

Regulation of Sex-Specific Selection of *fruitless* 5' Splice Sites by *transformer* and *transformer-2*

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In *Drosophila melanogaster*, the *fruitless* (*fru*) gene controls essentially all aspects of male courtship behavior. It does this through sex-specific alternative splicing of the *fru* pre-mRNA, leading to the production of male-specific *fru* mRNAs capable of expressing male-specific *fru* proteins. Sex-specific *fru* splicing involves the choice between alternative 5' splice sites, one used exclusively in males and the other used only in females. Here we report that the *Drosophila* sex determination genes *transformer* (*tra*) and *transformer-2* (*tra-2*) switch *fru* splicing from the male-specific pattern to the female-specific pattern through activation of the female-specific *fru* 5' splice site. Activation of female-specific *fru* splicing requires *cis*-acting *tra* and *tra-2* repeat elements that are part of an exonic splicing enhancer located immediately upstream of the female-specific *fru* 5' splice site and are recognized by the TRA and TRA-2 proteins in vitro. This *fru* splicing enhancer is sufficient to promote the activation by *tra* and *tra-2* of both a 5' splice site and the female-specific *doublesex* (*dsx*) 3' splice site, suggesting that the mechanisms of 5' splice site activation and 3' splice site activation may be similar.

Alternative pre-mRNA processing is widely used as a regulatory mechanism for the control of gene expression in higher eukaryotes. Developmentally regulated pre-mRNA processing can give rise to gene expression patterns that are specific with regard to tissue, developmental stage, and sex.

In *Drosophila melanogaster*, the *fruitless* (*fru*) gene regulates essentially all aspects of male courtship behavior, including sexual orientation (33, 54). It does this by functioning in about 0.5% of the neurons in the central nervous system (CNS). *fru* is part of the hierarchy of *Drosophila* sex determination genes (5), where it controls one of the two branches identified downstream of the genes *transformer* (*tra*) and *transformer-2* (*tra-2*) (63). The other branch controls all known aspects of somatic sexual differentiation outside the CNS, as well as certain aspects of sexual differentiation within the CNS, and is regulated by the *doublesex* (*dsx*) gene (13). Fundamental to the sex-specific functions of *fru*, transcripts from the distal *fru* promoter undergo sex-specific alternative splicing (54). In males, a male-specific 5' splice site (5' SS) is used that is located 1,590 nucleotides (nt) upstream of the female-specific 5' SS used in females. Both 5' SS are spliced to a common 3' splice site (3' SS) over 70 kb downstream. Male-specific and female-specific *fru* cDNAs contain a common coding region downstream of the common 3' SS. In males, usage of the distal male-specific 5' SS extends the open reading frame in the 5' direction such that an additional 101 amino acids are present at the N terminus.

Females mutant for either *tra* or *tra-2* exhibit male-specific *fru* splicing and male courtship behavior (54), indicating that *tra* and *tra-2* are required either directly or indirectly for female-specific *fru* splicing. *tra* and *tra-2* are known to activate directly the female-specific *dsx* 3' SS by binding to *cis*-acting *tra* and *tra-2* (*tra/tra-2*) repeat elements, (T/A)C(T/A)(T/A)C(A/G)ATCAACA (27, 30, 53). While *tra-2* is expressed in both sexes (2, 25), *tra*, which is expressed only in females (11), is one of the few cell-specific splicing regulators known to date. In

dsx, the *tra/tra-2* repeat elements are present in six copies which are part of an exonic splicing enhancer located 300 nt downstream of the female-specific *dsx* 3' SS (13, 48). Three copies of the *tra/tra-2* repeat elements are also present upstream of the female-specific 5' SS in *fru* (33, 54), suggesting that regulation of *fru* splicing by *tra/tra-2* may occur directly. However, it is not known whether the *tra/tra-2* repeat elements in *fru* are required for the regulation of *fru* sex-specific splicing.

tra and *tra-2* contain protein domains known to be involved in regulated and general splicing. *tra-2* contains a RRM-type RNA binding domain flanked by two SR domains, regions rich in serine and arginine residues, while *tra* contains a single SR domain. RRM-type RNA binding domains have been shown to be essential for the recognition of pre-mRNAs by a variety of RNA processing factors (12). SR domains have been implicated in protein-protein interactions between splicing factors (37, 68) and also in the intracellular localization of splicing factors to nuclear speckles (41), regions of the nucleus enriched in splicing factors. Other RNA processing factors containing RRM-type RNA binding domains and/or SR domains include the splicing factors of the SR protein family, general splicing factors which can also affect splice site choices (4, 15, 16, 18, 21, 23, 36, 39, 52, 55, 67, 72, 73), and the splicing factors U1-70K (64) and U2AF (74) involved in the general recognition of splice sites.

Recent studies have implicated additional proteins in splicing regulation by *tra/tra-2*. The TRA-2 protein has been reported to interact with the TRA protein (3, 32), the *Drosophila* SR protein RBP1 (29), human SR proteins (68), and the human U2AF³⁵ protein (68). The *Drosophila* SR protein RBP1 participates in the activation of the female-specific *dsx* 3' SS together with *tra/tra-2* by specifically binding to the *dsx* pre-mRNA in the proximity of the female-specific *dsx* 3' SS (28, 43). Mutations in the gene of another *Drosophila* SR protein, B52, inhibit female-specific *dsx* splicing, suggesting indirect or direct involvement of B52 in *dsx* splicing regulation (50). Unlike TRA, SR proteins appear to be ubiquitous, although there are differences in the relative abundance of members of this family in various tissues (71). Two other tissue-specific splicing factors, the soma-specific PSI protein in *Drosophila* (57) and a human neuron-specific 75-kDa protein (46), have been re-

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ported to regulate splicing of their target genes together with particular hnRNP proteins. These findings indicate that in several systems, tissue-specific splicing regulation involves both tissue-specific regulators and target-specific contributions from ubiquitous factors.

Recently, several mammalian *tra-2* homologs have been identified (6, 17, 44, 56). At least one human *tra-2* homolog can functionally replace *tra-2* in *Drosophila* (17), suggesting that the mechanisms governing splice site selection by *tra/tra-2* in *Drosophila* may also be significant in humans. The mechanism by which sex-specific *fru* splicing is regulated, is currently unknown. A priori, *tra/tra-2* could induce female-specific *fru* splicing by either blocking the male-specific 5' SS or activating the female-specific 5' SS. In *fru*, the *tra/tra-2* repeat elements are located between the two sex-specific 5' SSs, 38 nt upstream of the female-specific 5' SS and 1,352 nt downstream of the male-specific 5' SS (54). Thus, the close proximity of the *tra/tra-2* repeat elements to the female-specific *fru* 5' SS would suggest that the likely mechanism of *fru* regulation is 5' SS activation. In the case of *dsx*, instead of affecting 5' SS selection, *tra* and *tra-2* activate the female-specific *dsx* 3' SS (27, 30, 53). Furthermore, the activation model would predict that in *fru* the *tra/tra-2* repeat elements function as an upstream splicing enhancer, while in *dsx* their role is that of a downstream splicing enhancer. Unlike transcriptional enhancers, splicing enhancers can be position dependent. For example, shortening the distance of the *tra/tra-2* repeat elements to the female-specific *dsx* 3' SS inhibits *dsx* splicing regulation by *tra/tra-2* (65). Indeed, while other downstream splicing enhancers, generally containing multiple repeats of the sequence motif GAR, where R is a purine, have been identified in exons of vertebrate genes (14, 19, 24, 31, 40, 51, 62, 66, 69, 70), and several of these purine-rich downstream splicing enhancers are recognized by SR proteins (40, 51, 60, 61), to our knowledge no upstream splicing enhancer has been reported to date. Hence, an involvement of *tra/tra-2* and of the *tra/tra-2* repeat elements in the selection of *fru* 5' SSs would indicate a novel regulatory mechanism.

In order to determine whether *tra/tra-2* regulate *fru* sex-specific splicing by acting through the *tra/tra-2* repeat elements and, if they do, to determine the regulatory mechanism and its relationship to the mechanism of *dsx* splicing regulation, we developed a *fru* splicing assay system. Here we report that *tra* and *tra-2* regulate *fru* sex-specific splicing in transfected *Drosophila* cells. Our data show that *tra/tra-2* induce female-specific *fru* splicing by activating the female-specific *fru* 5' SS.

MATERIALS AND METHODS

Plasmid constructs. To make the *fru* minigene construct fruM+Fwt, a 1.5-kb *NotI-EcoRI* fragment from the class 1 *fru* cDNA (54), including the male-specific 5' SS, was ligated to a 0.645-kb genomic *EcoRI* fragment from phage 1H (54) spanning the *tra/tra-2* repeat elements and the female-specific 5' SS and cloned into pSK+ (Stratagene). A 1.2-kb genomic *PstI* fragment from cosmid HXI (54) including the common 3' SS was placed downstream. A 2.5-kb *BamHI* fragment including the actin 5C promoter (53) was placed upstream and a 0.5-kb *NotI* fragment encompassing the *tra-2* polyadenylation site (53) was placed downstream of the *fru* sequences, respectively. Constructs fruM+Fmut, fruM+FΔB-M, and fruFwt are derivatives of construct fruM+Fwt. In construct fruM+Fmut, the sequence of the *tra/tra-2* repeat elements was changed from TCAATCAACA to GGCAGCTTAC. In construct fruM+FΔB-M, a 1-kb *BbsI-MscI* fragment located between the male-specific *fru* 5' SS and the repeat elements was deleted. Construct fruFwt lacks the *NotI-EcoRI* fragment of construct fruM+Fwt, which contains the male-specific *fru* 5' SS. fruM+REwt and fruM+REmut are derivatives of construct fruM+FΔB-M. Both the 0.645-kb genomic *EcoRI* fragment and all *fru* sequences 4 bp upstream of the male-specific 5' SS were deleted, and PCR fragments encompassing the wild-type (wt) *fru* repeat elements and the mutated *fru* repeat elements, respectively, were inserted upstream of the male-specific 5' SS instead. The *fru* PCR primers used were 5'-TCCCCGGGGAAATTCGAGGACGTGTGA-3' and 5'-TAACCCGGGCGCCAGTTGGTGGGGAT-3'. Construct fruM+REds is a derivative of construct fruM+Fwt lacking the female-specific 5' SS in which the 0.645-kb genomic

EcoRI fragment was replaced by a PCR fragment containing the wt *fru* repeat elements. Construct dsxF+dsxRE contains genomic *dsx* sequences including the 114-bp intron between nt 2066 and 3048^f (13). Construct dsxF+fruRE is a derivative of construct dsxF+dsxRE in which the *dsx* repeat region between nt 2741^f and 3048^f (13) was deleted and a PCR fragment containing the wt *fru* repeat region was inserted instead. The *tra*, *tra-2*, *rbp1* and B52 expression constructs are as described previously (28, 53).

The following probes were used. For constructs fruM+Fwt, fruM+REmut, and fruM+FΔB-M, a 179-bp *BsaHI-BbsI* fragment spanning the male-specific *fru* 5' SS was placed upstream of the 0.645-kb genomic *EcoRI* fragment. For construct fruFwt, the 0.645-kb genomic *EcoRI* fragment was used. For construct fruM+REds, the 179-bp *BsaHI-BbsI* fragment was used. For constructs fruM+REwt and fruM+REmut, *EcoRI* fragments spanning the inserted repeat regions and the male-specific *fru* 5' SS were used. For constructs dsxF+dsxRE and dsxF+fruRE, a *dsx* genomic fragment spanning nt 2066 to 2711^f, including the 114-bp intron (13), was used as a probe.

Transfections and RNase protections. Transfections and RNase protections were performed essentially as described previously (28, 53). If not indicated otherwise, 10 μg of the minigene constructs and 30 to 100 ng of the cDNA expression constructs were transfected. Quantitations of RNase protection products were done by densitometry and were corrected for the different numbers of labeled residues in the protection products.

RT-PCR experiments. Reverse transcription (RT)-PCR experiments on RNA isolated from transfected SL2 cells were carried out using the primers m and c as described previously (54). Male-specific *fru* splicing gave rise to a 164-bp PCR product as expected. Upon cotransfection with *tra/tra-2*, a PCR product 1,754 bp long was detected, as expected for female-specific *fru* splicing. Several PCR products 350 to 1,600 bp long were also detected, possibly indicating cryptic splicing.

RESULTS

Regulation of *fru* sex-specific splicing by *tra* and *tra-2* in transfected *Drosophila* cells. To study the regulation of *fru* sex-specific splicing, we began by establishing a *fru* splicing assay system in transfected *Drosophila* Schneider (SL2) cells. As is characteristic for male somatic tissue, Schneider cells express endogenously a functional *tra-2* transcript, but no functional *tra* transcript (53). We constructed a *fru* minigene fruM+Fwt, driven by an actin promoter, which contains the *fru* genomic region spanning the male-specific 5' SS, the *tra/tra-2* repeat elements, and the female-specific 5' SS, fused to the downstream common 3' SS (see Fig. 2C). In this construct, the *fru* intron flanked by the female-specific 5' SS and the common 3' SS, over 70 kb long (Fig. 1), was shortened to 1.3 kb. Construct fruM+Fwt includes 300 bp of intron sequence downstream of the female-specific 5' SS and 1,000 bp of intron sequence upstream of the common 3' SS, respectively. Transient transfections into *Drosophila* Schneider (SL2) cells were carried out, and *fru* splicing products were detected by an RNase protection assay.

The male-specific *fru* 5' SS and the female-specific *fru* 5' SS are 1,590 nt apart. To visualize usage of the male and female 5' SSs simultaneously, the probe used contains two fragments synthesized in one piece which hybridize across the female 5' SS and the male 5' SS, respectively (Fig. 2C). Usage of the female and male 5' SSs are expected to generate the specific RNase protection products F and M, respectively (Fig. 1C). In addition, protection product MF will be generated from transcripts spliced in the female pattern, from unspliced RNA, and also in cases in which there is usage of a cryptic 5' SS located between the female and male 5' SSs. No endogenous *fru* transcript was detected in SL2 cells, as shown in Fig. 2A, lane 1. We observed that in the absence of cotransfected *tra/tra-2* expression constructs, the *fru* minigene fruM+Fwt is spliced mainly in the male pattern (Fig. 2A, lane 2) with a molar ratio of male (M) to female (F) protection products (M/F) of 6. The protection products were quantitated by densitometry, and quantitations were corrected for the different numbers of labeled residues in the protection products. No unspliced fruM+Fwt RNA was detected (not shown). A low ratio of protection product F to protection product MF (F/MF = 0.17) in the

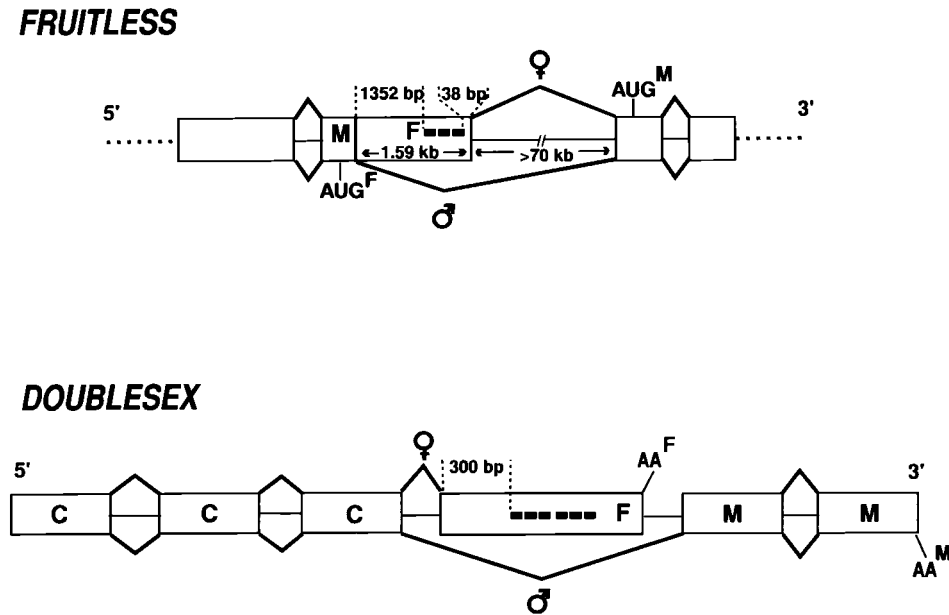


FIG. 1. Patterns of *fru* and *dsx* sex-specific splicing. Schematic drawings of *fruitless* (*fru*) and *doublesex* (*dsx*) splicing patterns are shown. Only part of the *fru* gene is depicted. Exons (boxes), introns (thin horizontal lines), and splices (thick lines) are indicated. Female-specific splicing patterns are indicated above and male-specific splicing patterns are indicated below each gene. Male- (M) and female- (F)-specific exon segments and common exons (C) are indicated. The *tra/tra-2* repeat elements are indicated by thick dashes, and distances of the *tra/tra-2* repeat elements to the sex-specific splice sites are given. Female-specific (AUG^F) and male-specific (AUG^M) translational start codons as well as female-specific (AA^F) and male-specific (AA^M) polyadenylation sites are shown.

absence of cotransfected *tra/tra-2* (Fig. 2A, lane 2) probably reflects the presence of cryptic splicing products (Fig. 1C), as also indicated by RT-PCR experiments (data not shown; see Materials and Methods).

To test for regulation of *fru* sex-specific splicing by *tra* and *tra-2*, we cotransfected *tra* and *tra-2* expression constructs. Significantly, when both *tra* and *tra-2* expression constructs were cotransfected together with the *fru* minigene, a shift to purely female-specific 5' SS usage occurs (Fig. 2A, lane 5), as indicated by an increase in spliced female product F, by the absence of detectable product M, and by a ratio of protection product F to protection product MF (F/MF) of 1.1. Switching from the male-specific 5' SS to the female-specific 5' SS by *tra/tra-2* was also confirmed by RT-PCR assay (data not shown; see Materials and Methods) and depends on the concentrations of the *tra/tra-2* expression constructs (compare Fig. 2B, lane 1, and Fig. 2A, lane 5). In Fig. 2B, lane 1, the ratio of product M to product F is 1.38 and the ratio of product F to product MF is 0.19. Expression of *tra* alone (Fig. 2A, lane 3) or *tra-2* (Fig. 2A, lane 4) induces only a slight shift to female-specific *fru* splicing, as indicated by a ratio of protection product F to protection product MF (F/MF) of 0.28. Thus, transfection of both *tra* and *tra-2* is required to switch *fru* splicing precisely and efficiently from the male-specific 5' SS to the female-specific 5' SS in cultured Schneider cells. These results in our assay system are consistent with results obtained in *Drosophila*, where female flies require both *tra* and *tra-2* for female-specific *fru* splicing (54).

We also cotransfected the *Drosophila* SR protein RBP1 in our *fru* splicing assay. Expression of RBP1 in transfected Schneider cells was shown to induce female-specific *dsx* splicing (28). We found that transfection of RBP1 can also induce some female-specific *fru* splicing (Fig. 2A, lane 6), suggesting that RBP1 may also play a role in the regulation of *fru* splicing. A potential RBP1 target site (ATCCCCA) (28) is present 12 nt upstream of the female-specific *fru* 5' SS. As a control, trans-

fection of the *Drosophila* SR protein B52 (16, 52) did not affect *fru* splicing (Fig. 2A, lane 7).

The *tra/tra-2* repeat elements are required for *fru* splicing regulation by *tra/tra-2*. To determine whether the *tra/tra-2* repeat elements are essential for regulation of *fru* splicing by *tra* and *tra-2*, we tested a construct, fruM+FREmut, in which the sequence of the conserved part of the *tra/tra-2* repeat elements was changed from TCAATCAACA to GGCAGCTTAC. The probe used is the same as in Fig. 2, and since it contains wt *tra/tra-2* repeat elements, generates short loop-outs when hybridized to the mutant *tra/tra-2* repeat elements in fruM+FREmut, resulting in a shortened female-specific RNase protection product (Fig. 3A, lanes 1 and 2). Inefficient cutting of these short loop-outs by RNase A also generates a larger protection product derived from spliced female product which is marked by an asterisk (Fig. 3A). We observed that in construct fruM+FREmut, switching to female *fru* splicing by *tra* and *tra-2* was almost completely blocked, as indicated by the presence of significant amounts of male splicing product M in the presence of cotransfected *tra* and *tra-2* (Fig. 3A, lane 2). In contrast, deletion of a 1-kb fragment between the repeat elements and the male-specific 5' SS, as in construct fruM+FΔB-M (Fig. 3B), did not affect regulation of *fru* splicing by *tra* and *tra-2* (Fig. 3A, lanes 3 and 4). These findings show that the *tra/tra-2* repeat elements are required for regulation of *fru* splicing by *tra* and *tra-2*, suggesting that *tra* and *tra-2* promote female-specific *fru* splicing by acting through these elements.

Evidence for activation of the female-specific *fru* 5' SS as the mechanism of *fru* splicing regulation. To determine whether *tra* and *tra-2* induce female-specific *fru* splicing by blocking the male-specific 5' SS or by activating the female-specific 5' SS, we tested constructs that contain upstream of the common 3' SS (i) either the male-specific 5' SS, including the *tra/tra-2* repeats (construct fruM+REds [Fig. 4C]), or (ii) the female-specific 5' SS, including the *tra/tra-2* repeats (construct fruFwt [Fig. 4C]). The *tra/tra-2* repeat elements were included at the

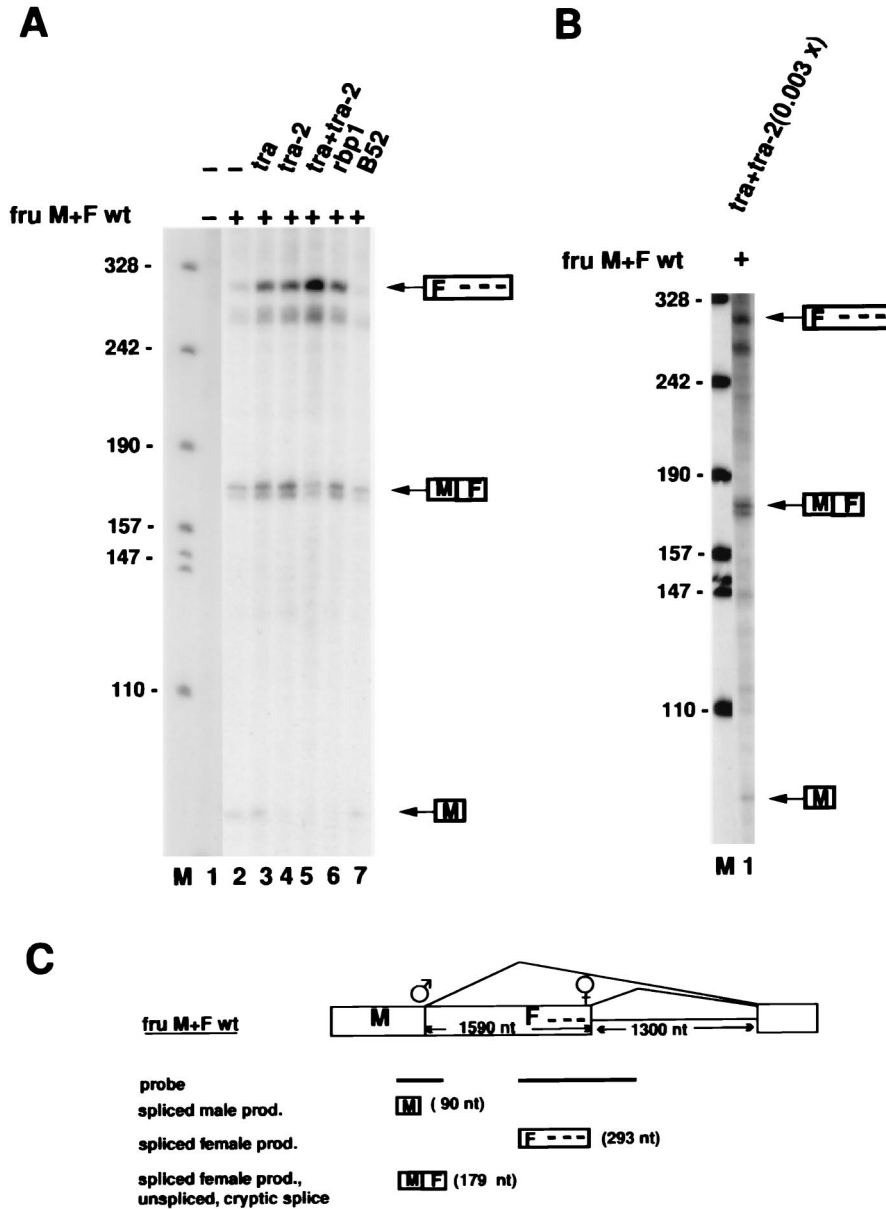


FIG. 2. Regulation of *fru* sex-specific splicing in transfected Schneider cells. (A) The *fru* minigene construct fruM+Fwt was transfected together with various combinations of cDNA expression constructs encoding *tra*, *tra-2*, *rbp1*, and B52 as indicated (28, 53) into Schneider cells, and *fru* splicing was analyzed by RNase protection assays. The presence (+) or absence (-) of construct fruM+Fwt and of the *tra*, *tra-2*, *rbp1*, and B52 expression constructs in the transfection experiments is indicated above each lane. Splicing products are identified to the right of the autoradiograph. Lane M contains pSK+ DNA cut with *Hpa*II and used as a nucleotide length marker. The positions of size markers (in nucleotides) are shown to the left of the autoradiographs in panels A and B. (B) The amount of cotransfected *tra* and *tra-2* expression constructs was titrated down to 0.3% (0.1 ng) of the amount transfected in panel A. (C) Depiction of construct fruM+Fwt. Exons are represented by boxes, and the intron is represented by a thin horizontal line. The positions of the male-specific and female-specific 5' SSs are indicated, and male-specific (M) and female-specific (F) exon segments are identified. The three dashes upstream of the female-specific 5' SS represent the *tra/tra-2* repeat elements. The antisense RNase protection probes are indicated directly below the map by horizontal bars. RNase protection products (prod.) derived from male-specific and female-specific *fru* splicing are given at the bottom of the panel, and the expected product lengths are given in parentheses. The distance between the male-specific and female-specific *fru* 5' SSs is 1,590 nt. The intron is 1,300 nt long. Note that the RNase protection probes and the protection products are not drawn to scale. Protection product MF can be obtained as a consequence of female splicing or use of cryptic 5' splice sites or from unspliced pre-mRNA.

authentic position relative to the splice sites, 1,352 nt downstream of the male-specific 5' SS and 38 nt upstream of the female-specific 5' SS, respectively. We did observe activation of the isolated female-specific 5' SS by *tra/tra-2*, as indicated by the disappearance of unspliced RNA upon cotransfection with *tra/tra-2* (Fig. 4A, lanes 1 and 2). A shorter exposure of the same gel (Fig. 4A, lanes 3 and 4) also shows an increase in

spliced female product by *tra/tra-2* (Fig. 4A, lane 4), suggesting that the effect observed in Fig. 4A, lane 2, is not due to instability of unspliced pre-mRNA. While spliced female product is seen to increase in Fig. 4A, lane 4, the background bands below the spliced female product in Fig. 4A, lanes 3 and 4, remain constant, suggesting equal loading of RNA. Usage of the isolated female-specific 5' SS in the absence of cotransfected *tra*

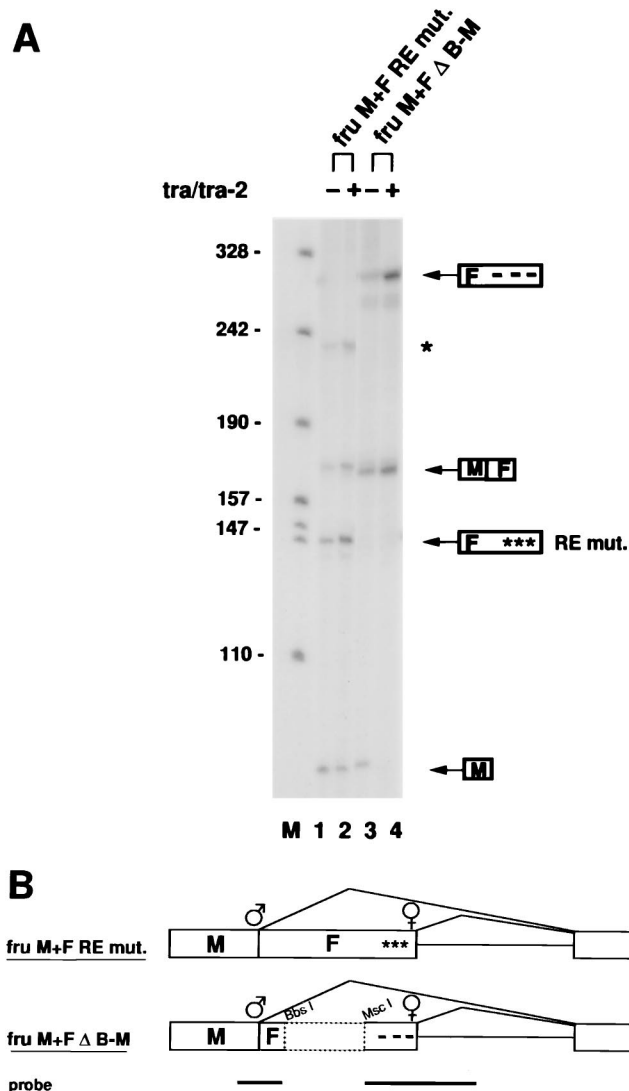


FIG. 3. *cis*-acting elements required for regulation of *fru* splicing by *tra/tra-2*. (A) Constructs carrying mutations within the *tra/tra-2* repeat elements (construct fruM+FREmut) or a deletion of sequences between the male-specific *fru* 5' SS and the *tra/tra-2* repeat sequences (construct fruM+FΔB-M) were transfected in the absence (-) or presence (+) of cotransfected *tra/tra-2* as indicated above the RNase protection assay autoradiograph. RNase protection products are indicated to the right of the autoradiograph and are as described in the legend to Fig. 2C. An additional RNase protection product (RE mut.) is derived from female-specific splicing of construct fruM+REmut. An incompletely cleaved protection product derived from female-specific splicing of construct fruF+MRE mut is indicated by an asterisk. The positions of size markers (in nucleotides) are shown to the left of the autoradiograph. Lane M contains pSK+ DNA cut with *Hpa*II. (B) Illustration of constructs fruM+FREmut and fruM+FΔB-M. fruM+FREmut contains mutated *tra/tra-2* repeat elements indicated by asterisks. In fruM+FΔB-M, a 1-kb *Bbs*I-*Msc*I fragment indicated by a broken-line box was deleted. The probe used in these experiments is as described in the legend to Fig. 2.

and *tra-2* (Fig. 4A, lane 1) is probably due to the lack of the competing male-specific *fru* 5' SS, since the deletion of sequences located between the male-specific *fru* 5' SS and the *fru* repeat region, as in construct fruM+FΔB-M (Fig. 3B), does not affect *fru* splicing. Similarly, the isolated female-specific *dsx* 3' SS is also used in the absence of cotransfected *tra/tra-2*, as shown previously (53) and as also reproduced in this study (see Fig. 6). In contrast, the male-specific *fru* 5' SS was found to be

unaffected by cotransfection with *tra/tra-2* (Fig. 4B, lanes 1 and 2). Taken together, these results suggest that *tra/tra-2* induce female-specific *fru* splicing by activating the female-specific *fru* 5' SS, as also suggested by the proximity of the *tra/tra-2* repeat elements to the female-specific 5' SS. Thus, in *fru*, the *tra/tra-2* repeat region functions as an upstream splicing enhancer.

The *fru* repeat region is sufficient to promote the activation of a 5' SS. We next wanted to address the question of whether the *tra/tra-2* repeat region in *fru* is sufficient to promote the activation of a 5' SS by *tra/tra-2*. Since we showed that the male-specific *fru* 5' SS is normally unaffected by *tra/tra-2* (Fig. 4), we inserted a 300-bp fragment of *fru* containing the *tra/tra-2* repeats 4 nt upstream of the male-specific *fru* 5' SS (construct fruM+REwt [Fig. 5B]). Interestingly, spliced male product was detected upon cotransfection with *tra/tra-2* (Fig. 5A, lane 2), suggesting activation of the male-specific *fru* 5' SS by *tra/tra-2* in this hybrid construct. For a control, when the same fragment carrying mutations within the *tra/tra-2* repeat elements was inserted 4 nt upstream of the male-specific *fru* 5' SS, as in construct fruM+REmut (Fig. 5B), no spliced male product in the presence of *tra/tra-2* was detected. Two separate probes, complementary to the constructs fruM+REwt and fruM+REmut, respectively, were used in these experiments. Thus, the *tra/tra-2* repeat elements are essential to promote the activation of a heterologous 5' SS by *tra/tra-2*. Lack of usage of the male-specific 5' SS in the constructs fruM+REwt and fruM+REmut in the absence of cotransfected *tra/tra-2* (Fig. 5A, lanes 1 and 3) was found to be due to the deletion of a stretch of sequence upstream of the male-specific 5' SS in these constructs. The insertion of the *fru* repeat region in either orientation does not affect the usage of the male-specific *fru* 5' SS in the absence of cotransfected *tra/tra-2* (data not shown).

The *fru* repeat region and the *dsx* repeat region are functionally interchangeable. Our study shows that in *fru*, the *tra/tra-2* repeat region functions as an upstream splicing enhancer required for the activation of the female-specific *fru* 5' SS by *tra/tra-2*. In contrast, *tra/tra-2* activate the female-specific *dsx* 3' SS by recognizing *tra/tra-2* repeat elements present downstream of this 3' SS (27, 30, 53). To address the question of whether the *tra/tra-2* repeat regions in *fru* and *dsx* are functionally interchangeable, we inserted the *tra/tra-2* repeat region from *fru* downstream of the female-specific *dsx* 3' SS (Fig. 6B). For the activation of the female-specific *dsx* 3' SS by *tra/tra-2* to occur, the *dsx* repeat region has to be positioned at a distance of 300 bp downstream of this 3' SS (65). To ensure positioning of the *fru* repeat region at this precise location, we placed the *fru* repeat region downstream of a *dsx* PCR fragment ending immediately upstream of the *dsx* repeat region, generating construct dsxF+fruRE (Fig. 6B), which lacks the competing downstream male-specific *dsx* 3' SS (compare diagram with that in Fig. 1). For a control, splicing of a construct dsxF+dsxRE (Fig. 6B) containing the *dsx* repeat region was tested. As shown previously, due to default usage of the isolated female-specific *dsx* 3' SS, activation of the female-specific *dsx* 3' splice site by *tra/tra-2* is indicated by a decrease in unspliced RNA in a construct lacking the male-specific *dsx* 3' SS (53) and is also demonstrated here (Fig. 6A, lanes 1 and 2). We found that the *fru* repeat region promotes the activation of the female-specific *dsx* 3' SS by *tra/tra-2* almost as efficiently (Fig. 6A, lanes 3 and 4) as the *dsx* repeat region (Fig. 6A, lanes 1 and 2), demonstrating that the *fru* and *dsx* repeat regions can be functionally interchangeable in terms of *dsx* regulation.

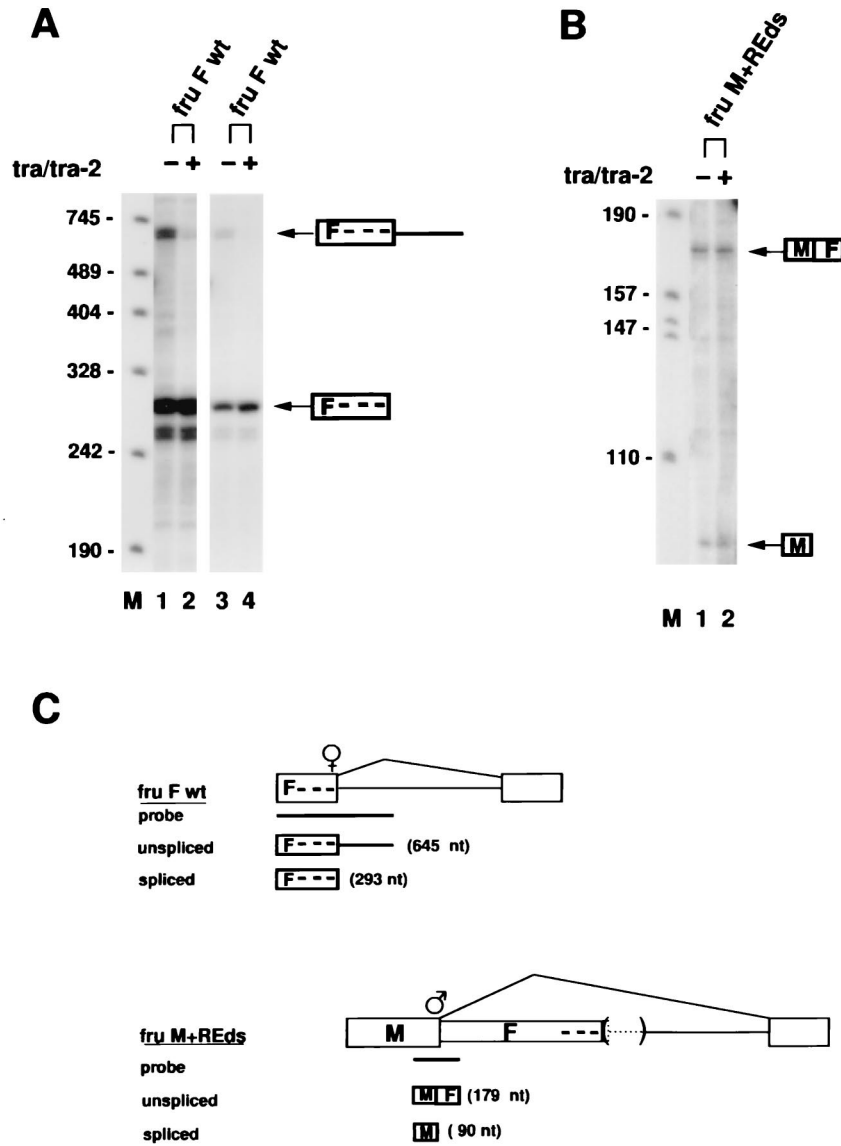


FIG. 4. Testing the activation and the blockage model of *fru* regulation. (A) Construct fruFwt containing the female-specific *fru* 5' SS and the *tra/tra-2* repeat elements was transfected in the absence (-) or presence (+) of cotransfected *tra/tra-2* as indicated above the RNase protection assay autoradiograph. Lanes 3 and 4 show a shorter exposure of lanes 1 and 2, respectively. Lane M contains pSK+ DNA cut with *Hpa*II. The positions of size markers (in nucleotides) are shown to the left of the autoradiographs in panels A and B. (B) Construct fruM+REds containing the male-specific *fru* 5' SS and the *tra/tra-2* repeat elements was transfected in the absence (-) or presence (+) of cotransfected *tra/tra-2* as indicated above the RNase protection assay autoradiograph. RNase protection products derived from spliced and unspliced pre-mRNAs of constructs fruFwt and fruM+REds are indicated to the right of the autoradiograph. (C) Illustration of constructs fruFwt and fruM+REds. fruFwt contains the female-specific *fru* 5' SS, and fruM+REds contains the male-specific *fru* 5' SS. The *tra/tra-2* repeat elements were included at the correct distance upstream of the female-specific 5' SS and downstream of the male-specific 5' SS, respectively. The deletion of the female-specific *fru* 5' SS in construct in fruM+REds is indicated by parentheses. The probes used in the RNase protection experiments are represented by horizontal bars below each construct. RNase protection products corresponding to spliced and unspliced pre-mRNAs are shown below each construct.

DISCUSSION

Here we report that *tra* and *tra-2* induce female-specific *fru* splicing by activating the female-specific *fru* 5' SS. Our results also suggest that *tra* and *tra-2* regulate *fru* splicing directly, since we show that *fru* splicing regulation requires the *tra/tra-2* repeat elements recognized by *tra* and *tra-2*. Hence, the *tra* and *tra-2* genes are functioning directly upstream of the *fru* gene. Although our results explain how female-specific *fru* splicing is achieved, it remains to be determined how male flies ensure exclusively male *fru* splicing. Several scenarios are conceivable. The female-specific *fru* 5' SS could be a weak 5' SS, causing default usage of the male-specific *fru* 5' SS in males when *tra* is

not expressed. This model is analogous to the situation in *dsx*, where the regulated female-specific *dsx* 3' SS is a weak 3' SS due to a purine-rich polypyrimidine tract (13). Both the sequences of the female (TCG/GTAAGT) and male (TAG/GTAAGC) *fru* 5' SSs match the *Drosophila* 5' SS consensus sequence MAG/GTRAGT (47) in 7 of 9 nt. However, the precise sequence context of the 9-nt consensus sequence of the 5' SS has been proposed to affect 5' SS usage (1). Alternatively, yet unidentified *trans*-acting factors could either block the female-specific *fru* 5' SS or activate the male-specific *fru* 5' SS in male flies. Our observation that the deletion of sequences upstream of the male-specific *fru* 5' SS inhibits male *fru* splic-

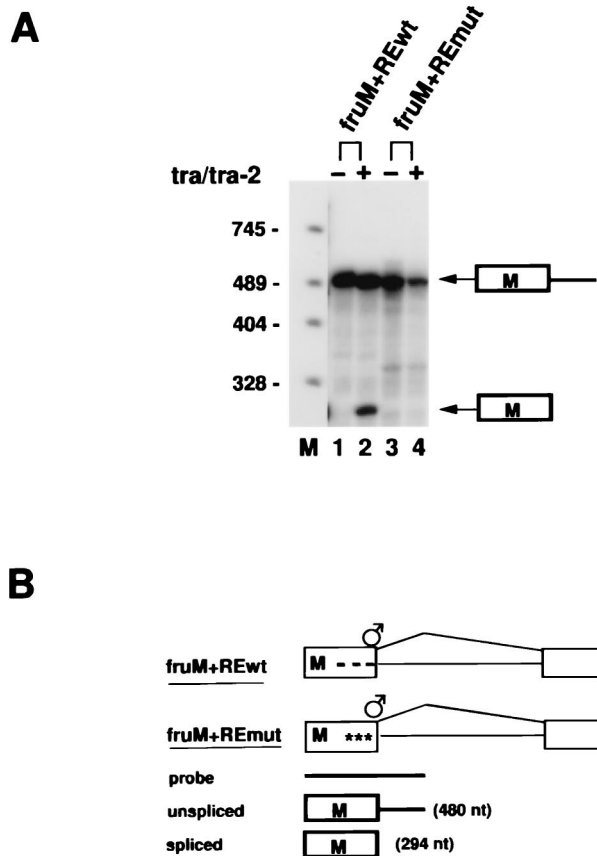


FIG. 5. Activation of a heterologous 5' SS by *tra/tra-2*. (A) Construct fruM+REwt containing wt *tra/tra-2* repeat elements upstream of the male-specific *fru* 5' SS and construct fruM+REmut containing mutant *tra/tra-2* repeat elements upstream of the male-specific *fru* 5' SS were transfected in the absence (-) or presence (+) of cotransfected *tra/tra-2* as indicated above the RNase protection assay autoradiograph. RNase protection products derived from spliced and unspliced pre-mRNAs are indicated to the right of the autoradiograph and are as described in the legend to Fig. 5B. Lane M contains pSK+ DNA cut with *Hpa*II. The positions of size markers (in nucleotides) are shown to the left of the autoradiograph. (B) Illustration of constructs fruM+REwt and fruM+REmut. The wt *fru* repeat region is indicated by three dashes, and the mutated *fru* repeat region is indicated by three asterisks. The repeat mutations in construct fruM+REmut are as in construct fruM+REmut. For each construct, a specific RNase protection probe hybridizing across the splice sites was generated and is indicated by a horizontal bar below the constructs. RNase protection products corresponding to spliced and unspliced pre-mRNAs are shown below the constructs.

ing could indicate that a mechanism for activating the male-specific *fru* 5' SS exists.

Activation of the proximal female-specific *fru* 5' SS by *tra* and *tra-2* represents a previously unknown functional property of *tra/tra-2*. Although increasing the concentration of the human SR protein ASF/SF2 induces the usage of proximal 5' SSs (22, 38), the mechanism of action of ASF/SF2 appears to be fundamentally different from what we know about the mechanism that leads to the usage of a proximal 5' SS in *fru*. ASF/SF2 has been shown to have a general affinity to 5' SSs (77) and stabilizes the recognition of 5' SSs by U1 small nuclear ribonucleoprotein particle (snRNP) (20, 59). Increasing the affinity of U1 snRNP to all 5' SS in a pre-mRNA molecule has been proposed to lead to the preferential usage of the 5' SS closest to a 3' SS (20). Splicing to a distal 5' SS in the human caldesmon gene is promoted by a purine-rich downstream splicing enhancer, although the regulatory factor(s) involved is

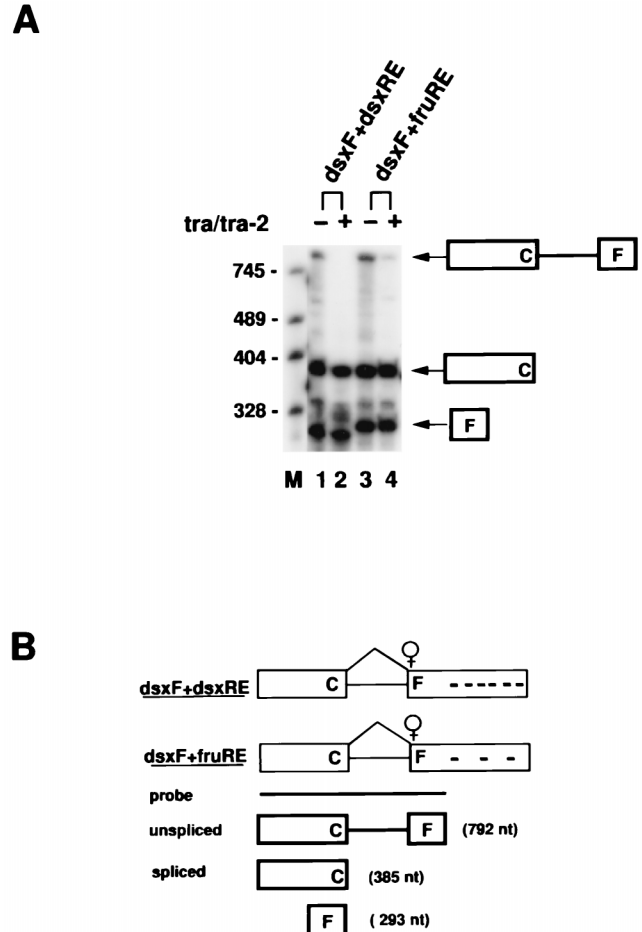


FIG. 6. The *fru* and *dsx* repeat regions are functionally interchangeable. (A) Construct dsxF+dsxRE containing the *dsx* repeat region downstream of the female-specific *dsx* 3' SS and construct dsxF+fruRE containing the *fru* repeat region downstream of the female-specific *dsx* 3' SS were transfected in the absence (-) or presence (+) of cotransfected *tra/tra-2* as indicated above the RNase protection assay autoradiograph. RNase protection products derived from spliced and unspliced pre-mRNAs are indicated to the right of the autoradiograph and are as shown in panel B. The slightly lower mobility of the protection product F derived from construct dsxF+fruRE (lanes 3 and 4) compared to that of product F derived from construct dsxF+dsxRE (lanes 1 and 2) is due to a short stretch of polylinker present in construct dsxF+fruRE and in the probe, but not in construct dsxF+dsxRE. Lane M contains pSK+ DNA cut with *Hpa*II. The positions of size markers (in nucleotides) are indicated to the left of the autoradiograph. (B) Illustration of constructs dsxF+dsxRE and dsxF+fruRE. The *fru* repeat region is indicated by three dashes, and the *dsx* repeat region is indicated by six dashes. The common (C) and the female (F) *dsx* exon are indicated (compare diagram with that in Fig. 1). The probe used in the RNase protection experiments is represented by a horizontal bar below the constructs. RNase protection products corresponding to spliced and unspliced pre-mRNAs are shown at the bottom of the panel.

not known (31). In contrast, *tra/tra-2* specifically select a 5' SS for activation depending on the presence of the upstream *tra/tra-2* repeat elements.

Involvement of *tra/tra-2* in both 5' SS activation in *fru* and 3' SS activation in *dsx* shows that *tra* and *tra-2* are multifunctional splicing regulators. *tra-2* (but not *tra*) is also involved in regulating splicing of the *tra-2* pre-mRNA (45) and the *exuperantia* pre-mRNA (26) in the male germ line, although it is not known whether these *tra-2* functions involve *tra/tra-2* repeat elements. Other examples of multifunctional splicing factors have been described both in *Drosophila* and in humans. The *Drosophila* *Sxl* protein regulates splicing of the *Sxl* (9), *tra* (11, 49, 58), and

msl-2 (7, 34, 75) pre-mRNAs and affects *msl-2* translation (8, 35). The human U1 snRNP-specific protein A is also involved in pre-mRNA polyadenylation (10, 42). Functional activity of the *fru* repeat region in promoting *dsx* 3' SS activation by *tra/tra-2* suggests that the mechanisms of 5' SS activation and 3' SS activation are surprisingly similar. 5' SSs and 3' SSs are defined by distinct sequence motifs and are specifically recognized early in spliceosome assembly by general splicing factors. While 5' SSs are recognized by U1 snRNP (76) and by the SR protein ASF/SF2 (77), U2 snRNPs and U2AF bind to the 3' ends of introns (74). SR proteins have also been shown to engage in protein-protein interactions both with U1 snRNPs and with U2AF (37, 68). Since *tra* and *tra-2* can interact with U2AF and with SR proteins (29, 68), it is conceivable that by binding to *tra/tra-2* repeat elements, *tra/tra-2* can stabilize the recognition of both a specific 5' SS and a 3' SS by general splicing factors. Thus, activation of a 5' SS versus a 3' SS by *tra/tra-2* could depend solely on the interactions of *tra/tra-2* with general splicing factors recognizing 5' SSs on the one hand and 3' SSs on the other. Alternatively, additional regulatory factors specific to *fru* 5' SS activation and/or *dsx* 3' SS activation could be involved. There is evidence that the regulation of *dsx* splicing involves *dsx*-specific features. The position of the *dsx* repeat region 300 nt downstream of the female-specific *dsx* 3' SS is conserved between *D. melanogaster* and *Drosophila virilis* (12a) and is essential for *dsx* regulation by *tra/tra-2* (65). In contrast, purine-rich downstream splicing enhancers appear to be generally found significantly closer to the upstream intron, at distances ranging from 3 to 117 nt (24, 40, 51, 66), and placing a purine-rich splicing enhancer beyond 293 nt downstream of the 3' SS inhibits splice site activation (40). Furthermore, activation of the female-specific *dsx* 3' SS involves the *Drosophila* SR protein RBP1 which recognizes evolutionarily conserved RNA target sequences present within the unusual purine-rich polypyrimidine tract of the female-specific *dsx* 3' SS and within the *dsx* repeat region (28). Further experiments are needed to identify possible additional players in *fru* and *dsx* splicing regulation and to define the molecular interactions involved.

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