The Drosophila *fl(2)d* Gene, Required for Female-Specific Splicing of *Sxl* and *tra* Pre-mRNAs, Encodes a Novel Nuclear Protein With a HQ-Rich Domain

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ABSTRACT

The Drosophila gene *female-lethal(2)d* [*fl(2)d*] interacts genetically with the master regulatory gene for sex determination, *Sex-lethal*. Both genes are required for the activation of female-specific patterns of alternative splicing on *transformer* and *Sex-lethal* pre-mRNAs. We have used *P*-element-mediated mutagenesis to identify the *fl(2)d* gene. The *fl(2)d* transcription unit generates two alternatively spliced mRNAs that can encode two protein isoforms differing at their amino terminus. The larger isoform contains a domain rich in histidine and glutamine but has no significant homology to proteins in databases. Several lines of evidence indicate that this protein is responsible for *fl(2)d* function. First, the *P*-element insertion that inactivates *fl(2)d* interrupts this ORF. Second, amino acid changes within this ORF have been identified in *fl(2)d* mutants, and the nature of the changes correlates with the severity of the mutations. Third, all of the phenotypes associated with *fl(2)d* mutations can be rescued by expression of this cDNA in transgenic flies. Fl(2)d protein can be detected in extracts from Drosophila cell lines, embryos, larvae, and adult animals, without apparent differences between sexes, as well as in adult ovaries. Consistent with a possible function in posttranscriptional regulation, Fl(2)d protein has nuclear localization and is enriched in nuclear extracts.

IN *Drosophila melanogaster*, the gene *Sex-lethal* (*Sxl*) controls the processes of sex determination, sexual behavior, and dosage compensation (the products of the X-linked genes are present in equal amounts in males and females). The control of *Sxl* expression throughout development occurs by sex-specific splicing of its transcripts. The male transcripts are similar to their female counterparts, except for the presence of an additional internal exon (exon 3), which contains a translation stop codon. Consequently, the male late transcripts give rise to presumably inactive truncated proteins. In females, this exon is spliced out and functional Sxl protein is produced (Bell *et al.* 1988; Bopp *et al.* 1991).

The gene *Sxl* encodes an RNA binding protein that regulates its own RNA splicing by binding to poly-U stretches in introns 2 and 3 (Sakamoto *et al.* 1992; Horabin and Schedl 1993). Genetic analyses have revealed three other genes important for female-specific splicing of *Sxl* pre-mRNA: *sans-fille* (*snf*), which is the Drosophila homologue of splicing factors U1A and U2B″ (Flickinger and Salz 1994); *virilizer* (*vir*; Hilfiker *et al.* 1995), which encodes a protein of 1878 amino acids (aa) without significant homologies to proteins in databases (D. Bopp and R. Nöthiger, personal communication); and *female-lethal-2-d* [*fl(2)d*] (Granadino *et al.* 1990).

Sxl controls sex determination and sexual behavior by inducing the use of a female-specific 3′ splice site in the first intron of *transformer* (*tra*) pre-mRNA. Use of the alternative, non-sex-specific 3′ splice site results in a transcript that encodes a nonfunctional truncated protein, while use of the female-specific site allows the synthesis of full-length functional Tra polypeptide (Boggs *et al.* 1987; McKeown *et al.* 1988). The products of genes *vir* (Hilfiker *et al.* 1995) and *fl(2)d* (Granadino *et al.* 1996) are required for proper splicing regulation of *tra* RNA.

Activation of *Sxl* is also required for normal female germ cell development (Cline 1983; Schüpbach 1985; Nöthiger *et al.* 1989; Steinmann-Zwicky *et al.* 1989). This is accomplished by expression of a germline-specific *Sxl* transcript and by regulation of the levels of one of the transcripts also found in the soma (Salz *et al.* 1989). As in the soma, *Sxl* activity is also maintained by autoregulation (Hager and Cline 1997). The functions of *snf* (Oliver *et al.* 1988; Steinmann-Zwicky 1988; Salz 1992), *vir* (Schutt *et al.* 1998), and *fl(2)d* (Gran-

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adino *et al.* 1992) are required for proper expression of *Sxl* in the germline.

EMS-induced mutations in *fl(2)d* have revealed that the gene has a dual function. *fl(2)d*^{1} is a recessive temperature-sensitive mutation that has stronger effects in females than in males: no homozygous females survive at 28°, and those that survive at 18° are sterile; homozygous males are fertile at both temperatures. This female-specific function of *fl(2)d* is related to its requirement for female-specific splicing of the *Sxl* and *tra* pre-mRNAs, because *fl(2)d* homozygous female larvae express the set of *Sxl* transcripts characteristic of males (Granadino *et al.* 1990, 1996). For this reason, loss-of-function mutations at either *fl(2)d* or *Sxl* are equivalent regarding sex determination and dosage compensation, as well as germline development. *fl(2)d* has also a second, non-sex-specific function, because another mutation, *fl(2)d*^{2}, is recessive mutant lethal in both sexes. Here we report the molecular identification of the gene *fl(2)d*, which encodes a novel nuclear protein containing an amino-terminal HQ-rich domain.

MATERIALS AND METHODS

Fly strains: Flies were cultured on standard food at 25° or 18°. For a description of the chromosomes and mutations see Lindsley and Zimm (1992).

Induction and genetic mapping of *fl(2)d*^{p} mutation: The *fl(2)d*^{p} mutation was induced following standard genetic procedures by transposition of the pLAC92 element in flies that carry in addition the pP[*ry*$^{+}$(Δ2-3)] element, which provides the transposase activity (Ashburner 1989). The number of second chromosomes analyzed in the mutagenesis was 5448. Its mapping was performed by analyzing the recombinant chromosomes from females of genotype *Bl L*^{2}/*fl(2)d*^{p}.

***In situ* hybridization to polytene chromosomes:** This was performed as described by Segarra and Aguadé (1993).

***P*-element-mediated germline transformation:** The *fl(2)d* cDNA coming from clone LD19472 (Berkeley Drosophila Genome Project, BDGP) was inserted into the *P*-element transformation vector pCaSpeR 4 containing a β-tubulin promoter. Germline transformants were obtained by standard procedures (Spradling 1986).

Construction of genomic libraries, screening and analysis of positive clones, and accession numbers: Total genomic DNA from flies was isolated according to Maniatis *et al.* (1982). The construction of the libraries was performed according to Pirrotta (1986). Identification of positive clones, plaque purification, preparation of phage DNA, Southern blot analysis, subcloning in plasmid vectors, and isolation of plasmid DNA were performed using the protocols described by Maniatis *et al.* (1982). Accession numbers of *fl(2)d* cDNAs and genomic sequences in the EMBL Nucleotide Sequence Database are as follows: EST LD19472, AJ243599; EST GH08722, AJ243607; genomic sequence (bases 13702–18607), AC005643.

DNA sequencing: Sequencing was performed using an automatic ABI377 DNA sequencer from Applied Biosystems (Foster City, CA).

RNA preparation and Northern analysis: RNA preparation, electrophoretical fractionation, blotting to nylon membranes, prehybridization, and hybridization were performed as previously described (Penalva *et al.* 1996). The probe corresponded to the *fl(2)d* cDNA sequences present in the insert of clone LD19472.

RT-PCR analyses: Total RNA (20 μg) was used for cDNA synthesis using Expand Reverse Transcriptase (Hoffmann-La Roche, Nutley, NJ) following the manufacturer's instructions. A total of 20% of the cDNA was used for 25–30 cycles of PCR amplification using 1 μl of Amplitaq (Perkin Elmer, Norwalk, CT) in a 50-μl reaction. The conditions of amplification were as follows: 94° for 1 min, 53° for 1 min, and 72° for 30 sec, followed by a final extension at 72° for 10 min. A total of 10 μl of the PCR product was loaded on a 2% agarose gel. The following sets of primers were used in reverse transcriptase (RT)-PCR reactions. Sense primer 1B (AGCAGCAAACGA GAAATCAG) and antisense primer 2B (GCATCCCGTCGT CAATCT) were used to detect the short transcript. Primers 1 (CCATCATCACCATCAGGAG)-sense and 2 (ACCTGTTC CAGCTTGAGATT)-antisense were used to detect the long transcript. Detection of both products was achieved using primers 1B and 2. The quantitative nature of the amplification reactions was assessed by comparing the products obtained from increasing concentrations of internal standards.

Transient transfections: Transient transfections were performed using Effectene (QIAGEN, Chatsworth, CA) according to the manufacturer's instructions. Schneider SL3 cells were usually transfected with 3 μg of plasmid DNA. Expression was induced by heat shock for 2–3 hr. Plasmid pBSHS-Fl(2)d was obtained by PCR cloning the long *fl(2)d* open reading frame (ORF) fused at the 5′ end to a sequence encoding an influenza virus hemagglutinine (HA) epitope (Alkhatib and Briedis 1986) in the Drosophila translation consensus (Cavener 1987) into the vector pBSHSPCAT (Inoue *et al.* 1990; kindly provided by K. Inoue).

Production of antibodies: A Fl(2)d carboxy-terminal (last 55 aa)-GST fusion and Fl(2)d amino-terminal (first 55 aa)-GST fusion were generated by cloning a PCR product from the LD19472 clone into the pGEXCS expression vector. The proteins were purified by their affinity to glutathione beads (Sigma, St. Louis) as described (Smith *et al.* 1993). The purified proteins were dialyzed against 1× PBS 20% glycerol and injected into rabbits together with RIBI Adjuvant System (RAS; RIBI ImmunoChem Research). The sera used in this study correspond to bleeds obtained after four boosts with the same antigen.

Protein preparation and Western analysis: Protein extracts were prepared from Drosophila Schneider cells, embryos, brains, and imaginal discs of larvae, and adult heads and ovaries, by homogenization in a buffer containing 20 mm Tris, pH 8.0, 80 mm NaCl, 20 mm EDTA, 1 mm DTT, 1% NP40, and 0.1 mm PMSF. Embryo nuclear extracts were prepared according to Becker and Wu (1992). Appropriate amounts of extract were fractionated by elecrophoresis in 10% polyacrylamide-SDS Laemmli gels. Proteins were transferred to nitrocellulose membranes using a semidry transfer cell (Bio-Rad, Hercules, CA). After blocking with 5% nonfat milk in PBS-Tween 20 buffer, the membranes were incubated with anti-FL(2)D rabbit antiserum at a 1:500 dilution or mouse monoclonal anti-β tubulin clone DM1A (Sigma) at 1:10,000 dilution or mouse monoclonal hemagglutinine HA-probe (F-7) from Santa Cruz Biotechnology at a 1:100 dilution. Anti-rabbit or anti-mouse horseradish-peroxidase-conjugated IgGs (Amersham) were used as secondary antibodies at a 1:2000 dilution. The blot was developed using an ECL detection kit (Amersham, Piscataway, NJ) and exposed to film for 2–5 min.

Immunofluorescence assays: Schneider cells were seeded onto cover slips and after 24 hr the cells were transfected with plasmid pBSHS-Fl(2)d. At 24 hr after transfection the cells were fixed with methanol and kept at −20° until use. Fixed cells were permeabilized with 0.2% Triton X-100 in PBS for

10 min, blocked with 1% BSA in PBS for 30 min, and then incubated with primary antibodies, anti-HA (F-7) monoclonal IgG2a antibody (Santa Cruz Biotechnology) or anti-FL(2)D antiserum, at a 1:500 dilution in PBS 1% BSA for 30 min. Cells were washed with PBS 1% BSA and incubated with the appropriate FITC-conjugated secondary antibodies (Amersham) at a 1:50 dilution in the dark for 30 min. The preparations were observed under a Zeiss (Thornwood, NY) Axioplan fluorescence microscope with a standard FITC filter.

Computer analysis: The DNA and protein databases (Swissprot, BDGP, GenBank, and EBI) were searched for homologies using BLAST and Fasta programs. Search of the ESTs databases (protein query against DNA database) was performed with eframe p2n (EMBL Bioccelerator). Similarities with known protein domains were analyzed using the Pfam and Prosite programs. All of these searches were performed using the EMBL Bioccelerator. Analysis of possible coiled coil regions was performed using the programs COILS (EMBnet) and BCM Search Launcher.

RESULTS

The gene *fl(2)d* and its products: To identify the molecular nature of *fl(2)d* gene, a *P*-induced *fl(2)d* mutation, $fl(2)d^{P}$, was produced. Males and females homozygous for this mutation are lethal. Several lines of evidence indicate that the *fl(2)d* gene was inactivated in $fl(2)d^{P}$. First, $fl(2)d^{P}$ was unable to complement the previous EMS-induced mutations $fl(2)d^{1}$ and $fl(2)d^{2}$. Second, genetic mapping was consistent with $fl(2)d^{P}$ being a new *fl(2)d* allele rather than a mutation in a different gene that synergistically interacts with *fl(2)d*. Third, *in situ* hybridization to polytene chromosomes of larvae $fl(2)d^{P}/+$ with a probe corresponding to the *lacZ* gene present in the *P* element of $fl(2)d^{P}$ showed a single hybridization signal in the 50C cytogenetic region of one of the two chromosomes 2 (data not shown), precisely the region described as the *fl(2)d* locus (Granadino *et al.* 1992).

Genomic DNA from flies heterozygous for $fl(2)d^{P}$ was used to generate a genomic library cloned in phage λEMBL4 (see materials and methods). This library was subsequently screened with two probes: the *P*-element pΠ25.1 (O'Hare and Rubin 1983) and a *Hin*dIII-*Hin*dIII fragment containing the *rosy* gene, which is present in the *P* element (Ashburner 1989) used to generate $fl(2)d^{P}$. A phage clone, λpr1.1, was identified whose DNA hybridized with both probes. Restriction mapping and Southern blot hybridization experiments indicated that the genomic DNA inserted in this clone contains a fragment of the *rosy* gene as well as a piece of genomic sequence presumably adjacent to the site of *P*-element insertion (Figure 1). The precise site of insertion was determined by direct sequencing (see Figure 4A).

A 5.8-kb *Eco*RI-*Eco*RI fragment of phage λpr1.1 (Figure 1) was used as a probe to screen a λEMBL4 Canton-S genomic library (see materials and methods). A phage, λR4A, was isolated. Restriction mapping and Southern blot hybridization indicated that it overlaps with λpr1.1

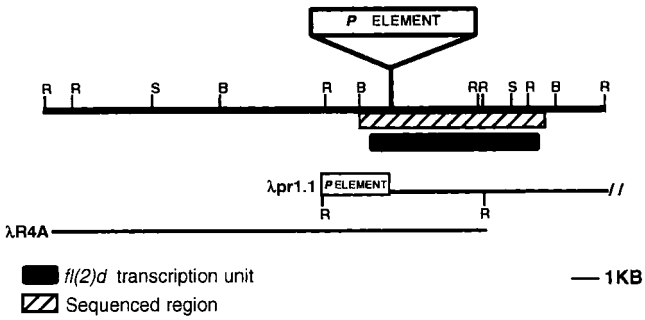

Figure 1.—Genomic localization of *fl(2)d*. Schematic representation of a 20-kb genomic fragment that contains the *fl(2)d* gene. The regions of overlap between this genomic fragment and sequences present in phages λpr1.1 and λR4A are represented as lines. The positions of restriction endonuclease cleavage sites are indicated as follows: R (*Eco*RI), S (*Sal*I), and B (*Bam*HI). The *Eco*RI-*Eco*RI fragment indicated in phage λpr1.1 was used as a probe to isolate phage λR4A. The *fl(2)d* transcription unit is represented as a solid bar. The site of *P*-element insertion is indicated by a triangle. The hatched bar indicates the genomic region that has been sequenced and contains the *fl(2)d* transcription unit.

(data not shown; Figure 1). *In situ* hybridization of clone λR4A to salivary gland polytene chromosomes showed a single hybridization signal to the 50C cytogenetic band, consistent with this phage containing sequences of the *fl(2)d* locus (data not shown).

To identify transcriptional units, a 5.9-kb genomic fragment containing the region of *P*-element insertion was sequenced (Figure 1). This sequence was then used to search the EST database of the BDGP. Among several clones found, two (LD19472 and GH08722) were sequenced. Comparison of these sequences and the genomic sequence indicated that these cDNAs correspond to two alternatively spliced transcripts generated by the use of two alternative 5′ splice sites (ss) in exon 1 (Figure 2A). Use of the upstream 5′ ss results in inclusion of exon 2B. This exon is skipped when the downstream 5′ ss is used.

Northern analysis of total RNA from male and female larvae, hybridized with a probe corresponding to cDNA LD19472, identified a 2.5-kb transcript of similar abundance in both sexes (Figure 3). The size of this RNA is compatible with that expected for the longer product of *fl(2)d* transcription. Using this type of approach we failed to detect the short transcript. Analysis of the *fl(2)d* EST clones present in the database suggests that this transcript could be less abundant. Among the 21 described clones, only one corresponds to this particular form. Quantitative RT-PCR was used to analyze the presence of these transcripts in embryos, larvae, and both male and female adult flies. The assignment of the amplification products was confirmed by sequencing. Consistent with the results of Northern analysis (Figure 3) and with the relative representation of the alternatively spliced mRNAs in libraries, the short transcript was

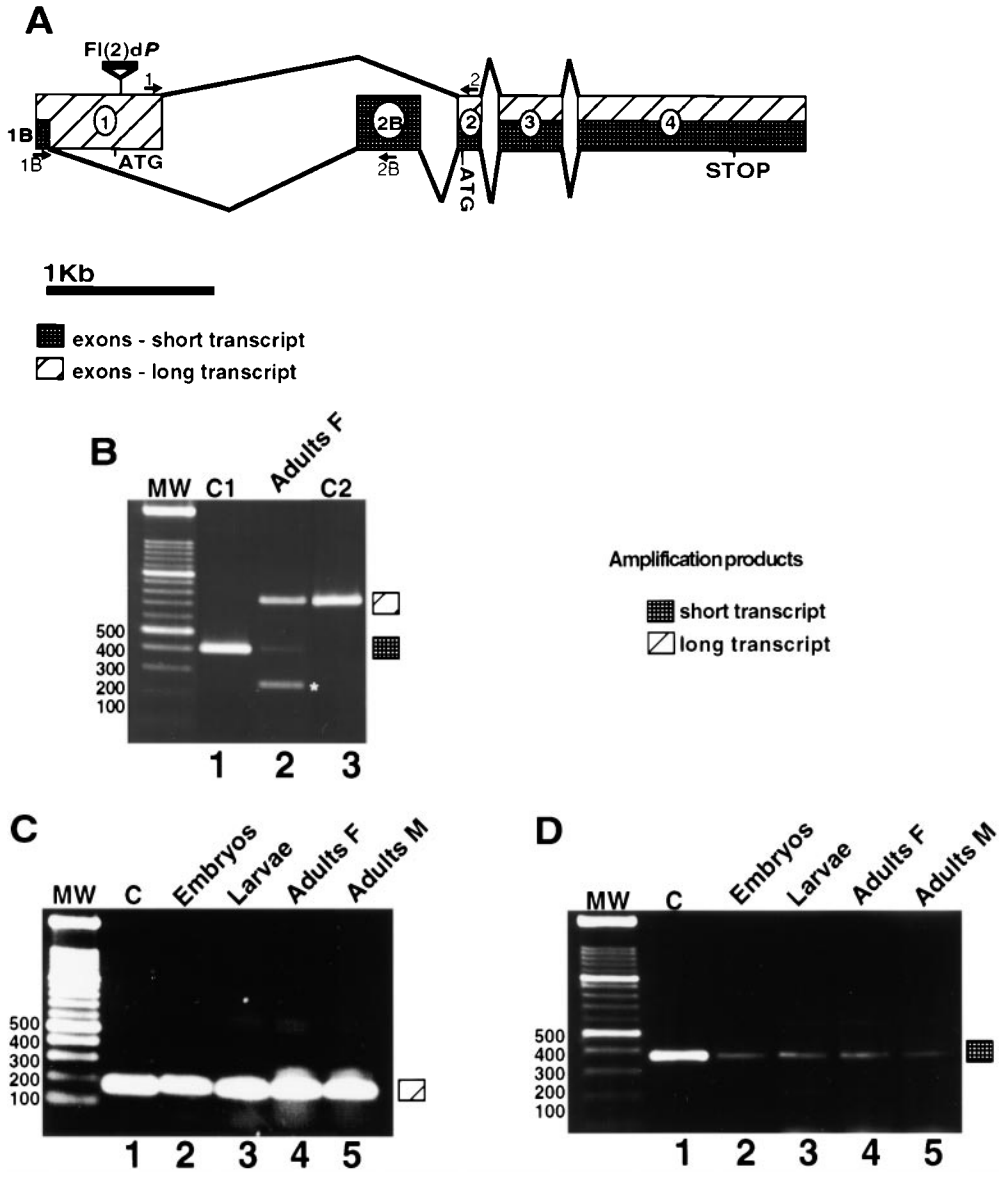

Figure 2.—Analysis of *fl(2)d* transcripts. (A) Schematic diagram of *fl(2)d* transcripts. Solid blocks correspond to exons. The alternative patterns of premRNA splicing for the long and the short transcripts are indicated above and below the blocks, respectively, and in addition by the striped or dotted patterns filling the exons. The position of the initiation codons (ATG), stop codon (STOP), *P*-element insertion (triangle), and the primers used in the RT-PCR analysis (arrows) are also represented. (B) RT-PCR analysis (30 cycles of amplification) of both *fl(2)d* transcripts from adult female fly RNAs using primers 1B and 2. The positions of the amplification products corresponding to the long and short transcripts are indicated. C1 and C2 indicate the amplification products from control plasmids containing cDNAs from each of the alternatively spliced RNAs. The band marked with an asterisk was cloned and sequenced; it corresponds to amplification of a sequence unrelated to *fl(2)d*. (C) RT-PCR analysis (25 cycles of amplification) of *fl(2)d* long transcripts during development and in different sexes, using primers 1 and 2. C indicates amplification from a control plasmid. (D) RT-PCR analysis (30 cycles of amplification) of *fl(2)d* short transcripts during development and in different sexes, using primers 1B and 2B. C indicates amplification from a control plasmid.

found to be less abundant than the long one (Figure 2B). The levels of both transcripts did not vary significantly during development or between sexes (Figure 2, C and D).

Conceptual translation of the alternatively spliced transcripts resulted in a single long ORF (Figure 4A). The transcript using the downstream 5′ ss (clone LD19472; Figure 2A) has the capacity to encode a protein of 539 amino acids, with a predicted molecular mass of 59,916 kD, whose most conspicuous feature is the presence of long stretches of histidine (H) and glutamine (Q) residues at the amino-terminal region (between residues 56 and 96). A schematic representation of Fl(2)d protein is shown in Figure 4B. The initiation codon of this ORF is skipped in the transcript that uses the upstream 5′ ss (clone GH08722; Figure 2A). This transcript could be translated in the same reading frame if a downstream AUG codon was used, thus generating a smaller polypeptide lacking the amino-terminal 127 residues that include the HQ-rich region. This initiation codon, however, is not in a good sequence context for initiation of translation in Drosophila (Cavener 1987), and no evidence was found for expression of this protein isoform (see below). Database searches identified regions of homology between the putative *fl(2)d* ORF and the products of conceptual translation of EST clones obtained from a variety of human and mouse cDNA libraries. Some of these correspond to a previously described cDNA (accession no. D14661) derived from the human male myeloblast cell line KG-1 (Nagase

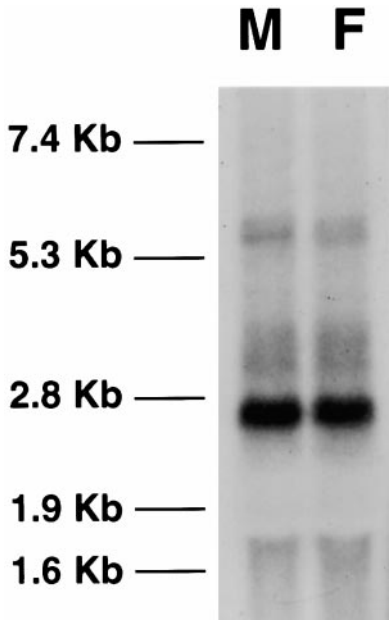

Figure 3.—RNA expression of *fl(2)d*. Northern blot of total RNA from male (M) and female (F) larvae. A total of 20 μg of purified RNA was loaded in each lane. The blot was probed with *fl(2)d* cDNA clone LD19472. The sizes of a molecular weight marker ladder are indicated.

et al. 1995). The stretch of homology with the putative Fl(2)d protein is shown in Figure 4C.

Evidence that the transcriptional unit identified corresponds to *fl(2)d*: Several lines of evidence indicate that the transcriptional unit described above corresponds to *fl(2)d*. First, the site of *P*-element insertion in the $fl(2)d^{p}$ mutant disrupts the longer Fl(2)d ORF (Figures 2A and 4A). Second, sequence analysis of genomic DNA from $fl(2)d^{1}$ flies, a recessive temperature-sensitive mutant of *fl(2)d*, revealed a single G to A nucleotide change at nucleotide position 939. This results in an amino acid change from aspartic acid to asparagine at position 180 of the longer version of Fl(2)d ORF (Figure 4, A and B). Third, sequence analysis of genomic DNA from $fl(2)d^{2}/+$ heterozygous flies also revealed DNA alterations that are consistent with the stronger, non-sex-specific phenotype associated with this mutation (Granadino *et al*. 1992). Because $fl(2)d^{2}$ is lethal in homozygosis, DNA from $fl(2)d^{2}/+$ heterozygous flies was amplified by PCR to generate products that span the complete ORF. The profile of the sequencing products revealed a single position of heterogeneity at nucleotide 615, which is a C in wild-type flies and is a mixture of C and T in $fl(2)d^{2}/+$ heterozygous flies. To confirm this, a 300-bp fragment spanning the region containing the putative change was amplified by PCR and cloned. Out of six independent clones sequenced, two had a C and four had a T at the position where the double peak was observed by direct sequencing of the PCR products (data not shown). This C to T transition would result in substitution of glutamine 72 by a stop codon (Figure 4, A and B). Therefore, flies homozygous for this mutation would produce only a truncated, presumably nonfunctional Fl(2)d protein, in agreement with the stronger character of the $fl(2)d^{2}$ mutation compared to $fl(2)d^{1}$ (Granadino *et al*. 1992).

Finally, and most importantly, all of the phenotypic effects associated with *fl(2)d* mutations were rescued in transgenic flies transformed with a cDNA corresponding to the longer transcript under the control of a constitutive β-tubulin promoter. Table 1 summarizes the results of viability analysis of the progeny of crosses set up to generate males and females homozygous for $fl(2)d^{2}$ (cross A), or $fl(2)d^{1}$ (cross B), which carry the *P[fl(2)d cDNA]* transgene inserted in the third chromosome. A single copy of the *P[fl(2)d cDNA]* transgene was sufficient to recover the viability of males and females homozygous for $fl(2)d^{2}$, or homozygous for $fl(2)d^{1}$ raised at the restrictive temperature. Moreover, both females and males were fertile in both genetic backgrounds. These results demonstrate that the transgenic Fl(2)d protein supplies $fl(2)d^{+}$ activity for both the female-specific and the non-sex-specific functions of *fl(2)d* in the soma, as well as the activity of this gene in the development of the female germline.

Taken together, the genetic and molecular data demonstrate that the gene *fl(2)d* has been cloned.

The gene *fl(2)d* encodes a nuclear protein: To verify whether the putative polypeptides encoded by the *fl(2)d* transcripts are indeed expressed, polyclonal antisera were obtained from rabbits immunized with purified recombinant proteins corresponding to either the amino-terminal 55 amino acids, or the 55 carboxy-terminal residues, of Fl(2)d fused to glutathione-*S*-transferase (GST). Two different antisera were obtained for each antigen. The four antisera, but not preimmune sera, detected a single major polypeptide of ~80 kD in extracts from Drosophila Schneider cells (Figure 5, A and B), total and nuclear extracts from embryos (Figure 5C), larvae (Figure 5D), adult flies (Figure 5E), and ovaries (Figure 5F). The protein was also detected with antibodies affinity-purified using the Fl(2)d portion of the antigen (data not shown). Although the apparent mobility of the polypeptide recognized by the different antisera is substantially different from the size predicted by conceptual translation of the long *fl(2)d* ORF, three lines of evidence indicate that the protein recognized by the antisera corresponds to the predicted Fl(2)d product. First, Schneider cells transfected with a plasmid containing a *fl(2)d* ORF fused to an influenza hemagglutinin epitope (HA) expressed a protein product of ~80 kD, as detected using anti-HA antibodies (Figure 5A, lanes 8 and 9), that was not detected in nontransfected cells (lane 7). Second, overexpression of *fl(2)d* resulted in an increase in the signal detected with the anti-Fl(2)d antisera, compared to the signal from untransfected cells (Figure 5A, compare lane 4 with lanes 5 and 6). Third, *in vitro* translation of *fl(2)d* ORF in reticulocyte

extracts resulted in the synthesis of a protein product of ~80 kD (data not shown). We conclude that the different antisera recognize Fl(2)d protein and that the protein has an anomalous mobility in SDS-polyacrylamide gels, perhaps related to the long stretches of histidine and glutamine residues.

The band marked with an asterisk in Figure 5, A and C, is unlikely to correspond to the product of translation of the short *fl(2)d* transcript, or to a degradation product of the complete Fl(2)d protein, because, first, it is detected with antibodies against both the amino and the carboxy terminus of the protein and, second, it is not detected with affinity-purified anti-Fl(2)d antibodies. It may correspond to cross-reaction of antibodies directed against the GST part of the antigen. Although we have not found evidence for expression of the Fl(2)d isoform encoded by the shorter *fl(2)d* transcript, we cannot rule out that this isoform is expressed in small amounts or only in particular cells or tissues.

To test whether differences in *fl(2)d* expression could be detected between male and female flies, Western blots of protein extracts from male and female heads were probed with antibodies against the carboxy terminus of Fl(2)d and β-tubulin. Two different amounts of total protein were loaded to verify the linear response of the signal detected. The results of Figure 5E indicate that equivalent amounts of Fl(2)d were present in both sexes. Similar results were obtained with antibodies against the amino terminus of Fl(2)d (data not shown).

Consistent with the requirement of *fl(2)d* function for the development of the female germline (Granadino *et al.* 1992), Fl(2)d protein was detected in total protein extracts from adult ovaries (Figure 5F).

Next we analyzed the subcellular localization of the Fl(2)d polypeptide. Figure 6a shows an immunofluorescence analysis of Schneider cells transfected with an expression vector containing *fl(2)d*-cDNA fused to an HA epitope. The anti-HA antibody revealed fluorescent signal in the nucleus. The anti-Fl(2)d antibodies did not allow detection of the endogenous Fl(2)d protein by immunofluorescence in Schneider cells. Nuclear fluorescent signal identical to that detected with the anti-HA antibody, however, could be detected with anti-Fl(2)d antibodies in cells overexpressing *fl(2)d* (Figure 6b). The nuclear localization of Fl(2)d is in agreement with the enrichment of the protein in nuclear extracts (Figure 5C, compare lanes 1–3) and is consistent with the possibility that Fl(2)d is involved in assisting Sxl in posttranscriptional regulation of gene expression. A putative nuclear localization signal is present between

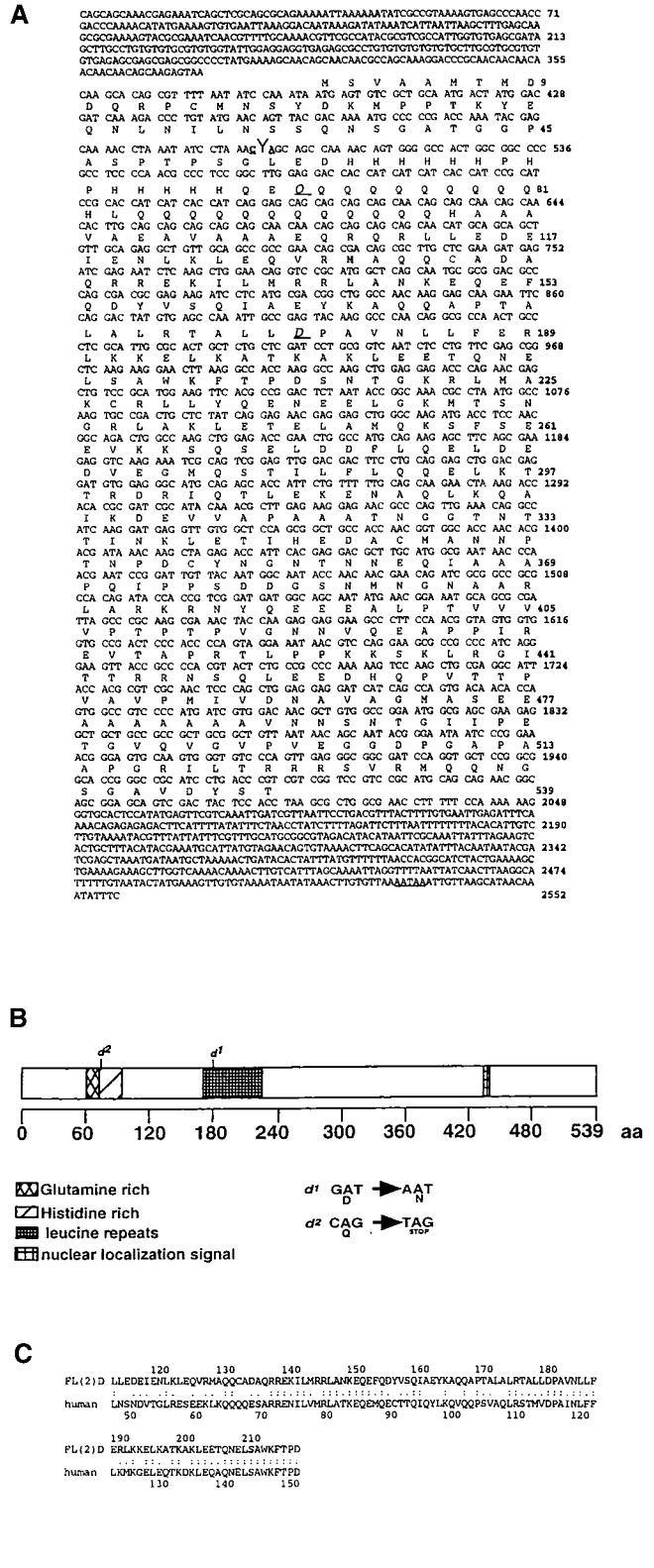

Figure 4.—Structure of the Fl(2)d protein. (A) Nucleotide sequence of *fl(2)d* cDNA and amino acid sequence of the putative Fl(2)d proteins. The nucleotide sequence corresponds to clone LD19472. The amino acid changes in the *fl(2)d*^{1} and *fl(2)d*^{2} mutants are italicized and underscored. The methionine corresponding to the first amino acid of the shorter Fl(2)d protein is indicated in boldface type. The site of *P*-element insertion is indicated by a Y. (B) Schematic representation of Fl(2)d protein. Relevant regions and *fl(2)d*^{1} (d1) and *fl(2)d*^{2} (d2) mutations are indicated in the figure. Numbers present below the bar (protein) refer to the relative position of the amino acids. (C) Alignment between Fl(2)d residues 112–216 and putative protein potentially encoded by a human cDNA (accession no. D14661).

TABLE 1

The *P[fl(2)d-cDNA], w*$^+$ transgene supplies the *fl(2)d*$^+$ function

Cross A: *cn fl(2)d*^{2} *bw/CyO* × *w/Y; cn fl(2)d*^{2} *bw/CyO, P[fl(2)d-cDNA],w*$^+$ */TM3, Sb*		
No. of control females		331
Viability of experimental females homozygous for *fl(2)d*^{2}	Without *P[fl(2)d-cDNA]*	0%
	With *P[fl(2)d-cDNA]*	78%, fertile
No. of control males		291
Viability of experimental males homozygous for *fl(2)d*^{2}	Without *P[fl(2)d-cDNA]*	0%
	With *P[fl(2)d-cDNA]*	78%, fertile
Cross B: *cn fl(2)d*^{1} *bw/CyO* × *w/Y; cn fl(2)d*^{1} *bw; P[fl(2)d-cDNA],w*$^+$ */TM3, Sb*		
No. of control females		186
Viability of experimental females homozygous for *fl(2)d*^{1}	Without *P[fl(2)d-cDNA]*	1.1%, sterile
	With *P[fl(2)d-cDNA]*	82%, fertile
No. of control males		174
Viability of experimental males homozygous for *fl(2)d*^{1}	Without *P[fl(2)d-cDNA]*	55%, fertile
	With *P[fl(2)d-cDNA]*	90%, fertile

In both crosses, control males and females referred to the average of males and females, respectively, of phenotype CyO and phenotype CyO plus Stubble. Cross A was at 25° and cross B was at 28°.

residues 434 and 437 (KKSK; Chelsky *et al.* 1989; Figure 4, A and B).

DISCUSSION

In this report we present the molecular identification of the gene *fl(2)d*. The transcriptional unit identified can generate two alternatively spliced transcripts detectable at all developmental stages. Nevertheless, only the protein encoded by the long transcript could be detected in extracts from Schneider cells, embryos, larvae, and heads and ovaries of adult animals. This suggests that the short *fl(2)d* transcript is not translated. We cannot rule out, however, that the levels of the short protein may be low and restricted to a few cells. The fact that all the phenotypic effects associated with *fl(2)d* mutations could be reversed by expression of a cDNA corresponding to the long transcript in transgenic flies further challenges the physiological significance of the short *fl(2)d* transcripts. Expression of *fl(2)d* throughout development is also consistent with the observation that the *fl(2)d*^{1} mutation shows its thermosensitive phenotype at all stages of development (Granadino *et al.* 1992) and is also in agreement with the idea that the gene *fl(2)d* is needed for *Sxl*$^+$ function, since this gene is continuously required for the development of female flies (Sánchez and Nöthiger 1982; Cline 1984).

The long *fl(2)d* ORF can encode a polypeptide of 539 amino acids. The most apparent features of the primary sequence are two stretches of five and six histidines separated by prolines (residues 56–69) and two adjacent stretches of 10 glutamines (residues 72–95), followed by a glutamine-rich region. Glutamine repeats can dimerize or oligomerize by forming β-sheets that in antiparallel configuration can establish multiple hydrogen bonds and form a polar zipper (Perutz *et al.* 1993). Polyglutamine stretches are found in a variety of genes, from receptors like *Notch* to >30 different transcription factors, including TFIID, Sp1, and the protein SRY, which is involved in sex determination in the mouse (Koopman *et al.* 1991; Gubbay *et al.* 1992; Goodfellow and Lovell-Badge 1993). Indeed, glutamine-rich domains constitute one of the three main classes of transcriptional activation domains and are often associated with histidine-rich stretches in transcription factors. These domains promote protein-protein interactions that facilitate the recruitment of transcription initiation complexes (Ptashne and Gann 1997). Consistent with the classical domain distribution of this type of transcription factors, the Q-rich domain of SRY is not involved in DNA binding (Nasrin *et al.* 1991; Giese *et al.* 1992), but is involved in protein-protein interactions (Zhang *et al.* 1999). This role is essential for the sex determination function of SRY, as indicated by the correlation between polymorphisms within this Q-rich domain and the sex determination activity of SRY among different *Mus musculus* strains (Coward *et al.* 1994). Polymorphic polyglutamine stretches are also associated with CAG trinucleotide expansions that have been implicated in the molecular pathogenesis of several human genetic diseases, including Huntington's disease (Perutz 1999).

A second feature of Fl(2)d sequence suggests that protein-protein interactions can be important for its function. Secondary structure prediction analyses sug-

gest three regions with potential to form coiled coils (around residues 100–125, 190–210, and 290–320). Coiled coils facilitate homo- and heterodimerization of multiple families of proteins, including transcription factors with classical leucine zippers like GCN4 or Fos/Jun, as well as factors with other arrangements of residues like the serum response factor Srf (Lupas 1996). Interestingly, the region between residues 172 and 208 contains six leucines that display an almost perfect heptad arrangement characteristic of leucine zippers (Pathak and Sieger 1992). Alternatively, this putative amphipathic α-helix could be packed against the rest of the protein fold, without being involved in dimerization through the formation of a coiled coil.

Therefore, two structural features often combined in transcription factors, a glutamine-rich region and a putative dimerization domain, are present in Fl(2)d. The absence of recognizable DNA binding domains and the fact that *fl(2)d* mutations result in post-transcriptional, rather than transcriptional, deregulation of its two known target genes (*Sxl* and *tra*) make the possibility that Fl(2)d is a transcription factor less likely. We cannot exclude, however, that Fl(2)d is important for transcription of a yet unidentified factor involved in the processing of *Sxl* and *tra* RNAs.

fl(2)d has two functions that can be separated genetically by mutations that are either female-lethal or both male- and female-lethal. In addition, the gene *fl(2)d* is also needed for proper expression of *Sxl* in the female germline (Granadino *et al.* 1992). Our data indicate, however, that the female-specific and the non-sex-specific functions, as well as the germline function of *fl(2)d*, are performed by a unique Fl(2)d protein. What are the molecular bases for this dual function of the protein? The thermosensitive-lethal phenotype associated with the $fl(2)d^{1}$ mutation is stronger in females than in males (Granadino *et al.* 1992). Molecular analyses of these mutants revealed a change from aspartic acid to asparagine at position 180 of Fl(2)d amino acid sequence. The *ts* phenotype of the $fl(2)d^{1}$ mutant would be compatible with D180 forming a salt bridge important for proper folding or stability of the protein (Schulz and Schirmer 1979). Mutation to asparagine can weaken its capacity to form salt bridges and therefore make the protein less stable or less active at higher temperatures. D180 is included in the region with potential to form a leucine zipper as referred to above.

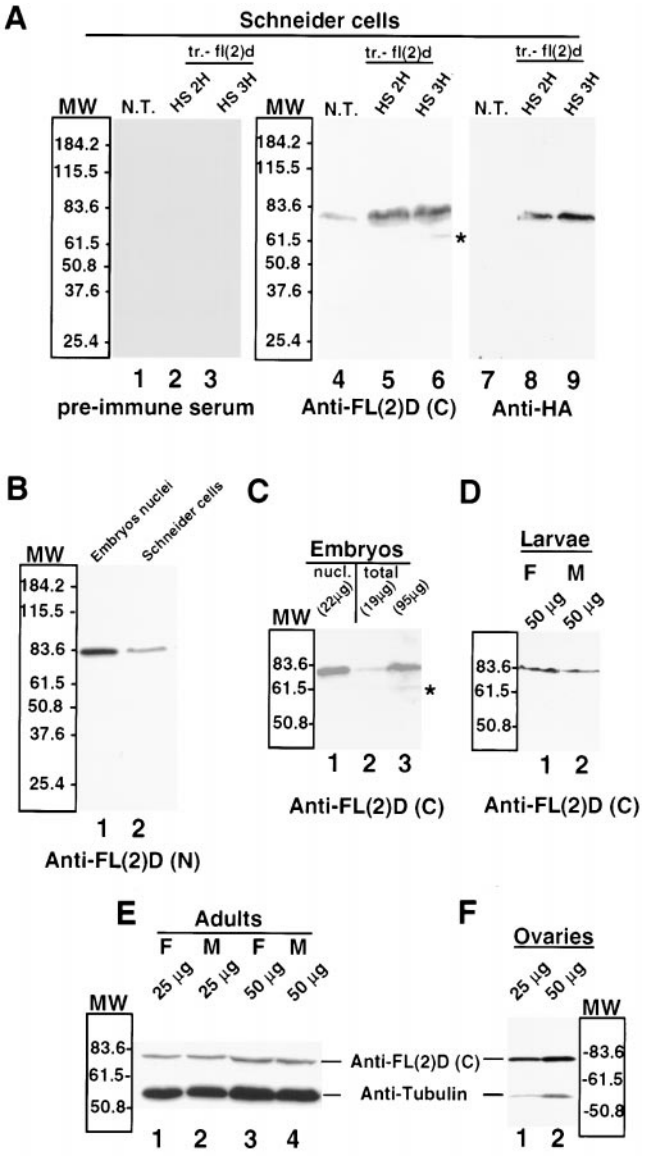

Figure 5.—Expression of Fl(2)d protein. (A) Analysis of *fl(2)d* expression in Schneider cells. Extracts containing 120 μg of protein from Schneider cells nontransfected (lanes 1, 4, and 7), or transfected with pBSHS-Fl(2)d, a plasmid containing an HA-tagged *fl(2)d* cDNA under a heat-shock promoter (lanes 2, 3, 5, 6, 8, and 9), were fractionated on a 10% SDS-polyacrylamide gel and blotted onto nitrocellulose membranes. The blots were probed with preimmune serum (lanes 1–3), antisera against the carboxy terminus of Fl(2)d (lanes 4–6), or anti-HA mouse monoclonal antibody (lanes 7–9). The asterisk indicates a cross-reactive species (see text). (B) Western blot analysis of embryo nuclear extracts (22 μg of protein loaded in lane 1) and nontransfected Schneider cells (120 μg of protein loaded in lane 2) probed with antisera against the amino-terminal part of Fl(2)d. (C) Expression in embryos. Lane 1 corresponds to embryo nuclear extract, and lanes 2 and 3 correspond to total embryonic extract (the amounts of protein are indicated above each lane). The blot was probed with antisera against the carboxy-terminal part of Fl(2)d. The asterisk indicates a cross-reactive species. (D) Presence of Fl(2)d protein in larvae female (F) and male (M) brain and imaginal discs. A total of 50 μg of protein extract was loaded in each case. The blot was probed with antisera against the carboxy-terminal part of Fl(2)d. (E) Analysis of *fl(2)d* expression in female (F) and male (M) heads of adult flies. Immunoblots containing equivalent amounts of protein extracts (as indicated above the lanes) were probed with antisera against the carboxy-terminal part of Fl(2)d and with anti-β-tubulin monoclonal antibodies. (F) Analysis of *fl(2)d* expression in ovaries. Immunoblots containing different amounts of protein extracts (as indicated above the lanes) were probed with antisera against the carboxy-terminal part of Fl(2)d and with anti-β-tubulin monoclonal antibodies.

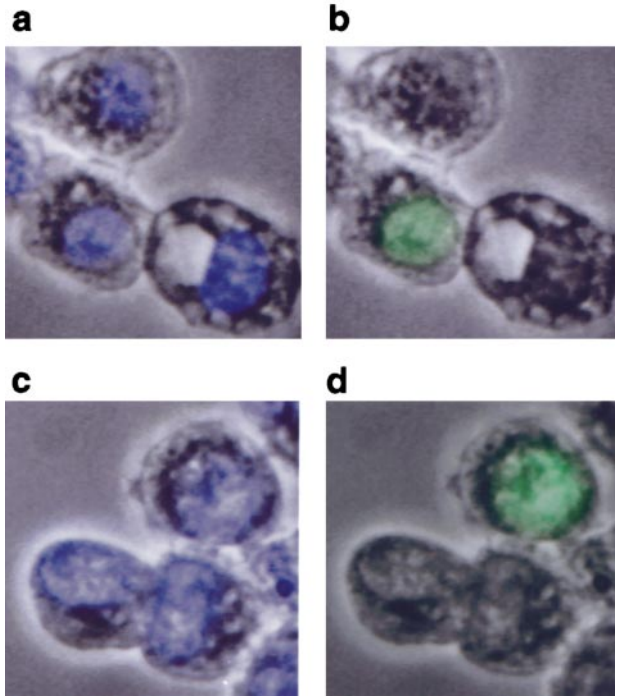

Figure 6.—Subcellular localization of Fl(2)d protein. Schneider cells transfected with pBSHS-HA-Fl(2)d were fixed, permeabilized, and probed with either an anti-HA monoclonal antibody (b), or an anti-Fl(2)d polyclonal antiserum (d), followed by FITC-conjugated secondary antibodies. The preparations were visualized under a fluorescence microscope. a and c show DAPI nuclear staining and conventional light images corresponding to the fluorescence images of b and d, respectively.

An intriguing possibility would be that D180 is involved in determining the specificity of homo- or heterodimerization through this domain and that its mutation affects the ability of the protein to establish interaction with relevant partners.

The female-specific function of *fl(2)d* is related to its requirement for proper splicing regulation of the *Sxl* and *tra* RNAs by the protein Sxl (Granadino *et al.* 1990, 1996). How can Fl(2)d affect the function of Sxl? One possibility is that Fl(2)d plays an important role in post-translational modifications or proper subcellular localization of Sxl. A second possibility is that Fl(2)d has a direct role in the regulation of pre-mRNA splicing. It could act, for example, by facilitating Sxl binding to its target pre-mRNAs or by assisting its repressive activities. These putative functions could be based on direct interactions between Sxl and Fl(2)d. Alternatively, Fl(2)d could be part of a complex in which Sxl functions, which could also include the products of the genes *snf* and *vir*. Finally, a third possibility is that Fl(2)d facilitates the use of the distal splice sites in *Sxl* and *tra* that become activated when Sxl represses the use of the proximal ones. We cannot rule out more indirect effects of Fl(2)d, as is likely to be the case for the recently reported effects on *Sxl* expression of mutations in an aspartyl-tRNA synthetase gene (Stitzinger *et al.* 1999).

The non-sex-specific function of *fl(2)d* remains to be identified. Because *Sxl* activity is not required for male development (Salz *et al.* 1987), mutations that affect both male and female viability cannot be attributed to genetic interactions with *Sxl*. It is also very unlikely that Fl(2)d is a general splicing factor involved in an obligatory step in the splicing reaction. First, no aberrant splicing pattern is detected in *Sxl* and *tra* RNAs in *fl(2)d* mutant males or females (the normal, default sites are used; Granadino *et al.* 1990, 1996). Second, *fl(2)d* mutations are not cell lethal. Particularly interesting are the results of the clonal analysis of $fl(2)d^2$, a mutation that produces a truncated, presumably nonfunctional protein. Clones homozygous for $fl(2)d^2$ induced in $fl(2)d^2/+$ males and females are viable (Granadino *et al.* 1990, 1991), except that in females they develop male instead of female structures, due to the female-specific function of *fl(2)d*. Furthermore, transplanted male germ cells homozygous for $fl(2)d^2$ can develop into functional spermatozoa, whereas transplanted female germ cells homozygous for $fl(2)d^2$ follow an abortive spermatogenetic pathway, which is an indication of a sexual transformation of the mutant germ cells, due to the female-specific function of *fl(2)d* (Granadino *et al.* 1992). If Fl(2)d was a component of the general splicing machinery, neither $fl(2)d^2$ homozygous clones nor mutant germ cells would survive. One possible scenario is that Fl(2)d plays a role in splicing regulation of a gene(s) important for development in addition to those involved in sex determination. Recently, it has been reported that *fl(2)d* appears to be necessary for inclusion of mI and mII microexons in *Ubx* mRNAs (Burnette *et al.* 1999). Other examples of splicing factors that are essential for viability but that are dispensable for processing of multiple pre-mRNAs have been described in yeast, Drosophila, and mammals. For some of these, mutations have been identified that disrupt the splicing of only particular substrates, similar to the effects of the $fl(2)d^1$ mutation (Puig *et al.* 1999).

We hope that the molecular characterization of novel *fl(2)d* mutations generated by mobilization of the *P* element of $fl(2)d^P$ mutation, or the *P* element inserted at the 5′ UTR of a new mutant *fl(2)d* allele present in the Drosophila genome project *P*-element collection, as well as biochemical analyses of the properties of the Fl(2)d protein will provide insights into the molecular mechanism underlying *fl(2)d* function.

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