arose in the presence of 1 M KCl (*3*). U2AF activity was eluted in buffer containing 2 M KCl (*Drosophila* Kc cell nuclear extract) or 2 M guanidine hydrochloride (HeLa cell nuclear extract). These fractions were termed the eluate. Depletion of U2AF was checked by protein immunoblot analysis with the use of antibodies to dU2AF^{50} and pepC (*3*), to detect the large and small subunit of U2AF, respectively. Splicing reaction mixtures (25 μl) contained 20 mM Hepes-KOH (pH 8.0), 48 mM KCl, 4 mM $MgCl_2$, 3 mM ATP, 5 mM creatine phosphate, 0.1 mM EDTA, 0.25 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, RNasin (0.5 U/μl), BSA (25 μg/ml), 2.5% polyvinyl alcohol, 10% glycerol, and 45 μg of protein from nuclear extract.

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U2AF Homolog Required for Splicing in Vivo

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Several fission yeast temperature-sensitive mutants defective in pre-mRNA processing (*prp*$^-$ mutants) at the nonpermissive temperature have been identified. Here, the *prp2*$^+$ gene has been cloned by its ability to complement the temperature-sensitive growth defect of a *prp2*$^-$ mutant. The gene also corrects the pre-mRNA splicing defect of *prp2*$^-$ mutants and encodes a 59-kilodalton polypeptide (PRP2). A molecular characterization indicates that PRP2 is a previously uncharacterized yeast splicing factor with extensive similarity to the mammalian splicing factor U2AF^{65}. Thus, this study provides evidence that a U2AF homolog participates in RNA processing in vivo.

Removal of intervening sequences from pre-mRNA is a multistep process requiring the participation of numerous RNA and protein factors that make up a large complex termed the spliceosome (*1*). The small nuclear ribonucleoprotein (snRNP) particles that make up the spliceosome are composed of small nuclear RNAs and several polypeptides. In addition, several non-snRNP proteins are essential for splicing; the functions of these trans-acting factors are now being elucidated.

The basic mechanism of pre-mRNA splicing has been evolutionarily conserved (*2*). The yeast *Saccharomyces cerevisiae* model system has been utilized to identify numerous splicing factors through classical and molecular genetic approaches (*2*). Biochemical analysis of mammalian splicing systems has identified additional factors, but a role for most of these putative splicing components has not been established in vivo. One of these factors, U2 auxiliary factor (U2AF), is a non-snRNP protein identified in mammalian cells that is required in vitro for pre-spliceosome assembly (*3*). It has been suggested that U2AF functions to facilitate binding of U2 snRNP to the pre-mRNA, but no genetic evidence demonstrating the importance of U2AF in RNA processing in vivo has been obtained.

The fission yeast *Schizosaccharomyces pombe* is a useful model for the study of splicing because its gene structure is similar to that of metazoans; multiple introns are not unusual, and consensus sequences at the intron-exon borders and the branchsite sequence are similar to those of mammalian introns. In addition, SV40 small t-antigen pre-mRNA is spliced correctly in *S. pombe*, suggesting a strong evolutionary conservation of the splicing machinery (*4*).

In order to identify trans-acting splicing factors in fission yeast, we initiated a search for splicing mutants (*5*). Several *prp*$^-$ mutants were identified from collections of temperature-sensitive fission yeast strains (*5*, *6*). These mutants block pre-mRNA splicing in vivo at the nonpermissive temperature. One mutant, *prp2*$^-$, is defective in an early step of pre-mRNA splicing, before lariat formation (*5*). To clone the *prp2*$^+$ gene, we used a genomic DNA library to transform a *prp2.1 leu1.32* strain of *S. pombe*. Plasmid from one transformant that grew at 37°C carried a 13.6-kb insert. Instability of the plasmid after growth under nonselective conditions resulted in coordinate loss of prototrophy and the ability to grow at high temperature, indicating that the rescue of the high-temperature growth defect was due to plasmid-borne DNA. Fragments of the insert were subcloned into expression vectors to determine the smallest insert capable of rescuing the temperature-sensitive growth defect (Fig. 1). A 3.3-kb Sst I fragment complemented the growth defect, whereas smaller subclones did not.

In a study to determine whether the Sst I fragment contained the wild-type *prp2*$^+$ gene or a multicopy number suppressor, a leucine plasmid bearing the cloned fragment was integrated by homologous recombination into the chromosome. The plasmid was linearized within the open reading frame with Nhe I and transformed into an *h*$^+$ *prp2.1 leu1.32 ade6.210* strain. A stable clone that grew at 37°C on minimal medium plus adenine was isolated. Integration was confirmed by Southern (DNA) analysis (*7*). The integrant was mated with an *h*$^-$ *ura4.D18* strain. Random spore analysis showed that all *leu*$^+$ spores were *ts*$^+$, indicating a tight genetic linkage between the leucine marker and the *prp2* locus. The *ura4* and *ade6* markers recombined freely. These results indicate that the wild-type gene was cloned.

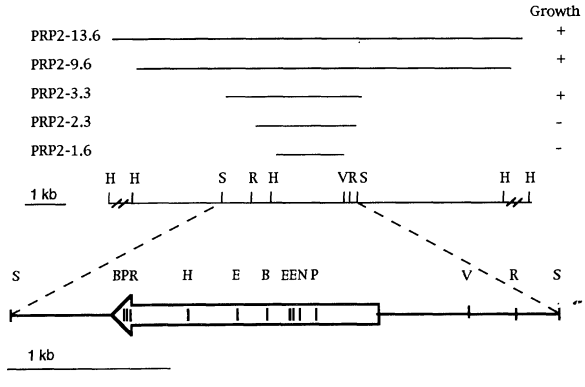

Fig. 1. Subcloning of the fragment containing the *prp2*$^+$ gene. Fragments of the 13.6-kb Hind III clone that rescues the temperature-sensitive growth defect of a *prp2*$^-$ strain were subcloned into fission yeast vectors. The numbers indicate the approximate length (in kilobases) of the restriction fragments containing the *prp2*$^+$ gene that were subcloned. Each plasmid was transformed into a *prp2*$^-$ strain and tested for ability (+) or inability (−) to grow at the nonpermissive temperature. The extent and direction of the *prp2*$^+$ open reading frame is indicated by the single broad arrow. B, Bgl II; E, Eco RI; H, Hind III; N, Nhe I; P, Pst I; R, Eco RV; S, Sst I; V, Pvu II. The original 13.6-kb Hind III fragment was partially digested with Hind III, and a 9.6-kb fragment was subcloned into the Hind III site of the yeast vector pWH5 (*20*) to produce pPRP2-9.6. A 3.3-kb Sst I fragment was inserted into the Sst I site of the expression vector pIRT3 (*20*) to create pPRP2-3.3. A 2.3-kb Eco RV fragment was inserted at the Sma I site of pIRT3 after end-filling with Klenow fragment to produce pPRP2-2.3. A 1.6-kb Hind III–Pvu II fragment was cloned into the Sma I site of pIRT3 after Klenow fragment treatment to produce pPRP2-1.6.

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To determine whether the *prp2*$^+$ gene complements the pre-mRNA splicing defect, we prepared RNA from *prp2*$^-$ mutants and transformants carrying pPRP2-3.3 after growth at either the permissive or nonpermissive temperature. An intron-specific oligonucleotide recognizing the fourth intron of the β-tubulin transcript was used to probe an RNA blot for accumulation of pre-mRNA. The temperature-sensitive mutant accumulated pre-mRNA at 37°C but not at 23°C (Fig. 2). In the transformants, there was no accumulation of precursor at either temperature, indicating that pPRP2-3.3 rescued the pre-mRNA splicing defect of the mutant. To verify that equivalent amounts of RNA were loaded in each lane, we reprobed the RNA blot with an exon-specific oligonucleotide that recognizes the last 15 nucleotides of exon 4 and the first 15 nucleotides of exon 5 of the β-tubulin transcript. Under stringent conditions, the exon-specific probe detected mRNA in the temperature-sensitive strain at the permissive temperature and the transformants grown at both temperatures.

The nucleotide sequence of the 3.3-kb Sst I fragment was determined by the dideoxy method from an exonuclease III deletion series (*8*). The largest open reading frame is 1575 nucleotides. If translation starts at the first ATG codon, the PRP2 protein is predicted to be 517 amino acids in length with a calculated molecular size of 59 kD and isoelectric point of 6.8.

A 787-nucleotide gap was created in the *prp2*$^+$ gene by removal of a Bgl II fragment (Fig. 1). The gapped molecule was used to clone the mutant allele by gap repair (*9*). The cloned *prp2*^{ts} gene did not complement the *ts*$^-$ phenotype of the *prp2.1* strain. A single nucleotide change was identified; the G at position 1160 was mutated to an A. As a result of this mutation, the predicted amino acid sequence would be changed from a cysteine (C) to a tyrosine (Y) residue (C387Y, Fig. 3).

Two regions of the predicted PRP2 protein sequence share similarity with motifs found in several RNA binding proteins. The NH_2-terminal region of PRP2 is arginine-serine–rich (Fig. 3). Of the first 111 amino acids, 31% are arginine and 17% are serine. Deletion and substitution experiments with the arginine-serine region from the *Drosophila su(w*^{a}*)* and *tra* proteins indicate that this motif is needed to direct these splicing factors to the subnuclear region that contains a concentration of splicing factors (*10*). Deletion studies on U2AF^{65} suggest that the arginine-serine–rich region is necessary for pre-mRNA splicing in vitro, but not for binding to pre-mRNA (*11*). Proteins with an arginine-serine–rich motif may play a role in the regulation of alternative splicing (*12*).

In the COOH-terminal region of the PRP2 protein there are three RNA recognition motifs (RRMs, Fig. 3). The RRMs are regions consisting of approximately 80 amino acids; their conserved primary structure contains two elements, RNP1 and RNP2, that have aromatic and basic residues (*13*). In addition to the conservation of primary structure, RRMs share a predicted β-α-β-β-α-β secondary structure. A Chou-Fasman analysis (*14*) of PRP2 predicts that the RRMs of PRP2 share this structure. The RRM region has been shown to be essential for the binding of several snRNP-specific proteins to their respective snRNA (*15*). The altered amino acid in the *prp2*$^-$ strain is between the second and third RRM.

A search for similar sequences in several databases revealed that PRP2 is 50% similar to the large 65-kD subunit of the mammalian splicing factor U2AF over the entire protein with the exception of the first 45 amino acids of PRP2, which extend beyond the initiating methionine of U2AF^{65} (Fig. 3). An alignment of the two sequences indicated that 28% of the amino acids are identical and 22% differ by conservative changes. The order of the NH_2-terminal arginine-serine–rich region and the COOH-terminal RRMs is identical in both proteins and opposite to that found in all other splicing factors that contain both motifs (*16*). The arginine-serine–rich region of both proteins is small and scattered, in contrast to the repetitive extensive region present in other splicing factors (*16*). The COOH-terminal region of both proteins contains three RRMs and shares 61% similarity that extends beyond the RRMs.

In a test for functional complementation, the human U2AF^{65} gene, subcloned adjacent to the SP6 and human β-globin promoters, was transformed into a *prp2.1* strain, and transformants were plated at 37°C. Transformants formed slow-growing colonies, indicating that there is weak rescue of the temperature-sensitive growth defect. Microscopic examination of the cells indicates that they are elongated and swollen as compared with a *prp2.1* strain

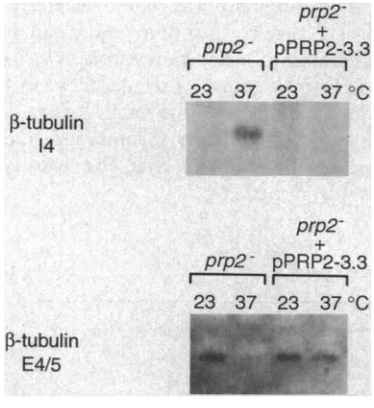

Fig. 2. Northern (RNA) blot analysis of the accumulation of β-tubulin pre-mRNA in a *prp2*$^-$ strain and mutants transformed with the plasmid pPRP2-3.3. Total cellular RNA (20 μg per sample) was analyzed by Northern blotting after cells were incubated at 23°C or 37°C. We probed the blot with I4 (*5*), an intron-specific probe, to test for the accumulation of pre-mRNA (upper panel). We subsequently removed the probe from the blots and reprobed the nylon membranes with E4/5 (*5*), an exon-specific probe, to observe the mature transcript (lower panel). The blots were autoradiographed for 3 days at −70°C with an intensifying screen. The I4 and E4/5 oligonucleotides were labeled with [γ-^{32}P]adenosine 5′-triphosphate (>7000 Ci/mmol; ICN Biomedical, Inc.).

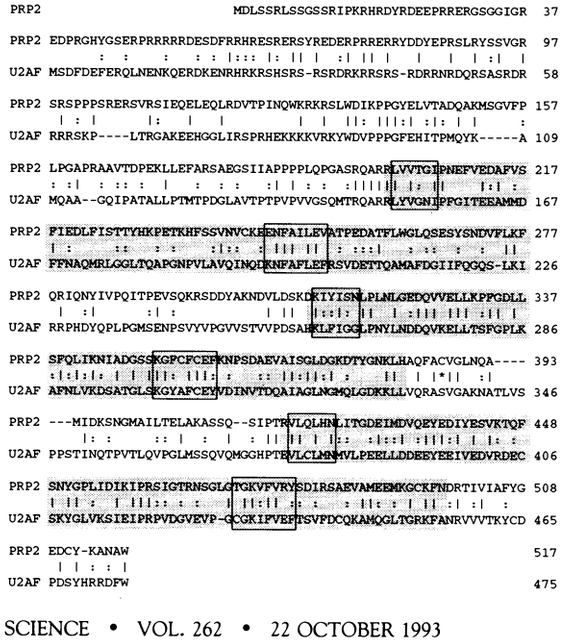

Fig. 3. Comparison between the predicted amino acid sequence of the *prp2*$^+$ gene product and the 65-kD subunit of U2AF (*11, 21*). Solid bars indicate identical amino acids, and colons indicate amino acids that have similar physiochemical properties (*22*). The three RRM domains are highlighted in gray, with the conserved RNP1 and RNP2 elements boxed. The amino acid altered in the *prp2.1* strain is marked with an asterisk. The GenBank, EMBL, and SwissProt databases were searched using the GCG software package (version 7.0, Genetics Computer Group, Inc.) and by GenBank E-mail service with the FASTA and BLAZE search programs.

transformed with the *prp2*$^+$ gene. These preliminary results indicate that the human U2AF^{65} gene functionally complements the mutant yeast *prp2.1* strain.

U2AF is composed of two polypeptides of 65 and 35 kD. The U2AF 65-kD polypeptide is the active component that binds the characteristic long polypyrimidine stretch between the branchsite sequence and the 3′ end of mammalian introns (*17*). In contrast, the distance between the branchsite and the 3′ end of the intron is short in *S. pombe* (*18*). Some fission yeast introns have short stretches of pyrimidines between the branchsite and the 3′ splice site, whereas others have a balanced purine and pyrimidine ratio. Generally, fission yeast introns are very pyrimidine-rich, with more U-runs present at the 5′ end of the intron as compared with the 3′ end of the intron (*19*). Identification of a homolog of U2AF^{65} in fission yeast suggests at least two possibilities: (i) either U2AF^{65} in fission yeast does not need to interact with a polypyrimidine stretch or it interacts with a polypyrimidine stretch at the 5′ end of the intron, or (ii) U2AF^{65} is essential for the splicing of some fission yeast introns (those containing polypyrimidine stretches) and not others (introns with a balanced purine-pyrimidine ratio). Perhaps fission yeast splicing could be regulated by the differential need for U2AF. It should now be possible to differentiate between these possibilities by studying U2AF genetically in *S. pombe*.

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Potentiation by the β Subunit of the Ratio of the Ionic Current to the Charge Movement in the Cardiac Calcium Channel

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The voltage-activated rabbit cardiac calcium channel α_1 subunit was expressed in *Xenopus* oocytes. The charge movement of its voltage sensor was measured and related to the opening of the ion-conducting pore. The half-activation potential for charge movement was 35 millivolts more negative than that for pore opening. Coexpression of the cardiac calcium channel β subunit reduced this difference without affecting charge movement. Thus, intramolecular coupling between the voltage sensor and the channel pore opening can be facilitated by a regulatory subunit.

Voltage-activated ion channels are membrane proteins with at least two functional domains: a pore through which ions flow and charged structures that serve as voltage sensors. The understanding of the molecular events coupling the voltage sensor to the pore opening requires not only measurements of ion fluxes through the pore (ionic currents) but also measurements of current associated with the movement of the voltage sensor (gating currents). One difficulty in assessing gating currents is the coexistence of multiple types of ion channels, each with its own set of voltage sensors. In some specialized cells, one particular channel type may be dominant, but even then contamination by other channels restricts the analysis to certain voltages. For example, in studies of Ca^{2+} channel gating currents in cardiac cells, Na^+ channels contribute nearly half the total gating charge (*1*) and voltage protocols designed to immobilize Na^+ channel gating current also alter the voltage dependence and kinetics of charges thought to come from Ca^{2+} channels (*2*). Molecular cloning of channels and expression in *Xenopus* oocytes is one solution to this problem, provided that voltage-clamped currents can be measured within the time immediately before channel opening in which the movement of the voltage sensor occurs. A technique, referred to as the cut-open oocyte voltage clamp (COVG), was developed to measure both the gating and the ionic currents of cloned K^+ channels (*3*, *4*). For these channels, the voltage-sensing structures were found to move with high cooperativity for channel opening (*5*).

We investigated whether the COVG

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