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Genetics

Mutations in the small subunit of the *Drosophila* U2AF splicing factor cause lethality and developmental defects

(fruit fly/pre-mRNA splicing/RNA-binding proteins/RS domains)

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ABSTRACT **The essential eukaryotic pre-mRNA splicing factor U2AF (U2 small nuclear ribonucleoprotein auxiliary factor) is required to specify the 3′ splice site at an early step in spliceosome assembly. U2AF binds site-specifically to the intron polypyrimidine tract and recruits U2 small nuclear ribonucleoprotein to the branch site. Human U2AF (hU2AF) is a heterodimer composed of a large ($hU2AF^{65}$) and small ($hU2AF^{35}$) subunit. Although these proteins associate in a tight complex, the biochemical requirement for U2AF activity can be satisfied solely by the large subunit. The requirement for the small subunit in splicing has remained enigmatic. No biochemical activity has been found for $hU2AF^{35}$ and it has been implicated in splicing only indirectly by its interaction with known splicing factors. In the absence of a biochemical assay, we have taken a genetic approach to investigate the function of the small subunit in the fruit fly *Drosophila melanogaster*. A cDNA clone encoding the small subunit of *Drosophila* U2AF ($dU2AF^{38}$) has been isolated and sequenced. The $dU2AF^{38}$ protein is highly homologous to $hU2AF^{35}$ containing a conserved central arginine- and serine-rich (RS) domain. A recessive *P*-element insertion mutation affecting $dU2AF^{38}$ causes a reduction in viability and fertility and morphological bristle defects. Consistent with a general role in splicing, a null allele of $dU2AF^{38}$ is fully penetrant recessive lethal, like null alleles of the *Drosophila* U2AF large subunit.**

Generation of functional mRNA in eukaryotes requires the removal of noncoding regions (introns) from pre-mRNA by a process termed RNA splicing (1–3). Pre-mRNA splicing takes place in the spliceosome, a dynamic RNA–protein complex that assembles in a stepwise ATP-dependent manner on the pre-mRNA (1–3). The spliceosome is composed of small nuclear ribonucleoprotein particles (snRNPs) and extrinsic (non-snRNP) protein factors. Studies with human cell (HeLa) nuclear splicing extracts have shown that targeting of U2 snRNP to the branch site on the pre-mRNA, an early step in spliceosome assembly, requires a protein factor called U2AF (U2 snRNP auxiliary factor) (4). U2AF binds site-specifically to the intron polypyrimidine tract located between the branch site and the 3′ splice site of the pre-mRNA (5). U2AF recruits U2 snRNP to the branch site in the first ATP-dependent step in spliceosome assembly. Recruitment of U2 snRNP to the branch site defines the 3′ splice site since in most cases the first AG dinucleotide downstream of the branch site is used as the 3′ splice site (6). Thus, U2AF plays a critical role in 3′ splice site selection.

Human U2AF (hU2AF) consists of two polypeptides, a 65-kDa large subunit ($hU2AF^{65}$) and a 35-kDa small subunit ($hU2AF^{35}$) (7). $hU2AF^{65}$ contains three carboxyl-terminal ribonucleoprotein consensus sequence (RNP-CS) domains that mediate RNA binding and an amino-terminal arginine- and serine-rich (RS) domain (5). All domains are essential for splicing *in vitro* (5). $hU2AF^{35}$ contains a central RS domain and a carboxyl-terminal glycine-rich region (8). The *Drosophila* large subunit homolog ($dU2AF^{50}$) is 75% identical to $hU2AF^{65}$ and is essential for viability (9). A temperature-sensitive mutation (prp2-1) in a putative homolog of $hU2AF^{65}$ from *Schizosaccharomyces pombe* causes a splicing defect *in vivo* (10). Splicing in HeLa cell nuclear extracts depleted of U2AF activity can be reactivated by the addition of either $hU2AF^{65}$ or $dU2AF^{50}$ (5, 9). Hence, the small subunit seems to be dispensable for splicing *in vitro*. It has been implicated in splicing only indirectly by its interaction with known splicing factors (11). A study of the small subunit in a genetically tractable organism may provide insight into its function *in vivo*. Immunochemical assays have shown a 38-kDa protein that is antigenically related to $hU2AF^{35}$ is present in the fruit fly *Drosophila melanogaster* (7).

We have isolated and sequenced a cDNA clone encoding $dU2AF^{38}$. The $dU2AF^{38}$ protein is highly homologous to $hU2AF^{35}$, containing a conserved RS domain and a glycine-rich carboxyl terminus. We have identified a recessive *P*-element insertion mutation affecting $dU2AF^{38}$ that causes a reduction in viability and fertility and causes morphological bristle defects. No splicing defect could be detected in the surviving homozygous mutants. We have created a null allele of $dU2AF^{38}$ by imprecise excision of the *P*-element. As with null alleles of the *Drosophila* large subunit gene, the $dU2AF^{38}$ null allele is fully penetrant recessive lethal.

MATERIALS AND METHODS

$dU2AF^{38}$ Purification and Gene Cloning. Affinity-purified anti-$dU2AF^{50}$ rabbit polyclonal antibody (R.K. and D.C.R., unpublished results) was coupled to protein G-Sepharose (12) and used to immunoaffinity-purify the dU2AF heterodimer from Q-Sepharose fractions of Kc nuclear extract (13). The immunoaffinity-purified proteins were separated by denaturing SDS/polyacrylamide gel electrophoresis, electroblotted, and proteolyzed, and peptides were sequenced as described (14). Degenerate oligonucleotides were designed from amino acids 37 to 46 and amino acids 136 to 143 and used for PCR as described (14). The resulting PCR product was used to screen a 4- to 8-h *Drosophila* embryo cDNA library in plasmid pNB40 (15). Positive cDNA clones were characterized by *in vitro* transcription–translation and both strands of a full-length cDNA insert were completely sequenced. $dU2AF^{38}$ genomic clones were isolated from a λFix *Drosophila* genomic DNA library (obtained from G. M. Rubin, University of California, Berkeley). Nitrocellulose filter lifts were hybridized using the

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Abbreviations: snRNP, small nuclear ribonucleoprotein; hU2AF, human U2AF; dU2AF, *Drosophila* U2AF; RS, arginine- and serine-rich.
Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U67066).

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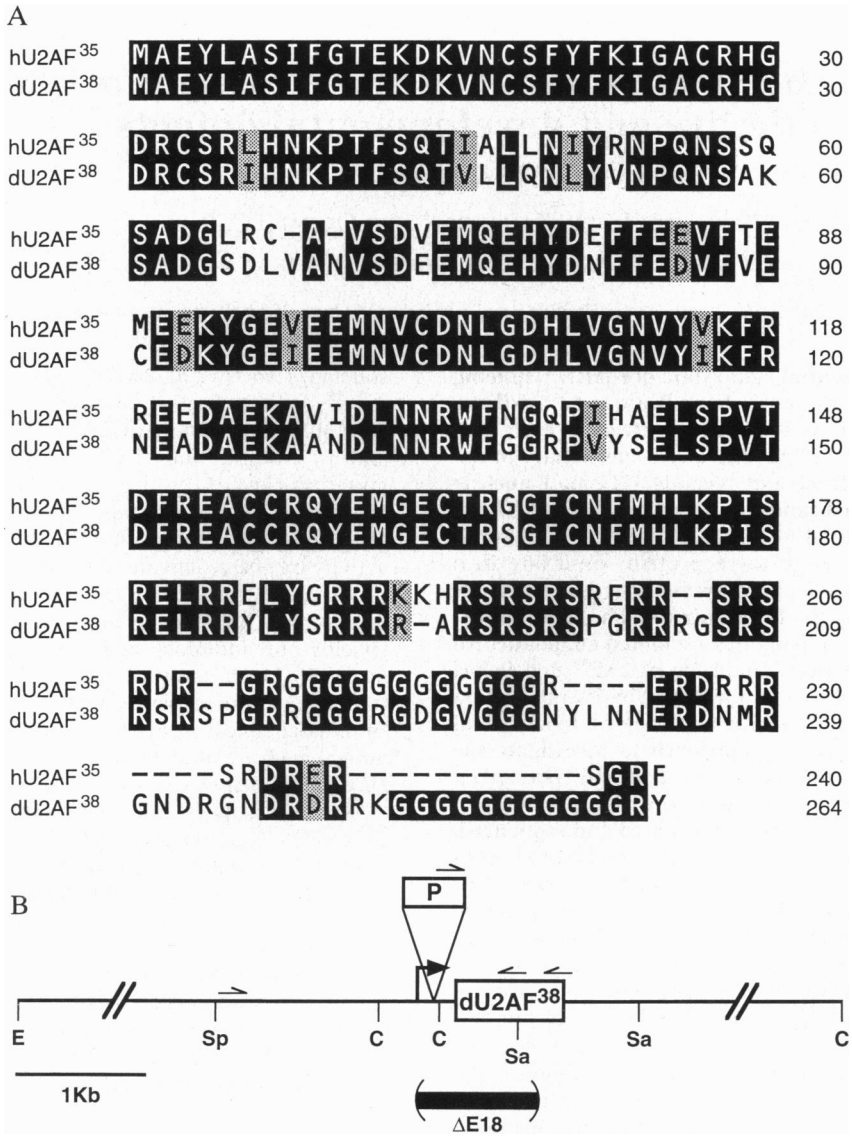

FIG. 1. Comparison of the deduced amino acid sequences of *Drosophila* and human U2AF small subunits and the organization of the dU2AF^{38} genomic locus. (*A*) The complete amino acid sequence of the small subunit of U2AF from the two organisms is shown. Amino acid identities and similarities are shown in black and grey boxes, respectively. Dashes denote gaps. Amino acid positions are shown on the right. The following amino acids are considered to be similar: V, L, and I; D and E; K and R. (*B*) The structure of the dU2AF^{38} genomic locus. A schematic diagram of the dU2AF^{38} locus is shown. The *P*-element insertion *P*[l(2)-06751] is indicated by a triangle. The *P*[l(2)-06751] insertion strain was obtained from the Berkeley *Drosophila* genome project (G. M. Rubin, personal communication). The 950-bp deletion mutation ΔE18 is indicated in parentheses. Oligonucleotide primers used for mapping the *P*-element insertion and the deletion are shown as arrows above the map. The restriction sites for *Eco*RI (E), *Sph*I (Sp), *Cla*I (C), and *Sal*I (Sa) sites are indicated.

dU2AF^{38} cDNA as a probe under standard conditions (16) and were characterized by restriction mapping, hybridization with the dU2AF^{38} cDNA, and PCR to localize the dU2AF^{38} coding sequence within the genomic DNA fragments.

***In Situ* Hybridization to Polytene Chromosomes.** Biotinylated DNA probes were prepared by nick translation (16) and hybridized to polytene chromosomes as described (17).

Analysis of dU2AF^{38} Alleles. The *P*-element *P*[l(2)-06751] was mapped relative to the dU2AF^{38} coding sequence by PCR using primers complementary to the *P*-element inverted repeat and to the dU2AF^{38} coding sequence. The extent of the ΔE18 deletion was mapped by PCR using a primer downstream of the *Sph*I site and a nested set of primers within the dU2AF^{38} coding sequence.

Complementation of dU2AF^{38} Mutant Alleles. w^{1118}/Y; *P*[l(2)-06751] or ΔE18/Cyo; *P*[w^+; dU2AF^{38}] males were crossed to w^{1118}; *P*[l(2)-06751] or ΔE18/Sm6β females in single pair. Viability of homozygous mutant progeny with and without the transgene was compared with viability of the heterozygous mutant siblings. More than 400 total progeny were scored in each complementation cross. Three independent transformants of each construct were tested. Fertility of the homozygous mutants was assayed by single pair matings to wild-type virgin females or males. All crosses were performed in single pairs at 25°C on standard *Drosophila* food unless indicated otherwise.

Isolation of a dU2AF^{38} Null Allele. Deletion mutations were created by imprecise excision of *P*[l(2)-06751], which is marked with rosy (ry^+). Dysgenic ry^+ males, *P*[l(2)-06751]/*Sp*; Δ2-3(99B), *Sb*/ry^{506} were crossed to +/CyO; ry^{506} females. Three hundred excision male progeny, "+"/CyO; ry^{506} were crossed to *P*[l(2)-06751]/CyO; ry^{506} females in single pairs. The viability of the heteroallelic progeny (non-CyO) in these crosses was compared with the viability of homozygous *P*[l(2)-06751] flies.

Genetic Analysis of dU2AF^{38}. *P*[l(2)-06751]/Sm6β females were crossed to *P*[l(2)-06751], $tra2^B$/Sm6β; Δ*tra*, *Ki*/+ males.

The intersexual phenotype of the *P*[l(2)-06751], $tra2^{B}$/*P*[l(2)-06751]; Δ*tra*, *Ki*/+ females was compared with the intersexual phenotype of their *P*[l(2)-06751], $tra2^{B}$/Sm6β; Δ*tra*, *Ki*/+ sisters.

Genetic Analysis of the Sex Comb Defect. *y cm* Sxl^{7B0}/Bins; *P*[l(2)-06751]/CyO females were crossed to *P*[l(2)-06751]/Sm6β males. The sex combs of the *y, cm,* Sxl^{7B0}/Y; *P*[l(2)-06751]/*P*[l(2)-06751] males were compared with the sex combs of their Bins/Y; *P*[l(2)-06751]/*P*[l(2)-06751] brothers. *P*[l(2)-06751]/Sm6β; dsx^{MR27}/TM3Ser females were crossed to +/Y^{B}; *P*[l(2)-06751]/Sm6β; dsx^{D}, *Sb*/+ males. The sex combs of +/Y^{B}; *P*[l(2)-06751]/*P*[l(2)-06751]; dsx^{D}, *Sb*/dsx^{MR27} males were compared with the sex combs of their +/Y^{B}; *P*[l(2)-06751]/*P*[l(2)-06751]; dsx^{MR27}/TM3Ser siblings.

RNA Analysis. RNA from 500 adult males was isolated using guanidinium thiocyanate and a CsCl step gradient (16). RNA was poly(A)$^{+}$-selected and 2.5 μg was electrophoresed on a 1.5% formaldehyde agarose gel, transferred to nylon membrane, and hybridized with a $dU2AF^{38}$ cDNA probe and then with an actin 5C DNA probe. Hybridization conditions were as described (18). Transcript levels were determined by PhosphorImager analysis (Molecular Dynamics).

RESULTS AND DISCUSSION

The dU2AF heterodimer was immunoaffinity-purified using anti-$dU2AF^{50}$ antibodies coupled to protein G-Sepharose. Tryptic peptides of the $dU2AF^{38}$ protein were sequenced and degenerate oligonucleotides were used in the polymerase chain reaction (PCR) to amplify an internal fragment of the $dU2AF^{38}$ cDNA. The amplified $dU2AF^{38}$ fragment was used to screen a 4- to 8-h *Drosophila* embryo cDNA library. A full-length cDNA was identified by *in vitro* transcription–translation of the cDNA clones and completely sequenced (data not shown). The $dU2AF^{38}$ cDNA contained a 792-bp open reading frame predicted to encode a 264-amino acid protein with a calculated molecular mass of 29.8 kDa. The predicted amino acid sequence of $dU2AF^{38}$ was 82% identical (87% similar) to its human homolog and contained a conserved central RS domain, glycine-rich carboxyl terminus, and the possible degenerate RNA recognition motif found in $hU2AF^{35}$ (Fig. 1*A*) (19). The $dU2AF^{38}$ protein was more similar to its human homolog than $dU2AF^{50}$ is to $hU2AF^{65}$ large subunit (75% identical) (9). Even though the small subunit seems to be dispensable for splicing *in vitro*, its high degree of evolutionary conservation suggests an important function *in vivo*.

The $dU2AF^{38}$ gene was localized cytologically to the left arm of the second chromosome at position 21B7-8 by *in situ* hybridization to polytene chromosomes. A *P*-element insertion had previously been localized to this cytological region. We mapped this *P*-element to approximately 200 bp upstream of the $dU2AF^{38}$ coding region by PCR and DNA blot hybridization (see Fig. 1*B*). Flies homozygous for the *P*-element insertion were severely reduced in viability (Fig. 2*A*, columns 1 and 3). The surviving homozygous escapers were infertile (Fig. 2*A*, columns 5 and 7) and exhibited bristle defects. The most prominent bristle defects were abnormal sex comb morphol-

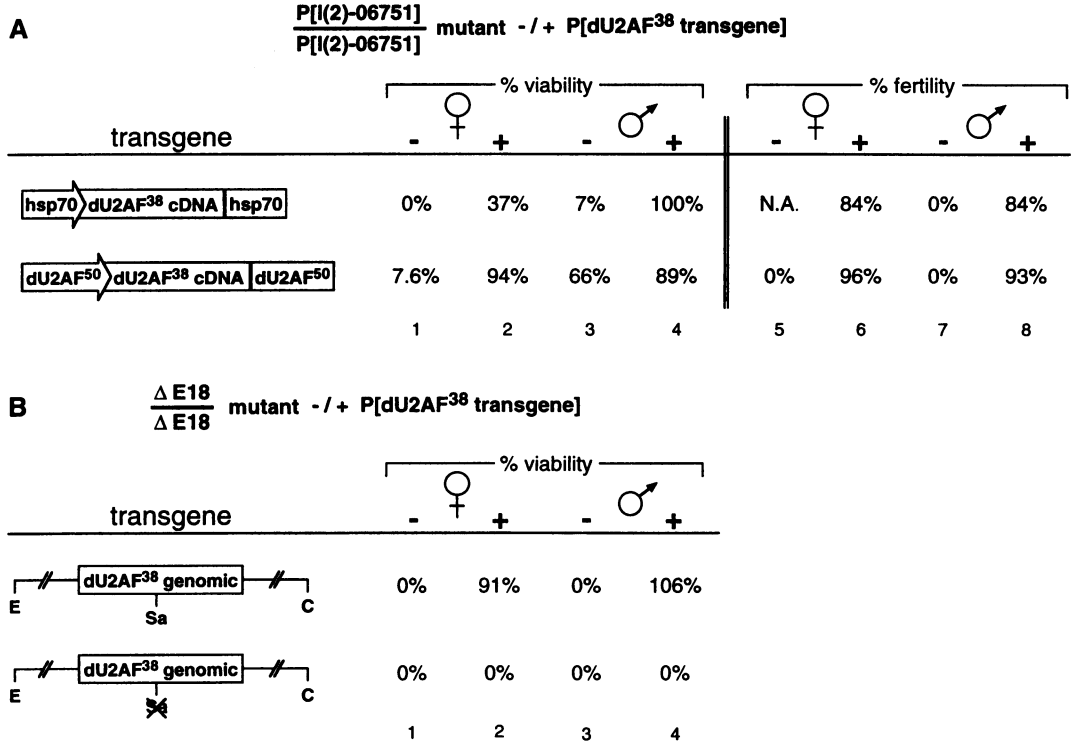

FIG. 2. *P*-element $dU2AF^{38}$ transgenes rescue the phenotypes associated with the *P*-element insertion mutation and the ΔE18 null allele. (*A*) The $dU2AF^{38}$ cDNA was cloned into a heat-inducible (hsp70) expression construct pCasper-hs (16) or a genomic $dU2AF^{50}$ promoter construct (D.Z.R., K.S.B., and D.C.R., unpublished results) in pw8 (16). In the complementation cross using the hsp70-$dU2AF^{38}$ cDNA gene fusion, the flies were heat shocked at 37°C for 30 min, every 6 h for the duration of the cross. This accounts for the unusually low viability of the homozygous mutants lacking the transgene. In the absence of heat shock no complementation was observed (data not shown). The viability of homozygous mutant males ranged from 35–60% (data not shown). Under crowded conditions male viability dropped to 1–4% (data not shown). In all crosses performed, the viability of the female homozygous *P*-element mutants lacking the $dU2AF^{38}$ transgene was 3–9 times lower than the males. Increasing or decreasing the gene dose of $dU2AF^{50}$ had no effect on the viability of the homozygous *P*-element mutants (data not shown). (*B*) A *P*-element containing a 10-kb genomic (*Eco*RI–*Cla*I) DNA fragment shown to contain the $dU2AF^{38}$ gene (see Fig. 1*B*) was used in the complementation of the null allele (ΔE18). A frameshift mutation in a *Sal*I (Sa) site within the $dU2AF^{38}$ coding sequence was also tested for complementation of the null allele. Neither $dU2AF^{38}$ cDNA transgene could complement the null allele (data not shown). The combination of the genomic frameshift transgene and the $dU2AF^{50}$ promoter cDNA transgene together partially rescued (17%) the null allele, consistent with the deletion affecting an adjacent gene (data not shown).

ogies (Fig. 3*B*). The sex comb is a unique set of very large bristles on the male foreleg used for mounting females during copulation. Interestingly, inappropriate overexpression of another splicing factor with an RS domain, SRp55, causes morphological bristle defects on the thorax, head, and legs (20).

Since the *P*-element was inserted 200 bp upstream of the dU2AF^{38} coding sequence, we tested whether it was affecting dU2AF^{38} transcript levels. RNA from homozygous and heterozygous mutant flies was isolated and analyzed by RNA blot hybridization. The dU2AF^{38} gene encodes a 1.3-kb mRNA whose level was substantially reduced in the homozygous mutant flies (Fig. 4, compare lanes 1 and 4).

To determine whether the phenotypes associated with the *P*-element insertion were due to reduction in dU2AF^{38} gene expression, we created germ-line transformants containing the dU2AF^{38} cDNA under the control of either the hsp70 promoter or the *Drosophila* U2AF large subunit (dU2AF^{50}) promoter. Both transgene cDNA expression constructs rescued all the phenotypes associated with the *P*-element insertion, establishing that the small subunit is not dispensable *in vivo*.

The effect of the *P*-element mutation on the sex combs, a sexually dimorphic body part, raised the intriguing possibility that reduced levels of dU2AF^{38} affected the *Drosophila* sex determination pathway that is controlled by a cascade of sex-specific alternative splicing events (21). The Sex-lethal (*Sxl*) gene product is present in females but not in males. *Sxl* controls the alternative splicing of the transformer (*tra*) pre-mRNA. The female-specific *tra* mRNA encodes a protein that activates the alternative splicing of the double-sex (*dsx*) transcript. Female-specific *dsx* mRNA encodes a protein required for female differentiation. Since the small subunit has been implicated in splicing, it is possible that a reduced level of dU2AF^{38} might cause inappropriate activation of the female sex determination pathway. If a reduction in dU2AF^{38} expression in males affected the sexual identity of the prospective sex comb cells, then mutations that block female-specific alternative splicing should rescue the sex comb defect. Neither a deletion of the Sex-lethal (*Sxl*^{7B0}) (22) gene nor a dominant allele of the double-sex gene (*dsx*^{D}) (23) that can only be spliced to the male-specific splice site was able to suppress the sex comb defect in males homozygous for the *P*-element insertion (data not shown). We conclude that the aberrant sex combs in the dU2AF^{38} mutants are not due to a defect in the sex determination pathway but rather a morphological defect perhaps due to the inability to efficiently splice one or more critical genes required for proper sex comb development.

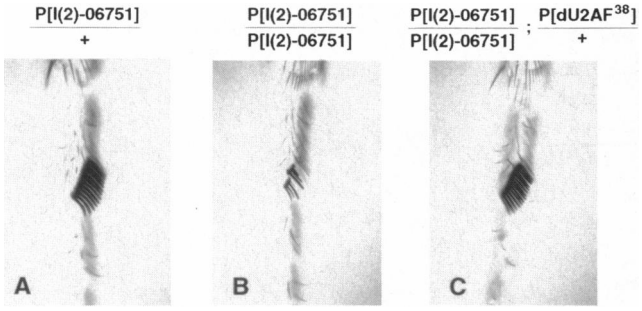

FIG. 3. Developmental defect in the sex comb of homozygous *P*-element insertion mutant males can be rescued by a dU2AF^{38} transgene. (*A*) Front foreleg from a heterozygous mutant male. (*B*) Front foreleg from a homozygous mutant male. (*C*) Front foreleg from a homozygous mutant male harboring a wild-type dU2AF^{38} transgene. The sex comb normally contains 12–14 teeth (machrocheates). In the homozygous mutant, the comb appeared broken with fewer teeth that were aberrantly shaped. This developmental defect could be rescued by any of the wild-type dU2AF^{38} transgenes but not a transgene with the genomic frameshift mutation (data not shown). The sterility in the mutant males was not caused by inability to mount females due to defective sex combs, as one particular transformant harboring the dU2AF^{38} cDNA transgene was incapable of rescuing the sex comb defect but was fully fertile (data not shown).

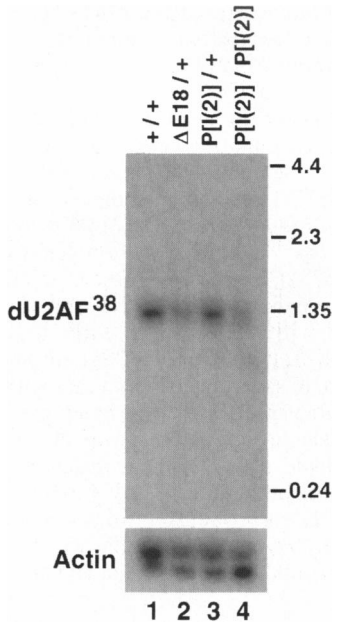

FIG. 4. Expression levels of dU2AF^{38} mRNA in wild-type and mutant flies. RNA samples are wild-type (lane 1), heterozygous for the null mutation (ΔE18) (lane 2), heterozygous for the *P*-element insertion (*P*[l(2)-06751]) (lane 3), and homozygous for the *P*-element insertion (lane 4). Heterozygous ΔE18 flies had 60% of the dU2AF^{38} transcript level relative to wild type. Heterozygous *P*-element insertion flies had 90% of the transcript level and homozygous mutant had 30% of the level compared with wild type. Actin mRNA was used as a loading control. We note that the ratio of the two predominant actin isoforms has shifted in the mutants compared with wild type. The two isoforms have different cleavage and polyadenylylation sites (28). RNAs were from adult male flies. Similar results were obtained using female flies (data not shown).

To determine the phenotype of a dU2AF^{38} null mutant, we created deletions by imprecise excision of the *P*-element. We isolated 12 excision mutations that had a more severe reduction in viability in combination with the *P*-element insertion compared with the viability of flies homozygous for the *P*-element. The sex comb defect associated with the heteroallelic survivors was also more extreme than in the homozygous *P*-element mutants (data not shown). Four of these excision lines produced no heteroallelic progeny. These four excisions were characterized by PCR using a series of nested primers. Excision line 18 (ΔE18) contained a 950-bp deletion that removed approximately two-thirds of the dU2AF^{38} coding sequence (Fig. 1*B*). To confirm that the excision mutation affected the dU2AF^{38} transcript level, RNA from heterozygous flies was isolated and dU2AF^{38} mRNA levels were assayed by RNA blot hybridization. The heterozygous deletion mutants had approximately 60% of the transcript level found in wild-type flies (Fig. 4, compare lanes 1 and 2). The deletion mutation was fully penetrant recessive lethal (Fig. 2*B*, columns 1 and 3); homozygous mutant progeny died in the first larval instar (data not shown), similar to dU2AF^{50} null mutants (9).

To determine whether the recessive lethality associated with this deletion was due to loss of dU2AF^{38}, we created germ-line transformants carrying a genomic DNA fragment containing the dU2AF^{38} gene. This genomic transgene rescued the lethality of the homozygous excision mutant (Fig. 2*B*). To confirm that the dU2AF^{38} gene was necessary for the observed rescue of the deletion mutation, we created a frameshift mutation at a *Sal*I site in the genomic DNA fragment. This *Sal*I site was within the central domain of the dU2AF^{38} coding

sequence (Fig. 1*B*). This mutant version of the dU2AF^{38} gene failed to rescue the deletion mutation, confirming that the null phenotype of dU2AF^{38} was recessive lethal (Fig. 2*B*). We conclude that the dU2AF^{38} gene provides an essential function *in vivo*.

Biochemical studies have implicated dU2AF^{38} in the alternative splicing of the double-sex pre-mRNA in females (11, 24). The alternative splicing factors transformer (tra) and transformer 2 (tra2) and SR proteins form a complex on the enhancer element of the *dsx* pre-mRNA *in vitro* (24). This enhancer complex has been proposed to stabilize U2AF binding to a weak female-specific polypyrimidine tract through interactions with the small subunit of U2AF (11). To investigate some of the genetic predictions of this model *in vivo*, we took advantage of a genetic background sensitized for the splicing of *dsx* in females. Female flies, heterozygous for *tra* and *tra2*, are fully female at 25°C but are partially intersexual at 29°C. A dominant mutation in the SR protein, SRp55 (*B52^{ED}*), enhances the intersexual phenotype by reducing female-specific splicing in this genetically sensitized background (25). A reduction in dU2AF^{38} levels is similarly predicted to increase the intersexual phenotype in this genetic background. We detected no increase in intersexuality in the surviving homozygous *P*-element mutant females that were heterozygous for *tra* and *tra2* (data not shown). Thus, our studies provide no genetic evidence to support the model that the enhancer complex interacts with dU2AF^{38} *in vivo*.

In an attempt to clarify the role of the small subunit in splicing, we looked for a splicing defect in flies homozygous for the *P*-element insertion. We analyzed the splicing efficiency of a panel of alternatively (*tra, Sxl*) and constitutively (actin 5C, *hsp83*, alcohol dehydrogenase) spliced introns. Total RNA was isolated from heterozygous and homozygous *P*-element mutant flies and the splicing efficiency of these test introns was analyzed using a reverse transcription-coupled polymerase chain reaction assay. We did not detect an alteration in splicing or accumulation of unspliced precursor RNA in the dU2AF^{38} mutant flies (data not shown). However, since we assayed splicing in viable flies with a reduced level of dU2AF^{38}, the defect in splicing may have been very modest. It is possible that unspliced pre-mRNA is rapidly degraded in the nucleus (26) and so this may not have been a good assay for detecting small decreases in splicing efficiency. Alternatively, dU2AF^{38} may not function in splicing or may only be required for the splicing of a subset of introns. There is precedent in *Drosophila* for failure of a mutation in a known splicing factor to show a defect in splicing *in vivo* (27). No impairment of splicing is observed in second instar larvae homozygous mutant for the splicing factor, SRp55, even though this is the developmental stage at which these animals die.

hU2AF^{35} interacts with factors that are involved in splicing, specifically the large subunit of U2AF, SR proteins, as well as tra and tra2 (8, 11). Yet, no biochemical activity has been assigned to the small subunit. Herein, we have shown that the *Drosophila* small subunit is more similar to its human homolog than dU2AF^{50} is to hU2AF^{65}. Although this protein seems to be dispensable *in vitro*, we have found that, like the large subunit, it is essential *in vivo*. A hypomorphic *P*-element insertion mutation that reduces the dU2AF^{38} transcript level impairs viability and fertility and causes morphological defects in the sex comb and other large bristles. In contrast to protein–protein interaction studies, no genetic interactions are observed among dU2AF^{38} and tra and tra2. The null mutation and a rescuing transgene described above will provide an invaluable assay to further study the function of dU2AF^{38} *in vivo*.

Note Added in Proof. Recently Zuo and Maniatis (29) have reported biochemical experiments providing evidence for a functional role for the small subunit of U2AF (hU2AF^{35}) in *in vitro* splicing assays.

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