the coding sequence was replaced by the *ura*4 gene, it was first necessary to produce a version of the selectable marker with *Pst*I sites on both ends. To this end, a 1.8-kb *Hin*dIII fragment carrying the *ura*4 gene was inserted into the same site of pBluescriptSK+ followed by digestion of this plasmid with *Bam*HI and *Sal*I to liberate the marker, which was subcloned

into pTZ19R digested with the same enzymes, generating pTZ19-ura4. Second, a recipient plasmid lacking a *Pst*I site in the polylinker was prepared by digestion of pBluescript with *Eco*RI and *Not*I, filling in the overhanging ends with Klenow fragment, and religating the backbone, generating pBS-Pst. The 3.3-kb *Sac*I genomic fragment containing the *prp*2 gene (Potashkin et al., 1993; a generous gift of Dr. J. Potashkin) was subcloned into the *Sac*I site of pBS-*Pst*I to generate the plasmid pBS-prp2. Finally, the 1.8-kb *Pst*I fragment carrying the selectable marker from pTZ19-ura4 was used to replace a 960-nt *Pst*I fragment in PBS-prp2 to generate the disruption/integration plasmid, pBS-prp2::ura4.

Mutagenesis of the *prp*2 gene carried on the *S. pombe* shuttle vector pIRT3 (Potashkin et al., 1993) was carried out with reagents supplied commercially (Amersham Corp., Arlington Heights, IL) using the following oligonucleotides:

E29A/R30A: 5′ CCCACCCGAACCAGCAGCTCGTCTA GGTTCT 3′
R105A/E106A: 5′ CCTTACGCTTCTAGCAGCGCTGGGA GGTGGAG 3′
R199A/R200A: 5′ CCAGTAACCACTAGAGCAGCAGCCT GTCTGCTAG 3′
E212A/D213A: 5′ GATAAATGATACAAAAGCAGCAGCAA CGAATTCGTTAGG 3′
F247D/I249D: 5′ CGACCTCAAGATCAGCATCGTTTTCT TCTTTACAG 3′
E256A/D257A: 5′ GAGGAAGGTGGCAGCAGCAGGAGT AGCGAC 3′
D310A/K311A: 5′ AATTTGAAATGTAAATAGCAGCTTTTG AATCTAAAACG 3′
E323A/D324A: 5′ CTCCACCACTTGAGCAGCGCCTAAA TTTAAAGGTA 3′
F353D/F355D: 5′ AGGATTTTTAAATTCGCAATCACAAT CACCCTTCGAAGATC 3′
C387Y/S: 5′ TTGATTGAGACCTACGXAAGCAAATT GTGCGTG 3′ (X = 50% T and 50% G)
D429A/E430A: 5′ CTTGTACATCCATAATAGCAGCCCCA GTAATTAAATTATG 3′
E438A/D439A: 5′ CAGACTCATATATAGCAGCATATTCTT GTACATCC 3′
F476D/Y479D: 5′ GATATCGGAGTCTCGTACATCAACCT TTCCAGTTC 3′
^{195}SRQARR/VALAEE^{200}: 5′ TCCAGTAACCACTAGCTCC TCAGCAAGTCTGACAGCACC TGGTTGTAAG 3′.

The identities of all mutations were confirmeed by sequencing with the neighboring downstream mutagenic primer except in the case of E29A/R30A, which was confirmed with the sequencing oligonucleotide E29R30Seq (5′ GTCTCGGC GACGTCTTC 3′) and E438A/D439A and F476D/Y479D, which were confirmed using the primer Prp2-Bam (5′ CCT TTTCATGGATCCTGAAATCACC 3′).

The coding region of the *prp*2 gene was placed under control of the *nmt*1 promoter by first introducing an *Nde*I site at its start codon and a *Bam*HI site just downstream from the stop codon via site-directed mutagenesis with the oligonucleotides Prp-Nde (5′ CAAATCCATATGAGTGAATGAATATG 3′, *Nde*I site underlined) and Prp-Bam (5′ CCTTTTCAT GGATCCTGAAATCACC 3′, *Bam*HI site underlined). The *Nde*I/*Bam*HI fragment was then inserted between the same sites of the plasmid pREP2 (Maundrell, 1990) to generate the plasmid pREP2-prp2. Similarly, to place the coding sequences of the large and small subunit of human U2AF (kindly provided by Drs. J. Valcarcel and M. Green) under control of the *nmt*1 promoter, *Nde*I sites (underlined) were first introduced at each start codons via site-directed mutagenesis with the oligonucleotides U2AF65-Nde (5′ GGCCGCCTCCATATG GTCGGACTTCG 3′) and U2AF35-Nde (5′ CTCCGCCATATG CCACCC 3′). Next, *Bam*HI sites (underlined) were inserted just downstream of each stop codon via site-directed mutagenesis with the oligonucleotides U2AF65-Bam (5′ CGGG ACTTCTGGATCCGGCGGCTGGGG 3′) and U2AF35-Bam (5′ GGTAAAAATGGGATCCCTCAGAATCGC 3′). Finally, the NdeI/*Bam*HI fragments were inserted between the same sites of pREP1 and pREP2 (Maundrell, 1990) to generate the plasmids pREP1-hU2AF65 and pREP2-hU2AF35, respectively.

To replace the RS/Hinge regions of fission yeast and human U2AF^{65} with *Kpn*I sites, we used site-directed mutagenesis with the oligonucleotides Prp2-ΔRS/hinge (5′ CACTAG TCGACGAGCCTGTCTGCTGGTACCATCCATATGTTAACA AAGCGACTATAAG 3′, *Kpn*I site underlined) and hU2AF-ΔRS/hinge (5′ GTAGAGGCGCCGGGCTTGTCTGGTACCCG ACATATGTTAACAAAGCGACTATAAG 3′, *Kpn*I site underlined), generating the plasmids pREP2-Prp2ΔRS/hinge and pREP2-hU2AFΔRS/hinge. To swap RS/Hinge domains, the appropriate regions of each coding sequence were amplified by PCR with the 5′ primers Prp2RS5′ (5′ CGCTTTGTTAA CATATGGATTTGTCT 3′) and hU2AFRS5′ (5′ CGCTTTGT TAACATATGTCGGACTTC 3′); *Nde*I sites underlined and the 3′ primers Prp2RS3′ (5′ GGGGGTACCTGGTTGTAAGGGC GGAGGGG 3′) and hU2AFRS3′ (5′ CGCGGTACCCTGGC TCCCGACCACGGGCAC 3′); *Kpn*I sites underlined.

To construct pREP1-cdc2, a 272-nt *Nde*I/*Bam*HI fragment containing the second intron of the fission yeast *cdc*2 gene together with its flanking exon sequences was subcloned from pREP2-cdc2 (Romfo & Wise, 1997) into the same sites of pREP1 (Maundrell, 1990). Similarly, pREP1-nda3 was constructed by moving a 150-nt *Nde*I-*Bam*HI fragment carrying the third intron of the *nda*3 (*β*-tubulin) gene together with its flanking exons from pREP2-nda3 (Alvarez et al., 1996) into pREP1 (Maundrell, 1990). To generate pREP1-cam1, the single intron from the *cam*1 (calmodulin) gene (Takeda & Yamamoto, 1987; Moser et al., 1995) and surrounding exonic sequences were amplified by PCR with the primers cam5′ (5′ CTGATTTTACCAGCATATGGTATGTTTATTAT 3′, *Nde*I site underlined) and cam3′ (5′ CCAATTCATTGGATCCGATAT TTCC 3′, *Bam*HI site underlined) and inserted between the *Nde*I and *Bam*HI sites of pREP1 (Maundrell, 1990). Construction of cgs2-Int1 is described elsewhere (C.M. Romfo, C.J. Alvarez, & J.A. Wise, in prep.).

To make expression constructs carrying the *adh* promoter and an *ade*6 selectable marker for *cdc*2-Int2, *nda*3-Int3, and *cgs*2-Int1, we first replaced the 2.2-kb LEU2 *Hin*dIII fragment in pART3 (Kelly et al., 1988) with the 3.0-kb *ade*6 *Hin*dIII fragment from pAD3 (Althoff et al., 1994) to create the plasmid pAD4. Next, *Nde*I-*Sac*I fragments from pREP2-cdc2, pREP1-nda3, and pREP1-cgs2, which contain intron and surrounding exon sequences from the indicated gene as well as the polyadenylation region of the *nmt*1 gene (Maundrell, 1990), were inserted into the *Nde*I and *Sac*I sites of pAD4. Finally, to replace the *adh* promoter and a portion of the *ade*6 marker

that were deleted in the last cloning step, a 2.0-kb *Nde*I fragment from pAD4 was inserted into the *Nde*I site of each of these plasmids to generate pAD4-cdc2, pAD4-nda3, and pAD4-cgs2.

Generation of the polypyrimidine-tract mutations in the second intron of *cdc*2 has been described previously (Romfo & Wise, 1997).

S. pombe manipulations

To construct a diploid strain to use as a host in the disruption of the *prp*2 gene, the two haploids SpDS1 and SpDS2 were crossed to produce the diploid SpDS3 (*ade6-M210/ade6-M216, leu1-32/leu1-32, ura4-d18/ura4-d18*). The SpDS2 strain (h$^+$, *ade6-M210*, *leu1-32*, *ura4-d18*) was constructed as described previously (Reich et al., 1992). To generate SpDS1, the corresponding haploid of opposing mating type (h$^-$, *ade6-M216*, *leu1-32*, *ura4-d18*), the strains FYC15 (h$^+$, *ade6-M216*) and FYC23 (h^{90}, *leu1-32*, *ura4-d18*), obtained from the Cold Spring Harbor Fission Yeast Course, were crossed. The DS3 diploid, which can be stably maintained through interallelic complementation at the *ade*6 locus (Alfa et al., 1993) was transformed with the linear, gel purified 5.1-kb *Sac*I fragment from pBS-prp2::ura4 containing the disrupted gene by the lithium acetate method. Screening for stable integrants and random spore analysis were performed essentially as described earlier (Porter et al., 1990).

To confirm that gene replacement had occurred at the correct location, Southern blot analysis was performed on genomic DNA isolated from four diploids that passed the stability test. The DNA was digested for 4 h at 37 °C with *Pvu*II and *Stu*I and restriction fragments resolved on a 0.7% agarose gel, transferred to nitrocellulose, and probed with a plasmid carrying full-length wild-type *prp*2, labeled by random priming. If the fragment has correctly integrated at the *prp*2 locus, digestion with *Pvu*II and *Stu*I should produce two bands, 1.2 kb and 2.0 kb in length, whereas the wild-type *prp*2 locus will produce a single 2.4-kb band. Subsequent complementation analyses were conducted using the diploid strain SpCR1, which is heterozygous for gene disruption at the *prp*2 locus.

Complementation assays with wild-type or mutant *prp*2 genes were performed by transforming SpCR1 with plasmids carrying the appropriate alleles. Sporulation and ascal wall digestion were performed as described previously (Liao et al., 1989). Spores were plated on EMM2 supplemented with uracil and adenine (100 mg/L each); haploid colonies were patched onto the same selective plate, which was used as a template for replica plating to test for leucine and/or uracil prototrophy as well as ploidy (Althoff et al., 1994).

To assay the effects of overexpressing *prp*2 and human U2AF as well as the domain-swap constructs, the haploid SpDS2 was transformed via the lithium-acetate method and plated on EMM2 supplemented with leucine + adenine (100 mg/L each) and thiamine (final concentration 2 μM). Transformants were restreaked on the same media with and without thiamine to score for growth upon derepression of the *nmt*1 promoter.

For splicing assays, an appropriate fission yeast strain was transformed by the lithium-acetate method using approximately 1 μg of purified plasmid DNA, which yielded $\sim 10^5$ colonies. The effect of the E256A/D257A mutation on splicing of *cdc*2-Int2, *nda*3-Int3, and *cgs*2-Int1 was assayed in a haploid derived from SpCR1, which carries the chromosomal disruption allele covered by the mutant *prp*2 gene on a *leu2* plasmid. The *prp*2.1 *leu*1.32 strain (a generous gift of Dr. J. Potashkin; Potashkin et al., 1993) was the recipient for assaying the effect of the C387Y mutation on the splicing of *cdc*2-Int2, *nda*3-Int3, *cam*1-Int1, and *cgs*2-Int1.

Plasmids were recovered from fission yeast charged-to-alanine mutants as previously described (Hoffman & Winston, 1987).

RNA preparation and analysis

To assay the effects of the two temperature-sensitive mutants of *prp*2 on splicing at the elevated temperature, cells were grown in selective minimal media to approximately mid-log phase at the permissive temperature (30 °C), at which time 100 O.D.$_{600}$ units of cells were harvested for preparation of RNA. The remaining cells were used to inoculate prewarmed selective minimal media followed by growth for various intervals at the nonpermissive temperature (37 °C) before cells were again harvested for RNA isolation. Preparation of RNA and primer-extension splicing assays of splicing were performed as described previously (Alvarez et al., 1996).

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