sln1-1 mutation, the (slowly growing) *sln1-1* segregants of IOY305 must almost always be Trp$^-$, whereas the (normally growing) *SLN1* segregants must almost always be Trp$^+$ (bearing the *TRP1* gene tightly linked to *SLN1*). Nineteen tetrads were dissected, and all of the large-colony (*SLN1*) segregants were found to be Trp$^+$, whereas all of the small-colony (*sln1-1*) segregants were Trp$^-$, indicating that the cloned ORF was indeed the *SLN1* gene.

14. Nucleotide sequence of the *SLN1* locus determined in this work encompassed 428 bp upstream of the (inferred) start codon of *SLN1*, the *SLN1* ORF, and 320 bp downstream of the *SLN1* stop codon. This sequence, determined on both strands with the use of the chain termination method and standard strategies (*24*), has been submitted to GenBank; its accession number is U01835. Hybridization of the ^{32}P-labeled ~4-kb Xba I fragment that contained the 5′ flanking and 5′ proximal regions of *SLN1* to the blot of *S. cerevisiae* chromosomes fractionated by pulse-field electrophoresis (Clontech) localized *SLN1* to Chromosome IX. More detailed mapping, with the use of an ordered set of lambda phages containing *S. cerevisiae* genomic DNA inserts [Prime Clone Blots; a gift from L. Riles and M. Olson] positioned *SLN1* at the left arm of Chromosome IX, between *SUC2* and *HIS5*.
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18. Yeast DNA inserts of the library-derived, *CEN4*, *LEU2*-based, *SLN1*-containing plasmids that complemented *sln1-1* were cut at different restriction sites within and beyond the *SLN1* ORF, and the resulting linear ("gapped") (*17*) fragments were introduced into IOY7 [*sln1-1 ubr1Δ::HIS3 leu2*, pYULU]. Leu$^+$ transformants were selected and tested for their sensitivity to FOA (that is, for their inability to lose the *URA3*, *UBR1*-expressing pYULU plasmid without losing viability). FOA-sensitive Leu$^+$ transformants would be expected to have been transformed with plasmids whose "gap" encompassed the mutation-containing region of *sln1-1*. The gap-repaired plasmids (*17*) were isolated and sequenced at the gap-encompassing regions. This analysis detected a G-to-A transition at nucleotide position 2671 that resulted in a Gly-to-Asp alteration at residue 891 of Sln1.
19. The *sln1-Δ1::HIS3* deletion allele was constructed by subcloning into pUC19 (*24*) the 655-bp Eco RI–Bam HI fragment containing the 5′ flanking sequence of *SLN1* and the first 527 bp of the *SLN1* ORF, as well as the 452-bp Bam HI–Sal I fragment containing the last 40 bp of the *SLN1* ORF, the *SLN1-UBI1* intergenic region, and part of the *UBI1* ORF [this fragment was produced with the use of the polymerase chain reaction (PCR) and standard protocols (*24*)], yielding pSLN-21. The ~1.8-kb *HIS3*-containing Bam HI fragment of YEp6 (*25*) was subcloned into the Bam HI site of the pSLN-21 insert, yielding pSLN-22. The ~2.9-kb Eco RI–Sal I fragment of pSLN-22 was introduced into the diploid DF5 [*his3/his3*] (*16*), and His$^+$ transformants were selected; Southern hybridization was used to confirm the replacement (*17*) of one of two copies of *SLN1* with *sln1-Δ1::HIS3*.
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The Conserved Pre-mRNA Splicing Factor U2AF from *Drosophila*: Requirement for Viability

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The large subunit of the human pre–messenger RNA splicing factor U2 small nuclear ribonucleoprotein auxiliary factor (hU2AF^{65}) is required for spliceosome assembly in vitro. A complementary DNA clone encoding the large subunit of *Drosophila* U2AF (dU2AF^{50}) has been isolated. The dU2AF^{50} protein is closely related to its mammalian counterpart and contains three carboxyl-terminal ribonucleoprotein consensus sequence RNA binding domains and an amino-terminal arginine- and serine-rich (R/S) domain. Recombinant dU2AF^{50} protein complements mammalian splicing extracts depleted of U2AF activity. Germline transformation of *Drosophila* with the dU2AF^{50} complementary DNA rescues a lethal mutation, establishing that the dU2AF^{50} gene is essential for viability. R/S domains have been found in numerous metazoan splicing factors, but their function is unknown. The mutation in *Drosophila* U2AF will allow in vivo analysis of a conserved R/S domain–containing general splicing factor.

Generation of functional mRNAs in eukaryotes requires removal of noncoding regions (introns) from pre-mRNAs by a process termed RNA splicing (*1*). Pre-mRNA splicing takes place in the spliceosome, a dynamic RNA-protein complex that assembles in a stepwise adenosine triphosphate (ATP)–dependent manner on the pre-mRNA (*1*). The spliceosome is composed of small nuclear ribonucleoprotein (snRNP) particles and extrinsic RNA binding proteins. Studies with human cell (HeLa) nuclear splicing extracts have shown that the targeting of U2 snRNP to the branch site on the pre-mRNA requires a protein factor called U2 auxiliary factor (U2AF) (*1*). U2AF binds site-specifically to the polypyrimidine tract located between the branch site and the 3′ splice site of the pre-mRNA (*2*). Human U2AF consists of two associated polypeptides with apparent molecular sizes of 65 and 35 kD, termed hU2AF^{65} and hU2AF^{35}, respectively (*3*). Splicing activity of HeLa cell nuclear extracts depleted of U2AF activity can be restored by the addition of hU2AF^{65} (*3*). Sequence analysis of the complementary DNA (cDNA) encoding hU2AF^{65}, in conjunction with biochemical experiments, have revealed that hU2AF^{65} contains three COOH-terminal ribonucleoprotein consensus sequence (RNP-CS) domains that mediate RNA binding and an NH_2-terminal arginine- and serine-rich (R/S) domain that is essential for splicing activity in vitro (*2*). Biochemical and immunochemical assays have shown that U2AF activity and antigenically related proteins of 50 and 38 kD, corresponding to hU2AF^{65} and hU2AF^{35}, respectively, are present in the fruitfly *Drosophila melanogaster* (*3*).

We isolated a cDNA encoding the *Drosophila* homolog of hU2AF^{65} by screening a 4- to 8-hour *Drosophila* embryonic cDNA library under low-stringency conditions with a radiolabeled hU2AF^{65} cDNA fragment (*4*). The dU2AF^{50} cDNA contains a 1248–base pair open reading frame predicted to encode a 416–amino acid protein with a calculated molecular size of 46,652 daltons. The predicted amino acid sequence of dU2AF^{50} indicates that it contains functional domains similar to those found in hU2AF^{65} (Fig. 1). Amino acids 93 to 408 contain three RNP-CS domains and amino acids 6 to 41 contain an R/S domain. Comparison of the *Drosophila* and mammalian proteins shows that the amino acid identity is greatest within the RNP-CS RNA binding domains (80% identity and 90% similarity). Optimal alignment of the amino acid sequences requires that gaps and insertions be added in the regions connecting the RNP-CS domains, but not within

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them. Compared to dU2AF^{50}, hU2AF^{65} contains a 24–amino acid insertion between the most NH_2-terminal RNP-CS domain and the R/S domain. Optimal alignment also requires the addition of gaps in the R/S domain of dU2AF^{50}. When the RNP-CS domains are excluded, the percent identity between dU2AF^{50} and hU2AF^{65} drops to 47% (48% similarity). Thus, there may be stronger evolutionary pressure to conserve the RNP-CS domains than there is to conserve other regions in the large subunit of U2AF.

Use of a membrane-immobilized protein binding assay (*5*) has demonstrated specific interaction between the two subunits of hU2AF. Using this assay and immunoblotting, we showed that this interaction is conserved among species (*6*). ^{35}S-methionine–labeled dU2AF^{50} bound specifically to nitrocellulose-bound hU2AF^{35} and to a 38-kD protein from *Drosophila* Kc cells. This 38-kD protein is likely to be the *Drosophila* equivalent of hU2AF^{35} because it is antigenically related to hU2AF^{35}. In addition, this 38-kD protein coimmunoprecipitates with antibodies to dU2AF^{50} (*6*).

To investigate whether the isolated cDNA encodes a protein with biochemical activity expected for U2AF, we purified recombinant dU2AF^{50} protein from *Escherichia coli* (*7*). Figure 2A shows a denaturing polyacrylamide gel of the purified recombinant protein. The recombinant protein is referred to as His-dU2AF^{50} because it contains a 4-kD NH_2-terminal tag (*7*). As a control, we purified recombinant hU2AF^{65} (His-hU2AF^{65}). We first tested site-specific binding of the recombinant proteins to an RNA polypyrimidine tract. Various concentrations of His-dU2AF^{50} were incubated with a ^{32}P-labeled, 22-nucleotide synthetic RNA polypyrimidine tract (MINX-WT) in the presence of a ~60,000-fold molar excess of total yeast tRNA (*7*). Protein-RNA complexes and unbound RNA were separated by native polyacrylamide gel electrophoresis (Fig. 2B). His-dU2AF^{50} bound specifically to MINX-WT RNA. Binding to a mutant version of the polypyrimidine tract RNA (MINX-MUT) was not observed. The apparent dissociation constant (K_d) is $\sim 3 \times 10^{-6}$ M (*7*). To control for the presence of the 4-kD tag on dU2AF^{50}, we carried out the same experiment with His-hU2AF^{65} (Fig. 2B). The apparent K_d of His-hU2AF^{65} for MINX-WT RNA is $\sim 4 \times 10^{-7}$ M. This value is in excellent agreement with a previously determined apparent K_d of $\sim 3 \times 10^{-7}$ M for a recombinant hU2AF^{65} fused to the 27.5-kD glutathione-S-transferase protein (*2*).

Next, we tested the ability of the protein encoded by the dU2AF^{50} cDNA to activate splicing in vitro. To do so, we depleted HeLa cell nuclear extract of U2AF activity by chromatography on polyU-Sepharose in the presence of 1 M KCl (*3*). The depleted nuclear extract (indicated by ΔU2AF) could no longer splice the *Drosophila fushi tarazu* (*ftz*) pre-mRNA in vitro (Fig. 2C, lane 3), whereas splicing by the nondepleted nuclear extract was quite efficient (Fig. 2C, lane 2). When U2AF activity was

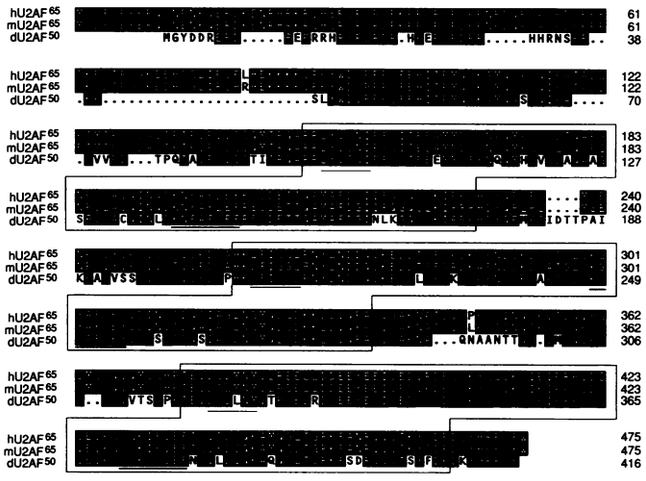

Fig. 1. Comparison of the amino acid sequences of the large subunits of human (*2*), mouse (*25*), and *Drosophila* U2AF. The complete amino acid sequences of the large U2AF subunit of the different organisms are shown in the single-letter code (*26*). Amino acid identities and similarities are shown in black and gray boxes, respectively. Dots denote gaps. Amino acid positions are shown on the right. The three RNP-CS domains are boxed and the RNP-1 and RNP-2 conserved elements are underlined with thick and thin lines, respectively (*27*). 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; and G and A. The GenBank accession number for the dU2AF^{50} cDNA sequence is L23404.

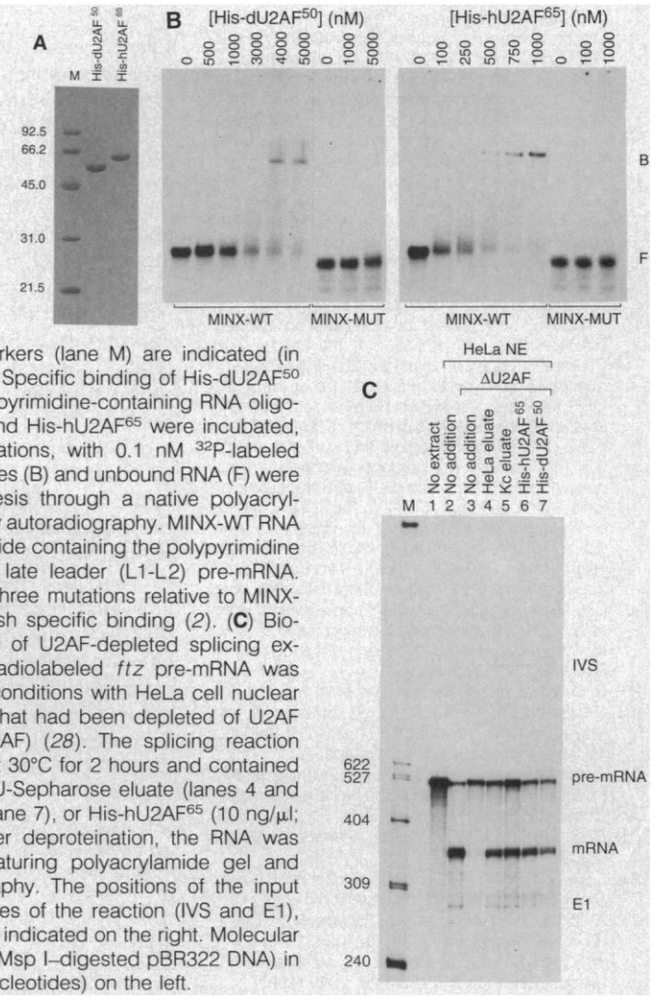

Fig. 2. Site-specific RNA binding and in vitro splicing activity of recombinant His-dU2AF^{50}. (**A**) SDS–polyacrylamide gel electrophoresis of recombinant His-dU2AF^{50} and His-hU2AF^{65} proteins. The His-dU2AF^{50} and His-hU2AF^{65} proteins (1.5 μg), purified from *E. coli*, were electrophoresed through a 12% SDS-polyacrylamide gel. The Coomassie blue–stained gel is shown. Molecular size markers (lane M) are indicated (in kilodaltons) on the left. (**B**) Specific binding of His-dU2AF^{50} and His-hU2AF^{65} to a polypyrimidine-containing RNA oligonucleotide. His-dU2AF^{50} and His-hU2AF^{65} were incubated, at the indicated concentrations, with 0.1 nM ^{32}P-labeled RNA. Protein-RNA complexes (B) and unbound RNA (F) were separated by electrophoresis through a native polyacrylamide gel and visualized by autoradiography. MINX-WT RNA is a 22-nt RNA oligonucleotide containing the polypyrimidine tract from the adenovirus late leader (L1-L2) pre-mRNA. MINX-MUT RNA contains three mutations relative to MINX-WT RNA that should abolish specific binding (*2*). (**C**) Biochemical complementation of U2AF-depleted splicing extracts by His-dU2AF^{50}. Radiolabeled *ftz* pre-mRNA was incubated under splicing conditions with HeLa cell nuclear extract (indicated by NE) that had been depleted of U2AF activity (indicated by ΔU2AF) (*28*). The splicing reaction mixtures were incubated at 30°C for 2 hours and contained either buffer (lane 3), polyU-Sepharose eluate (lanes 4 and 5), His-dU2AF^{50} (9 ng/μl; lane 7), or His-hU2AF^{65} (10 ng/μl; lane 6), as indicated. After deproteination, the RNA was analyzed on a 12% denaturing polyacrylamide gel and visualized by autoradiography. The positions of the input pre-mRNA, the intermediates of the reaction (IVS and E1), and the mRNA product are indicated on the right. Molecular size markers (^{32}P-labeled, Msp I–digested pBR322 DNA) in lane M are indicated (in nucleotides) on the left.

eluted from the polyU-Sepharose column with buffer containing 2 M guanidine hydrochloride (3) and added to the HeLa ΔU2AF nuclear extract, it restored splicing activity (Fig. 2C, lane 4). Using this assay, we tested the biochemical activity of His-dU2AF^{50} in pre-mRNA splicing. Purified His-dU2AF^{50} protein efficiently reconstituted splicing activity of HeLa ΔU2AF nuclear extract (Fig. 2C, lane 7), as did His-hU2AF^{65} (Fig. 2C, lane 6). His-dU2AF^{50} also reconstituted splicing activity of *Drosophila* Kc cell ΔU2AF nuclear extract (6). We conclude that the dU2AF^{50} cDNA encodes a biochemically active form of the large subunit of *Drosophila* U2AF.

We used the dU2AF^{50} cDNA as a probe and localized the position of the dU2AF^{50} gene by in situ hybridization to polytene chromosomes. The dU2AF^{50} gene was localized to the X chromosome region 14C1,2 (8). DNA (90 kb) from this region was previously cloned to identify the *no-on transient A* gene (9). This region also contains two lethal mutations, *9-21* and *i19e* (9). Two restriction fragments derived from the genomic DNA region that rescues the *9-21* mutation by transformation experiments hybridize to a 1.6-kb transcript (9). We showed that these same restriction fragments also hybridized to the dU2AF^{50} cDNA (6), suggesting that the dU2AF^{50} gene is identical to the gene defined by the *9-21* mutation. RNA blot hybridization analysis indicated that the dU2AF^{50} cDNA hybridized to an abundant 1.6-kb mRNA that is present in *Drosophila* Kc cells, in 0- to 12-hour embryos (Fig. 3), and which is present throughout development (*10*).

We used protein immunoblot analysis to test whether the dU2AF^{50} protein is affected in *9-21* mutants. Because *9-21* mutants die in the first instar larval period of development (9), we prepared nuclear extracts from 0- to 12-hour embryos (*11*). Embryos were collected from stocks carrying the original *9-21* mutation and from stocks carrying other alleles of *9-21*. A protein blot of nuclear extract from these embryos was probed with affinity-purified polyclonal antibodies to dU2AF^{50}. All nuclear extracts contained full-length dU2AF^{50} protein, which is encoded by the wild-type copies of the dU2AF^{50} gene in the mixed genotype embryo population and by the maternally deposited mRNA. However, the nuclear extract from the embryos carrying the *6b4* allele of the *9-21* mutation contained, in addition, truncated versions of dU2AF^{50} (*11*), suggesting that *9-21* mutations affect expression of the dU2AF^{50} protein.

If the *9-21* mutation is in the dU2AF^{50} gene, as suggested by the immunoblotting analysis, then the dU2AF^{50} cDNA should rescue the lethality of *9-21* mutants. We tested this directly using P element–mediated germline transformation with the dU2AF^{50} cDNA under control of the hsp70 promoter. We found that the transgene carrying the hsp70-dU2AF^{50} cDNA rescued the *9-21* mutation at a frequency that was 29% of the maximal theoretical value (26 of 672 total progeny scored; Fig. 4). Rescue depended on the presence of the dU2AF^{50} cDNA and was specific for the *9-21* mutation (*12*). No rescue was observed in the absence of heat shock. The hsp70 promoter is constitutively expressed at a low level, but heat shock markedly increases expression. The rescue of the *9-21* mutation by the dU2AF^{50} cDNA was not complete. This could be due to the lack of endogenous dU2AF^{50} expression signals in the cDNA expression construct. Indeed, complete rescue (100%) was observed when the dU2AF^{50} gene in the test cross was provided in the context of its endogenous expression signals on a ~10-kb genomic DNA fragment (Fig. 4). To prove that the dU2AF^{50} gene on this genomic DNA fragment was responsible for the observed rescue of the *9-21* mutation, we created a frameshift mutation at a Bst EII site in this genomic DNA fragment (*12*). This Bst EII restriction site is unique in the ~10-kb genomic DNA fragment, and our sequencing data indicated that the dU2AF^{50} cDNA contains a unique Bst EII site in the region connecting the second and third RNP-CS domains. This mutant version of the dU2AF^{50} gene failed to rescue the *9-21* mutation (Fig. 4). We conclude that the *9-21* gene encodes dU2AF^{50} and that the large subunit of U2AF provides an essential function in vivo.

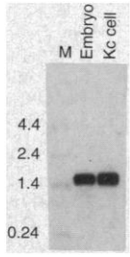

Fig. 3. RNA blot hybridization of dU2AF^{50} expression. Polyadenylate-enriched RNA was isolated from *Drosophila* Kc cells and 0- to 12-hour embryos as described (*24*). The RNA (1.5 μg) was electrophoresed through a 1.5% formaldehyde-agarose gel and transferred to a nylon membrane. Hybridization to the dU2AF^{50} cDNA probe and washing were done as described (*24*).

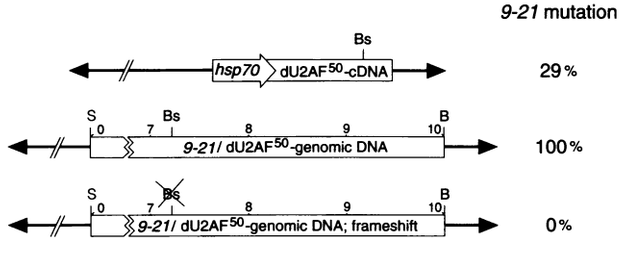

Fig. 4. Genetic rescue of the *9-21* mutation by the dU2AF^{50} gene. Shown are schematic representations of the P element constructs used in the rescue experiments. The P elements contained either the dU2AF^{50} cDNA or a ~10-kb genomic DNA fragment, shown to contain the *9-21* gene (*9*). A P element containing this same genomic DNA fragment carrying a frameshift mutation in the unique Bst EII site was also tested. The filled arrowheads indicate the inverted repeats required for P element integration. The hsp70 promoter and Sal I (S), Bst EII (Bs), and Bam HI (B) restriction sites are indicated. The scale on the genomic DNA constructs is in kilobases. On the chromosome the distal end would be to the right. The details of the transformation and test cross procedures are described (*12*).

Analysis of the function of R/S domain–containing general splicing factors has been limited to biochemical approaches because of the difficulty of genetic analysis in mammals and the lack of *Drosophila* mutants. Genetic analysis of pre-mRNA splicing has been extremely successful in the yeast *Saccharomyces cerevisiae* (*19*). However, none of the precursor RNA processing (*PRP*) mutants isolated to date have identified R/S domain–containing proteins, and the *S. cerevisiae* homolog of the mammalian U1 snRNP 70K protein does not contain an

R/S domains play a major role (or roles) in the mechanism of pre-mRNA splicing in metazoans. In addition to hU2AF^{65}, R/S domains are present in the general mammalian splicing factors SC35, SF2/ASF, and U1 snRNP 70K protein, as well as in the *Drosophila* splicing regulators su(w^a), tra, and tra-2 (*13*). More recently, a family of R/S domain–containing splicing factors, termed SR proteins, has been identified (*14*). Like the large subunit of U2AF, the SR proteins are conserved among metazoans and contain RNP-CS RNA binding domains. The SR protein family consists of, among others, the previously identified SF2/ASF and SC35 proteins, as well as the *Drosophila* proteins SRp55, its splice variant B52, and RBP1 (*15*). Splicing activity of cell extracts depleted of the SR protein family can be reconstituted by addition of any individual SR protein (*16*). In addition, SF2/ASF and SC35 behave identically in in vitro splicing assays (*17*). Thus, in vitro the SR proteins may be functionally redundant, although recent experiments suggest that individual SR proteins may have distinct functions in alternative splicing (*18*). The data presented here indicate that no other R/S domain–containing splicing factor can functionally substitute for dU2AF^{50} in vivo.

R/S domain (*20*). In addition, the recruitment of U2 snRNP to the branch point sequence in *S. cerevisiae* may proceed through a mechanism that does not involve a polypyrimidine tract binding activity, because the pre-mRNAs of *S. cerevisiae* lack the extensive polypyrimidine tracts found in mammalian pre-mRNAs. Instead, it has been suggested that if a U2AF-like activity exists in *S. cerevisiae*, it may bind the branch point sequence instead of the polypyrimidine tract (*21*). However, the presence of extensive polypyrimidine tracts can influence 3′ splice choice in *S. cerevisiae* (*22*). In this report we have shown that the previously uncharacterized lethal mutation *9-21* is in the large subunit of *Drosophila* U2AF and that the large subunit of *Drosophila* U2AF has biochemical properties identical to those of its mammalian counterpart. Thus, the combination of biochemical and genetic techniques available in *Drosophila* now provides powerful tools to investigate the mechanism of U2AF action in metazoan pre-mRNA splicing.

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4. A cDNA library derived from 4- to 8-hour embryos [N. H. Brown and F. C. Kafatos, *J. Mol. Biol.* **203**, 425 (1988)] (provided by N. Brown) was screened with ^{32}P-labeled hU2AF^{65} cDNA. Hybridization was done at 42°C in 5× sodium saline citrate–phosphate (SSCP), 10× Denhardt's solution, 25% formamide, 0.5% SDS, and denatured herring testis DNA (0.2 mg/ml) [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, ed. 2, 1989)]. The final wash was performed in 2× SSC and 0.5% SDS at 42°C. Plasmids containing inserts that hybridized to the hU2AF^{65} cDNA were isolated and tested by in vitro transcription-translation (pNB40-dU2AF^{50} 2 was sequenced).
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7. The cDNAs encoding dU2AF^{50} and hU2AF^{65} were subcloned into *E. coli* expression vector pRSETA (Invitrogen). The fusion proteins derived from these plasmids contain a 37–amino acid NH_2-terminal tag that includes six consecutive His residues. The proteins were purified from *E. coli* by chromatography through Q-Sepharose, Ni-NTA agarose (Qiagen), and MonoS columns and stored at −85°C in 20 mM Hepes-KOH (pH 8.0), 500 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 10% glycerol, and 0.05% Nonidet P40. Synthetic RNA oligonucleotides were chemically synthesized with phosphoramidite chemistry, purified on a Delta-Pak C_{18} column (Waters) with an 8 to 50% acetonitrile gradient in 0.1 M triethylammonium acetate (pH 7.0), and 5′ end-labeled with ^{32}P as described [L. Conway and M. Wickens, *EMBO J.* **6**, 4177 (1987)]. The sequence of the RNA oligonucleotides was 5′-CCUGUCCCUUUUUUUUCCACAG-3′ (MINX-WT) and 5′-CCUGUCCCAUUAUUAUCCACAG-3′ (MINX-MUT). Incubations were done for 15 min on ice in a volume of 20 μl containing the indicated concentrations of U2AF proteins, 0.1 nM RNA oligonucleotide, 15 mM Hepes-KOH (pH 8.0), 50 mM KCl, 1 mM EDTA, 1 mM DTT, bovine serum albumin (BSA; 50 μg/ml), yeast tRNA (150 μg/ml), 0.005% Nonidet P40, and 2% glycerol. One-fourth of the reaction mixtures was electrophoresed through a 4% polyacrylamide gel (60:1; 0.5× tris-borate EDTA) at 4°C for 100 min at 20 V/cm. Quantitation was done with a Molecular Dynamics PhosporImager. The affinity of the proteins for RNA is expressed in apparent K_d values obtained from the protein concentration at which 50% of the MINX-WT RNA is bound.
8. Polytene chromosome squashes were prepared as described (*23*) and subjected to hybridization with biotinylated dU2AF^{50} cDNA. Hybridization and detection were done as described [C. S. Zuker, A. F. Cowman, G. M. Rubin, *Cell* **40**, 851 (1985)].
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10. Polyadenylate-selected RNA isolated from embryos (0- to 2-, 2- to 5-, 5- to 8-, and 8- to 12-hour), larvae (first, second, and third instar), pupae (early and late), adults (female and male), and ovaries were analyzed as described in Fig. 3. The 1.6-kb RNA was present in all developmental stages that were tested, suggesting that dU2AF^{50} mRNA is maternally deposited into the embryo.
11. Nuclear extracts were prepared from 0- to 12-hour embryos as described (*24*). Antibodies to purified His-dU2AF^{50}, generated in rabbits, were affinity-purified on dU2AF^{50}-agarose as described [E. Harlow and D. Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989)]. The alleles of the *9-21* mutation used were *9-21* (obtained from K. Rendahl), *4a3-1*, *6b4*, *XR15*, *XR18*, and *XR26* (obtained from R. Stanewsky). As controls, nuclear extract from embryos of Canton *S*; *ry^{506}* [D. L. Lindsley and G. G. Zimm, *The Genome of* Drosophila melanogaster (Academic Press, San Diego, 1992)] and from *Drosophila* Kc cells were used.
12. The cDNA transformants were constructed as follows. The *w^{1118}* embryos were injected with pCaSpeRhs-dU2AF^{50} as described [A. C. Spradling, Drosophila: *A Practical Approach*, D. B. Roberts, Ed. (IRL Press, Oxford, 1986), p. 175]. The pCaSpeRhs-dU2AF^{50} transformant was constructed by subcloning of a 1.6-kb Hind III–Not I fragment, containing the dU2AF^{50} cDNA, from pNB40-dU2AF^{50} 2 into the Hpa I–Not I restriction sites of the *w*$^+$ transformation vector pCaSpeRhs [C. S. Thummel, A. M. Boulet, H. D. Lipshitz, *Gene* **74**, 445 (1988)]. Three independent transposon insertions (C31.4, C7-1, and C80.1) were tested for the ability to rescue the lethality of *9-21* by mating of transformant males, carrying a single transposon copy on one of their autosomes, with virgin females of the genotype I(1)*9-21*/FM7a. The FM7a balancer chromosome carries the markers *yellow* (*y*), *white apricot* (*w^a*), and *Bar* (*B*). The results of these three crosses were used to calculate the indicated percentage of rescue. Because *9-21* is an X-linked and lethal mutation, phenotypically wild-type males will only be observed in the test cross if the dU2AF^{50} cDNA rescues *9-21*. The number of progeny observed in the cross were 248 *wt* females, 216 *w*$^+$ or *w^a* semibar females, 182 *yw*$^+$*B* or *yw^{a}B* males (balancer males), and 26 *wt* males. Calculation of the percentage of rescue corrects for the fact that only the half of the *wt* males that carry the transposon are viable. To ensure that the rescue depended on the presence of the dU2AF^{50} cDNA, we did a control cross between *w^{1118}* males (the injection host strain) and I(1)*9-21*/FM7a females. To show that the rescue was specific for the *9-21* mutation, we performed a control cross between the transformant males and I(1)*i19e*/FM7a females. No phenotypically rescued males were observed in either of these control crosses. Crosses were done at 25°C. Every 6 hours the temperature was raised to 35°C for 10 min. The genomic DNA transformants were constructed as follows. The *w^{1118}* embryos were injected with pW8-genomic-dU2AF^{50} or the frameshift mutant pW8-genomic-dU2AF^{50}FS. The pW8-genomic-dU2AF^{50} was constructed by subcloning of a ~10-kb Sal I–Bam HI fragment from pHSX-211S12 (*9*) into pHSX. To create a frameshift, we filled in the unique Bst EII site that lies in the dU2AF^{50} coding sequence using the Klenow fragment of *E. coli* DNA polymerase I. A Not I fragment containing the wild-type or mutant dU2AF^{50} coding sequence was then transferred to pW8 [R. Klemenz, U. Weber, W. J. Gehring, *Nucleic Acids Res.* **15**, 3947 (1987)], to generate pW8-genomic-dU2AF^{50} and pW8-genomic-dU2AF^{50}FS, respectively. Transformant males containing pW8-genomic-dU2AF^{50} (F77.2) or pW8-genomic-dU2AF^{50}FS (G34.2) on one of their autosomes were mated with virgin females of the genotype I(1)*9-21*/FM7a. The number of progeny observed in the test cross with the genomic dU2AF^{50} gene were 101 *wt* females, 83 *w*$^+$ or *w^a* semibar females, 77 *yw*$^+$*B* or *yw^{a}B* males, and 40 *wt* males (the phenotypically rescued class). In the cross with the dU2AF^{50} gene containing the frameshift mutation, 91 *wt* females, 76 *w*$^+$ or *w^a* semibar females, 63 *yw*$^+$*B* or *yw^{a}B* males, and 0 *wt* males were observed. In each of these matings one *yw*$^+$ male was observed. They probably arose from recombination between the FM7a balancer chromosome and the chromosome containing *9-21*, and therefore these males could carry a wild-type copy of the *9-21* gene.
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26. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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28. ^{32}P-labeled *ftz* pre-mRNA was prepared as described [D. C. Rio, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2904 (1988)]. HeLa and *Drosophila* Kc cell nuclear extracts were prepared as described [J. D. Dignam, R. M. Lebovitz, R. G. Roeder, *Nucleic Acids Res.* **11**, 1475 (1983)] and were depleted of U2AF activity by chromatography on polyU-Seph-

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.

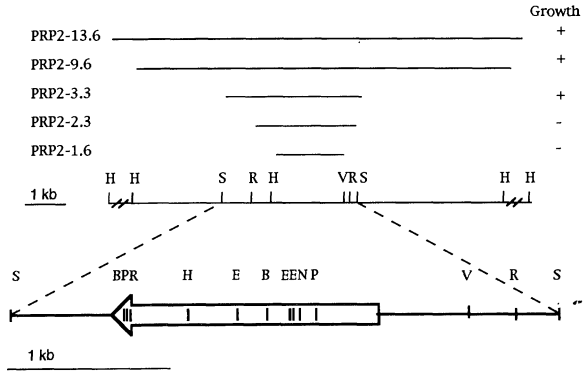

Fig. 1. Subcloning of the fragment containing the *prp2*$^+$ gene. Fragments of the 13.6-kb Hind III clone that rescues the temperature-sensitive growth defect of a *prp2*$^-$ strain were subcloned into fission yeast vectors. The numbers indicate the approximate length (in kilobases) of the restriction 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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