Regulation of *doublesex* pre-mRNA processing occurs by 3'-splice site activation

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Sex-specific alternative processing of the *doublesex* (*dsx*) pre-mRNA controls somatic sexual differentiation in *Drosophila melanogaster*. Processing in the female-specific pattern results from the utilization of an upstream 3'-terminal exon and requires the activities of both the *transformer* (*tra*) and *transformer-2* (*tra-2*) genes. Use of the more downstream male-specific terminal exons does not require the activities of these genes and is thus considered the default *dsx*-processing pattern. Here, we used transient expression of *dsx* pre-mRNAs in the presence or absence of *tra* and *tra-2* gene products in *Drosophila* tissue culture cells to investigate the molecular mechanism controlling this alternative RNA-processing decision. These studies reveal that female-specific processing of *dsx* pre-mRNA is controlled by *tra* and *tra-2* through the positive regulation of female-specific alternative 3'-terminal exon use. Delineation of *cis*-acting sequences necessary for regulation shows that a 540-nucleotide region from within the female exon is both necessary and sufficient for regulation. In addition, utilization of the female-specific 3'-splice site (3'SS) is regulated independently of female-specific polyadenylation. Regulated polyadenylation was obtained only in the presence of splicing, suggesting that activation of female-specific exon use occurs by 3'SS activation.

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Alternative splicing and polyadenylation of pre-mRNA are ubiquitous means by which higher eukaryotes generate protein diversity. The use of alternative RNA processing sites can result in cell type-specific expression of protein isoforms and can also serve to control gene expression by producing functional versus nonfunctional mRNA (for reviews, see Bingham et al. 1988; Smith et al. 1989; McKeown 1990; Maniatis 1991). A survey of known cases of alternative processing shows that nearly every possible combination and arrangement of 5'-splice sites (5'SS), 3' splice sites (3'SS), and polyadenylation sites can be used differentially to produce alternative transcripts (Smith et al. 1989). In addition, a number of pre-mRNAs are known to be spliced in alternative ways in different developmental contexts, implying the existence of both *cis*-acting sequences and *trans*-acting factors that regulate RNA-processing site selection.

Cis-acting sequences that influence splice-site selection include the location and sequence of the 5'-splice site (Aebi et al. 1987; Fu and Manley 1987; Zhaung et al. 1987; Peterson and Perry 1989; Lear et al. 1990; Kuo et al. 1991) and branch point/polypyrimidine tract (Fu et al. 1988; Noble et al. 1988; Reed and Maniatis 1988; Helfman and Ricci 1989; Nelson and Green 1989; Smith and Nadal-Ginard 1989; Sosnowski et al. 1989; Wu and Manley 1989; Zhuang and Weiner 1989; Goux-Pelletan et al. 1990; Mullen et al. 1991), as well as other less well-defined non-splice-site sequences that lie within exons or introns (for review, see Smith et al. 1989). In several

studies it has been demonstrated that the efficiency of splice-site recognition by the splicing machinery plays a significant role in the control of alternative splicing decisions (Zhuang et al. 1987; Noble et al. 1989; Kuo et al. 1991). However, the mechanisms by which splice-site usage is modulated in a cell type-specific manner are not understood.

Only a few *trans*-acting factors have been identified that regulate alternative RNA-processing choices. In two cases the reproduction of differential processing in vitro with nuclear extracts from different cell types allowed the characterization of factors that affect alternative processing choices. One involves differential usage of two alternative 5'-splice sites in the SV40 early pre-mRNA (Ge and Manley 1990), and the other involves retention of an intron in the *Drosophila* P transposable element pre-mRNA (Siebel and Rio 1990). For the case of SV40 early pre-mRNA, the ability to reproduce regulation in vitro has led to the isolation of a gene encoding a protein factor, ASF, that controls 5'-splice site choice (Ge et al. 1991). This same factor was isolated independently as a general splicing factor, called SF2 (Krainer et al. 1990; 1991).

Other genes known to regulate RNA processing include six genes: the *suppressor-of-white apricot* [*su(w^a)*; Zachar et al. 1987]; *Sex-lethal* (*Sxl*; Bell et al. 1991); *transformer* (*tra*; Nagoshi et al. 1988); and *transformer-2* (*tra-2*; Mattox and Baker 1991) genes of *Drosophila*, and genes encoding a yeast and a *Xenopus* ribosomal protein

(Bozzoni et al. 1984; Dabeva et al. 1986; Cafferelli et al. 1987; Eng and Warner 1991). With the exception of *tra*, the products of each of these genes regulate the splicing of their own pre-mRNAs. The *Sxl*, *tra* and *tra-2* genes also regulate the processing of pre-mRNAs from other genes (Nagoshi et al. 1988). *Sxl* and *tra-2* encode proteins that contain RNA-binding consensus sequences (Amrein et al. 1988; Bell et al. 1988; Goralski et al. 1989), strongly suggesting that their protein products play direct roles in regulating RNA processing. Insight into how the *Sxl* protein regulates pre-mRNA splicing has come from studies of the control of *Sxl* of the use of alternative 3'SS in the *tra* gene pre-mRNA (Boggs et al. 1987; Sosnowski et al. 1989; Inoue et al. 1990), where *Sxl* protein functions to repress use of the default acceptor (Sosnowski et al. 1989).

The product of the *tra-2* gene, in conjunction with the product of the *tra* gene, functions in somatic cells of females to regulate the processing of the pre-mRNA of the *doublesex* (*dsx*) gene (McKeown et al. 1988; Nagoshi et al. 1988). The female-specific *dsx* product is generated by the splicing of a common 5' region to a female-specific terminal exon that is located upstream of two male-specific terminal exons. In the absence of *tra* and/or *tra-2* activity (either in a wild-type male or a female carrying a mutation in the *tra* and/or *tra-2* genes), splicing to the male-specific terminal exons occurs, suggesting that this is the default pattern for *dsx* RNA processing. On the basis of the finding that all four mutations that lead to the default (or male-specific) pattern of *dsx* RNA processing are located within the female-specific *dsx* exon, it was suggested that regulation by *tra* and *tra-2* occurs by activating use of the female-specific exon rather than by blocking use of the male-specific exons. All four of these *dsx* mutations either displace or delete a region of the female-specific exon that contains six copies of a 13-bp sequence (three mutations are inserts of middle repeat sequences and one is a deletion). These findings led to the hypothesis that the 13-bp repeats may interact with *trans*-acting regulatory factor(s), namely *tra* and/or *tra-2*, to effect female-specific processing of *dsx* pre-mRNA (Burtis and Baker 1989; Nagoshi and Baker 1990).

We have employed a cotransfection assay to examine regulatory interactions between specific *cis*-acting sequence elements of *dsx* pre-mRNA and *tra* and *tra-2* gene products. The results establish that *tra* and *tra-2* function to activate use of the female-specific exon. In addition, we have identified a region within the female-specific exon that is both necessary and sufficient for regulation by *tra* and *tra-2*. Finally, we find that correct splicing of the female-specific exon occurs in the absence of the female polyadenylation site, suggesting that *tra* and *tra-2* control splicing by directly promoting the use of the female acceptor and not indirectly through control of polyadenylation.

Results

Dissection of the regulation of *dsx* pre-mRNA processing by *tra* and *tra-2* requires a derivative of the *dsx* gene

of manageable size (the pre-mRNA of the wild-type *dsx* gene is ~45 kb in length) that produces a transcript, the processing of which is *tra* and *tra-2* dependent. To this end we used transfection to transiently express pre-mRNA from a *dsx* minigene in *Drosophila* Schneider's line 2 (S-L2) tissue culture cells in the presence or absence of the products of cotransfected *tra* and/or *tra-2* genes.

Determination that Drosophila S-L2 tissue culture cells are male

We examined the types of products produced by the endogenous *dsx*, *tra*, *tra-2*, and *Sxl* genes in S-L2 cells by Northern blots (Fig. 1), because these products could influence the results and interpretations of transfection experiments. In addition, RNA protection analysis of endogenous S-L2 mRNA has been performed to distinguish somatic from germ-line type *tra-2* transcripts (W. Mattox, unpubl.). The results with respect to *tra*, *tra-2*, and *Sxl* expression show that S-L2 cells display the array of gene products characteristic of male somatic cells (non-functional *tra* and *Sxl* mRNAs and somatic type *tra-2* mRNAs). These cells should therefore be functionally male with respect to the regulation of *dsx* RNA processing. With regard to *dsx* itself, Northern blots showed that *dsx* is not transcribed at detectable levels in S-L2 cells (Fig. 1, lane 3). Hence, the analysis of *dsx* transcripts generated from transfected DNA will not be complicated by endogenously expressed *dsx* RNA.

Regulation of dsx pre-mRNA processing by tra and tra-2 in a cotransfection assay system

Our basic *dsx* minigene, *pdsx*, contains all *dsx* sequences from the last common 5' exon through the female-specific exon to the 3' end of the first male-specific exon and is driven by the *Drosophila* actin 5C distal promoter. The structure of this minigene and its relationship to the intact *dsx* gene are detailed in Fig. 2. The pre-mRNA generated from this construct consists of a common *dsx* 5' exon that can be spliced to either a female-specific terminal exon or a more downstream male-specific terminal exon.

To determine the types of *dsx* RNA processing products generated in S-L2 cells from the minigene, we used an RNase protection assay that allows direct comparison of the amount of RNA processed in the male- versus female-specific patterns. Specifically, a uniformly labeled riboprobe was generated that allows detection of use of the female 3'SS, male 3'SS, and common 5'SS by formation of RNA protection products of distinct sizes (Fig. 2B). In addition, protection products generated from transcripts with an unspliced female-specific intron or an unspliced male 3'SS can be detected.

The pre-mRNA of the *pdsx* minigene is processed predominantly in the male-specific pattern by the endogenous splicing machinery of S-L2 cells (Fig. 3A, lane 1). Some splicing of the female-specific 3'SS is also detected. Quantification of the use of the male versus fe-

Figure 1. Expression of endogenous *dsx*, *tra*, *tra-2*, and *Sxl* in S-L2 cells. Poly(A)⁺ RNA isolated from Canton-S male (m) and female (f) adult flies and S-L2 cells (SL2) was separated electrophoretically, blotted to filters, and hybridized with gene-specific DNA probes as indicated above each panel. The detected transcripts are labeled by their sizes in kilobases. Male-specific transcripts are labeled as 3.9 kb for *dsx* (lane 1), 1.2 kb for *tra* (lanes 4–6), and 4.3 and 3.3 kb for *Sxl* (lanes 10,12). Female-specific transcripts are labeled as 3.5 kb for *dsx* (lane 2), 1.0 kb for *tra* (lane 5), and 4.1, 3.1, and 1.9 kb for *Sxl* (lane 11). *tra-2* transcripts are labeled as ~1.6 kb. Note that the differences in the pattern of *tra-2* transcripts detected in adult male flies vs. those detected in adult female flies and S-L2 cells are the result of the presence of high levels of male germ-line-specific transcripts present in RNA isolated from whole adult male flies.

male 3'SS (by densitometry and correcting for the different numbers of labeled residues in the relevant portions of the probe) indicates that the *dsx* male-specific site is used ~75% of the time. Moreover, splicing of this minigene is quite efficient. The level of unspliced female intron detected in Figure 3A, lane 1, represents <10% of all *pdsx* transcripts. Unspliced male 3'SS cannot be resolved on this gel because of its close proximity to the prominent signal generated from the use of the common 5'SS. Use of a similar probe that only monitors the male 3'SS showed that <3% of RNA containing the male-specific exon is unspliced (Fig. 3A, lane 11). Thus, this minigene produces a good substrate for RNA processing, and these cells process it primarily in the male-specific manner, as expected from the types of *tra* and *tra-2* products that they contain.

To determine whether regulated processing can be obtained in this system, we cotransfected the *pdsx* minigene with plasmids capable of expressing *tra* and *tra-2* proteins (for the structure of these plasmids, see Materials and methods). The presence of *tra* and *tra-2* completely shifts splicing from predominant use of the male-specific 3'SS to exclusive use of the female-specific 3'SS (Fig. 3A, lane 2 and 12). Thus, *pdsx* contains sufficient sequences to obtain regulated processing of its transcript, and the simultaneous presence of functional *tra* and *tra-2* products are sufficient to switch from the male-specific to the female-specific pattern of *dsx* RNA processing.

Cotransfection with *pdsx* and either the *tra* or *tra-2* expression construct alone also caused a slight shift toward use of the female 3'SS (cf. the ratio of the female- vs. male-specific protection products in Fig. 3A, cf. lane 1 with lanes 3 and 4). The result from cotransfection

with *tra* alone is not surprising as there is probably endogenous *tra-2* protein in the S-L2 cells (see above). Moreover, the low level of female-specific processing generated in this situation is not unreasonable because the level of endogenous *tra-2* product is expected to be low compared to the levels of the products generated from the transfected *tra* and *dsx* genes. More interesting is the finding that cotransfection with *tra-2* alone caused a slight shift toward use of the female-specific 3'SS; genetic experiments have suggested that *tra* and *tra-2* are both required for female-specific *dsx* RNA processing (McKeown et al. 1988; Nagoshi et al. 1988) and S-L2 cells should not express functional *tra* protein (see above). The effects of expressing *tra* and *tra-2* singly were also examined in all derivatives of *pdsx* described below (Fig. 4, and data not shown). In all cases there were weak effects similar to those seen with *pdsx*. At present, the significance of these results is unclear.

The accuracy of splicing at the common 5'SS, female 3'SS, and male 3'SS was checked by S1 nuclease analysis with end-labeled single-stranded DNA probes. In all cases, the authentic processing sites were used (Fig. 3A, lanes 13–16, and data not shown). In addition, S1 nuclease analyses provided independent confirmation of the magnitude of effects on male- versus female-specific detected by the RNase protection assay. However, unlike the RNase protection assay, S1 analysis does not allow a direct comparison of the relative amounts of female- versus male-specific splicing.

Regulation of *dsx* pre-mRNA processing by *tra* and *tra-2* involves activation of female-specific exon use

To test the proposal (Nagoshi and Baker 1990) that the

Figure 2. Structure of the wild-type *dsx* minigene and RNase protection riboprobe. (A) The structure and approximate scale of the *dsx* gene are diagrammed. Open boxes numbered 1–3 represent the common 5' exons. The lightly shaded box (f) represents the female-specific 3'-terminal exon; the dark shaded boxes represent the male-specific terminal exons (m' and m''). DNA sequence present in the wild-type *dsx* minigene is indicated by the heavy bars below the drawing. (B) The structure of the wild-type *dsx* minigene, *pdsx*, is shown at the top. The arrow marks the transcriptional start site of the actin 5C promoter. The common (c), female-specific (f), and male-specific exons (m) are indicated. The structure of the riboprobe used for RNase protection assays is diagrammed below. Its relationship to the *dsx* minigene is indicated by the diagonal lines connecting the two structures. Potential RNA protection products and their sizes (nt) are shown.

regulation of *dsx* pre-mRNA processing is controlled by activating use of the female-specific exon rather than by repressing use of the male-specific exons, we asked whether *tra* and *tra-2* can regulate use of the female-specific exon in the absence of a competing male-specific exon. The minigene we used for this, *pdsxRI*, was constructed by truncating *pdsx* at an *EcoRI* restriction site downstream of the female-specific exon (Fig. 4B). If the female-specific exon requires activation for use, removal of the competing male-specific exon may not be sufficient to allow full use of the female-specific exon, resulting in the accumulation of unspliced RNA. In addition, if unspliced RNA does accumulate, then *tra* and *tra-2* should convert this to spliced product. However, if repression of male-specific exon use is the primary regulatory mechanism, full use of the female-specific exon is expected in the absence of the competing male-specific exon. The results (Fig. 4A, lanes 1,2,9,10) show that both of the expectations for an activation mechanism have been met. A significant amount of unspliced RNA does

accumulate (~50% is unspliced as determined by densitometry; Fig. 4A, lanes 1,9), and this drops significantly (to ~3%) when *tra* and *tra-2* are present (Fig. 4A, lanes 2,10). These results thus demonstrate that regulation of *dsx* pre-mRNA processing by *tra* and *tra-2* occurs by activating use of the female-specific exon. They show further that the *cis*-acting *dsx* sequences both necessary and sufficient for activation of the female 3'SS are in the female-specific exon or 5' to it.

Delineation of sequences within the female-specific exon necessary for regulation by tra and tra-2

Previous molecular and genetic studies have shown that *dsx* mutants that produce transcripts processed in the male-specific pattern in female flies contain either insertions or deletions of sequences within the female-specific exon, suggesting that sequences within the female-specific exon, perhaps the 13-bp repeats, are necessary for *tra*- and *tra-2*-dependent regulation of *dsx* pre-mRNA processing (Nagoshi and Baker 1990). To test whether the sequence requirements for regulated processing of *dsx* pre-mRNA were the same in S-L2 cells as in flies we introduced one of these mutations, *dsx^S*, into *pdsx* to produce *pdsxΔS* (see Fig. 3B). This mutation contains a deletion that removes 448 nucleotides of sequence, beginning 108 nucleotides downstream of the female 3'SS and contains a 5-nucleotide insertion at that position. Five of the 13-bp repeats closest to the female 3'SS are removed by this deletion. In S-L2 cells pre-mRNA from *pdsxΔS* undergoes very low levels of female-specific splicing relative to male-specific splicing (>92% is processed in the male pattern; Fig. 3A, lane 5). When *tra* and *tra-2* were cotransfected the ratio shifted slightly toward female-specific splicing, although still favoring male-specific splicing (~74% is processed in the male pattern; Fig. 3A, lane 6). That the *dsx^S* deletion has essentially the same effects on splicing in S-L2 cells as it does in flies provides compelling support for the conclusion that the regulation of splicing in S-L2 cells faithfully represents what is occurring in the intact organism. In addition, the occurrence of some use of the female 3'SS in *pdsxΔS* pre-mRNA, but very little response to the presence of the *tra* and *tra-2* products, provides strong evidence that sequences essential for regulating usage of the female 3'SS lie within the *dsx^S* deletion.

To examine further the role of sequences in this region, a minigene containing a somewhat larger deletion, *pdsxΔAvi*, which removes 540 nucleotides of sequence beginning 68 nucleotides downstream of the female-specific 3'SS, including all six of the 13-bp repeats, was also tested for its effects on sex-specific processing (for a diagram of this minigene, see Fig. 3B). In S-L2 cells pre-mRNA from *pdsxΔAvi* undergoes only male-specific splicing. No splicing of the female 3'SS was detected, even in the presence of *tra* and *tra-2* (Fig. 3A, lanes 7,8). Removal of the competing male-specific exon, by truncating *pdsxΔAvi* at the *EcoRI* site downstream of the female-specific exon (*pdsxΔAviRI*), enables the detection of some female-specific splicing, but this low level of

Regulation of *dsx* pre-mRNA processing

Figure 3. Analysis of RNA-processing products generated from *dsx* minigenes transfected in the presence or absence of *tra* and/or *tra-2*. (A) RNase protection products generated by hybridization of total S-L2 cell RNA from transfections with either the uniformly labeled riboprobe diagramed in Fig. 2B (lanes 1–10) or a similar riboprobe containing only the male portion of the construct (lanes 11,12) are shown after electrophoretic separation. Protection products from S1 nuclease analyses performed on the

same S-L2 cell RNA used in lanes 1–4 are shown in lanes 13–16. The 5'-end-labeled single-stranded probe used for S1 nuclease protections is diagramed at top right. This probe contained *dsx* sequence from 179 nucleotides upstream of the common 5'SS to 103 nucleotides downstream of the female 3'SS. The identity of protection products detected are indicated diagrammatically at the right of each panel. Size markers (M) are *HpaII*-digested pBR322 DNA. Representative markers are labeled by size in nucleotides. The *dsx* minigenes transfected are indicated at top: (Lanes 1–4), *pdsx* (wt); (lanes 5, 6), *pdsx*Δ*S* (Δ*S*); (lanes 7,8), *pdsx*Δ*Avi* (Δ*Avi*); (lanes 9,10), *pdsx*In*Avi* (In*Avi*). (B) The structures of *dsx* minigenes used in transfections are diagramed. Brackets delimit sequences deleted from *pdsx*. The lightly shaded region with an arrow denotes inverted sequence. Regions with diagonal lines represent sequence from the *tra-2* polyadenylation site. Dots along lower edges of the minigene diagrams indicate the positions of the 13-bp repeats.

female splice site use is not increased in the presence of *tra* and *tra-2* (Fig. 4A, lanes 5,6). This result indicates that the 540-nucleotide deletion disrupts sequences essential for regulation of the female 3'SS. However, it does not distinguish between the possibilities that regulation is obliterated as a result of the removal of sequences essential for regulation or the abnormal spacing of sequences remaining in the minigene.

To inquire whether the sequences within the 540-nu-

cleotide region deleted in *pdsx*Δ*Avi* are essential for regulation we tested a construct, *pdsx*In*Avi*, containing the 540-nucleotide *Avi* fragment reinserted at the same site but in the opposite orientation. With this construct the spacing of sequences outside of the 540-nucleotide region have been maintained. As with *pdsx* Δ*Avi*, when the competitor male 3'SS is present, female-specific splicing is not detected (Fig. 3A, lanes 9,10); and in the absence of the competitor male 3'SS (*pdsx*In*Avi*RI) a very low level

Figure 4. Analysis of RNA-processing products generated from truncated *dsx* minigenes. (A) RNase (lanes 1–8) and S1 nuclease (lanes 9–16) protection products generated as in Fig. 3A. The 3'-end-labeled single-stranded DNA probe used to detect use of the female-specific polyadenylation site (lanes 13–16) is diagramed *above right*. The probe covers the *dsx* sequence from 160 nucleotides upstream of the polyadenylation site to 590 nucleotides downstream. The *dsx* minigenes transfected are indicated at *top*: (lanes 1–4 and 9–14) *pdsxRI* (RI); (lanes 5,6,15,16) *pdsxΔAviRI* (Δ AviRI); (lanes 7,8) *pdsxInAviRI* (InAviRI). The lower portion of lanes 5–8 was taken from a longer exposure of the gel to show the presence of protection products representing use of the female-specific 3'SS. (B) The structures of the truncated *dsx* minigenes are diagramed as in Fig. 3B.

B

of female-specific splicing is detected (Fig. 4A, lanes 7,8). Moreover, as was the case with *pdsxΔAviRI*, female-specific splicing of *pdsxInAviRI* is not stimulated by *tra* and *tra-2* (Fig. 4A, lanes 7,8). The fact that regulation of the female 3'SS is obliterated in *pdsxInAvi* shows that this 540-nucleotide region contains at least some of the sequences essential for the regulation of female-specific processing via *tra* and *tra-2*; thus, the effects of the *dsx*^S and Δ Avi deletions cannot be attributed solely to effects on spacing.

Identification of sequences sufficient for regulation by *tra* and *tra-2*

To determine whether the 540-nucleotide *cis*-acting regulatory region identified above is sufficient for regula-

tion, we asked whether the region can impose regulation by *tra* and *tra-2* on the use of a heterologous exon. To do this we inserted the 540-nucleotide segment from the female-specific exon 35 nucleotides downstream of the male-specific 3'SS of the *pdsxΔS* minigene (*pdsxΔSTpAvi*) and asked whether *tra* and *tra-2* can regulate use of this chimeric male-specific exon. In addition, we inserted the 540-nucleotide segment into the same site in the male-specific exon but in the opposite orientation (*pdsxΔSTpInAvi*) to control for effects on male-specific splicing not related to the particular sequences inserted. The structures of both constructs are diagramed in Figure 5B. The reaction products from this experiment were analyzed both by RNase and S1 nuclease protection assays with probes that specifically detect use of the chimeric male-specific 3'SS (Fig. 5A). The

Figure 5. Analysis of RNA-processing products from *dsx* minigenes designed to test whether sequences from within the female-specific exon are sufficient for regulation. (A) RNase (lanes 1,2) and S1 nuclease (lanes 3–6) protection products generated as in Figs. 3A and 4A except that the probes contained a sequence homologous to the chimeric male exon of *pdsx*ΔSTpAvi and *pdsx*ΔSTpInAvi as indicated at top. Both of the 5'-end-labeled

single-stranded probes used for S1 nuclease analysis covered *dsx* sequence from 83 nucleotides upstream of the male 3'SS at an *Sfa*NI restriction site to the *Mlu*I site of the chimeric exons (for the exact structure of these minigenes, see Materials and methods). The *dsx* minigenes transfected are indicated at top. (Lanes 1–4) *pdsx*ΔSTpAvi (ΔSTpAvi); (lanes 5,6), *pdsx*ΔSTpInAvi (ΔSTpInAvi). (B) The structures of the *dsx* minigenes are diagrammed as in Fig. 3B. The orientation of the 540-nucleotide *Avi* fragment inserted into the male exon is indicated by the arrow.

results show that the chimeric exon is activated by *tra* and *tra-2* when the insert is in the correct orientation; products containing spliced male-specific 3'SS increase >10-fold relative to unspliced male-specific 3'SS when *tra* and *tra-2* are present (Fig. 5A, lanes 1–4). Thus, this 540-nucleotide segment of the female-specific exon contains sequences sufficient for activation of a 3'SS by *tra* and *tra-2*.

Note that the insertion of this 540-nucleotide segment in either orientation inhibits splicing of the male-specific 3'SS (cf. the levels of spliced relative to unspliced male exon in Fig. 5A, lanes 3 and 5, and Fig. 3A, lane 11). This may be a result of the normal use of this 3'SS re-

quiring sequences that have been displaced by the insert. This suggestion is supported by our analysis of an additional construct that contains all sequences of *pdsx* through the male-specific 3'SS and a substitution of all 3'-terminal sequences beginning 35 nucleotides downstream of the male-specific 3'SS. This construct also does not produce detectable amounts of spliced male-specific 3'SS (data not shown).

tra and *tra-2* activate female-specific processing by promoting 3'-splice site use

The experiments presented above demonstrate that reg-

ulation of *dsx* RNA processing seems to occur largely by activation of female-specific exon use. As a result of the arrangement of processing sites and *cis*-acting regulatory elements in the *dsx* pre-mRNA, it is possible that female-specific processing results from the activation of polyadenylation or splicing, or by the independent activation of both female-specific splicing and polyadenylation. To distinguish between these possibilities we examined whether female-specific splicing and polyadenylation could be regulated separately.

The simplest way that we could envisage activation of the female-specific exon is by promoting use of its polyadenylation site. Processing at the female-specific polyadenylation site would preclude use of the male-specific exon; splicing of the female-specific 3' SS would then follow because it would remain as the only option. Implicit in this type of mechanism is the proposition that the female-specific polyadenylation site is normally not used in the absence of *tra* and *tra-2* because of its weakness as a processing site.

To test this idea we constructed a minigene, *pdsxΔInt*, in which the potential for splicing, but not polyadenylation, has been eliminated by substituting a segment of DNA that does not contain splice sites for sequences including the common 5' exon, 5' SS and female-specific 3' SS of *pdsx* (Fig. 6B). In this construct the choice is whether to polyadenylate at the female-specific site or at the more downstream male-specific site. Processing of *pdsxΔInt* pre-mRNA in S-L2 cells shows that the female-specific polyadenylation site is used exclusively; no male-specific RNA is detected (Fig. 6A, lanes 3,4), and essentially all transcripts are cleaved at the female-specific polyadenylation site (Fig. 6A, lanes 1,2). Not surprisingly the addition of *tra* and *tra-2* had no effect on the utilization of this polyadenylation site because it was already used efficiently. These results show that the female-specific polyadenylation site in this construct does not require the activities of the *tra* and *tra-2* genes for its use.

An alternative model for activation of female-specific exon use is that *tra* and *tra-2* promote splicing at the female-specific 3' SS. To test this model we made a *dsx* minigene, *pdsxΔPA*, in which the female-specific polyadenylation site has been deleted (Fig. 6B). In this construct the choice of processing sites is then between two competing 3' SS's. If regulation occurs by activating use of the female-specific 3' SS, the presence of *tra* and *tra-2* should activate use of the female 3' SS in *pdsxΔPA*. The results show that this is the case; female-specific splicing is not detected in S-L2 cells but is activated fully by the presence of *tra* and *tra-2* (Fig. 6A, lanes 5,6), indicating that regulation of female-specific exon use involves promotion of 3' SS use.

In principle, utilization of the female 3' SS is all that is required to generate a transcript that encodes the female-specific protein product regardless of which polyadenylation site is used. However, only transcripts polyadenylated at the female-specific polyadenylation site are detected in female flies, indicating that use of this polyadenylation site is also stipulated by the activities of

tra and *tra-2*. Because the experiments described above suggest that the female-specific polyadenylation site is not regulated (since it is used efficiently in the absence of splicing) it seemed possible that splicing and polyadenylation of the female-specific exon are mechanistically coupled. Several additional experiments were consistent with this idea and support the proposal that regulation of *dsx* pre-mRNA processing works by promoting splicing.

First, a construct, *pdsxΔ3'SS* that has the same structure as *pdsx*—except that the female-specific 3' SS and branchpoint region were deleted—was tested (Fig. 6B). Processing of the pre-mRNA generated from *pdsxΔ3'SS* involves the choice between whether to splice in the male-specific pattern or polyadenylate at the female-specific site. Our initial intention for using this minigene was to test whether polyadenylation at the female-specific site can be regulated independently of splicing at the female 3' SS. The results show that use of the female-specific polyadenylation site was increased by *tra* and *tra-2* (data not shown) but that this occurred with the simultaneous activation of cryptic 3' SS's (Fig. 6A, lanes 7,8). The cryptic 3' SS's were identified by the presence of two novel RNA protection products whose sizes correspond to use of sequences within the female-specific exon that have good matches to 3' SS consensus sequences (including branchpoints, polypyrimidine tracts, and 3' SS's) found at 110 and 162 nucleotides downstream of the wild-type 3' SS (the site at 162 nucleotides was used predominately). Furthermore, the amount of cryptic splicing that is activated by *tra* and *tra-2* can account for the amount that polyadenylation increases at the female site (data not shown). These results provide further support for the idea that activation of the female-specific exon by *tra* and *tra-2* functions by promoting splicing.

We also analyzed use of the female-specific polyadenylation site of the construct *pdsxRI*, truncated downstream of the female-specific polyadenylation site, and found that even though the female-specific polyadenylation site is the only available site, its use was also regulated. In the absence of *tra* and *tra-2* the polyadenylation site was used ~80% of the time, the remaining 20% was uncleaved and poly(A)⁻ (Fig. 4A, lane 13, and data not shown); and when *tra* and *tra-2* were added its use increased to essentially 100% as did splicing of the 3' SS (Fig. 4A, lane 14). Note, however, that in the absence of *tra* and *tra-2* a much greater fraction of RNA was cleaved at the polyadenylation site than was spliced (~50% was spliced). Therefore, some polyadenylation occurs in the absence of splicing. Moreover, the truncated minigene containing the 540-nucleotide *AviII* deletion (*pdsxΔAviRI*), which showed very little female-specific splicing, was also cleaved inefficiently at the female-specific polyadenylation site (<50%; Fig. 4A, lane 15). In addition, neither splicing (Fig. 4A, lanes 5,6) nor polyadenylation (Fig. 4A, lanes 15,16) of the pre-mRNA of this construct was activated by *tra* and *tra-2*. These results with the truncated minigenes show that the regulation of polyadenylation is dependent on the presence of the same *cis*-acting region required for splicing. However,

Figure 6. Analysis of RNA-protection products from *dsx* minigenes designed to test whether splicing and/or polyadenylation is regulated by *tra* and *tra-2*. (A) S1 nuclease (lanes 1,2) and RNase (lanes 3–8) protection products generated as in Figs. 3A and 4A. The *dsx* minigenes transfected are indicated at top. (Lanes 1–4) *pdsx* Δ Int (Δ Int); (lanes 5,6) *pdsx* Δ PA (Δ PA); (lanes 7,8), *pdsx* Δ 3'SS (Δ 3'SS). (B) The structure of the *dsx* minigenes are diagrammed as in previous figures. The

black region represents substituted sequence (for the exact structure of this minigene, see Materials and methods). Brackets delimit deleted sequence.

because we were unable to design a construct in which polyadenylation could be regulated independently of splicing but the reverse was possible (with *pdsx* Δ PA), we favor the idea that the commitment to splice is the regulated step that dictates use of the polyadenylation site.

Discussion

We have described a transient expression system using a *Drosophila* cell line that substantially reproduces the regulated splicing of *dsx* pre-mRNAs characterized previously by molecular genetics experiments in whole flies. In particular, we find that ~75% of spliced transcripts of a transfected *dsx* minigene are normally pro-

cessed in the male-specific pattern in these cells. The ~25% of transcripts that are processed in the female pattern in the absence of *tra* and *tra-2* function is not representative of what is seen in wild-type male flies where no female processing is detected. This could be either because our “wild-type” minigene does not contain all elements necessary for complete regulation or because of differences between flies and the tissue culture system. Our finding with respect to *pdsx* Δ PA, suggests that the female-specific splicing seen in tissue culture cells may be the result of the abnormal use of the female-specific polyadenylation site, because the splicing of pre-mRNAs in the female pattern is eliminated by the deletion of the polyadenylation site in *pdsx* Δ PA. Re-

ardless of the validity of this explanation, the biological relevance of our findings is supported by our demonstrations that the *dsx* RNA processing pattern in this cell line shifts completely to the female-specific pattern upon coexpression of *tra* and *tra-2*, just as in whole organisms. Moreover, a *dsx* mutation, *dsx^S*, that prevents regulated processing in flies, has similar effects in this transient expression assay. Thus, by these criteria, the major aspects of regulation are reproduced in the tissue culture system.

A priori, regulation of *dsx* pre-mRNA processing could be either negative (repression of the default male processing pattern) or positive (activation of female processing pattern). It has been suggested that in all other cases where regulated splicing has been dissected sufficiently to delineate mechanism, that regulation is negative (Maniatis 1991). However, in the case of *dsx*, the location in the *dsx* female exon of all known *cis*-acting mutations that alter the processing of *dsx* pre-mRNA led to the suggestion that regulation of *dsx* RNA processing was by activation (Nagoshi and Baker 1990). Our finding that pre-mRNA from a *dsx* minigene truncated after the female exon, and thus lacking the male exons, is processed in a *tra*- and *tra-2*-dependent manner provides compelling support for the proposal that the regulation of *dsx* occurs by activation. This conclusion has also been reached recently by Hedley and Maniatis (1991) and Hoshijima et al. (1991) from similar experiments.

That processing of *dsx* minigene transcripts lacking the male-specific terminal exons is still under the control of *tra* and *tra-2* also establishes that *dsx* sequences sufficient for regulated processing must lie either within or immediately 5' to the female exon. Furthermore, the reduction in the ability of *tra* and *tra-2* to elicit use of the female 3' SS by the deletion (*dsx^S*) that begins 108 nucleotides downstream of the female 3' SS and removes the first five copies of the 13-bp sequence found repeated six times in the *dsx* female exon supports the previous suggestion that sequences in the female-specific exon, perhaps the 13-bp repeats, might be sites through which *tra* and/or *tra-2* proteins exert their functions (Burtis and Baker 1989; Nagoshi and Baker 1990). Transcripts containing a slightly larger deletion, which removed all six of the repeat sequences, showed no stimulation of female-specific processing upon coexpression of *tra* and *tra-2*, providing further support of this idea.

Recently, Hoshijima et al. (1991) and Hedley and Maniatis (1991) have also reported the results of cotransfection experiments consistent with the previous findings in flies that the region within the female-specific exon containing the 13-bp repeats was required for regulation. Hoshijima et al. (1991) also examined deletions of different subsets of the six repeats and found that the number of repeats correlated with the efficiency of activation, suggesting that it is the repeated sequence itself that is required for regulation. In addition, Hedley and Maniatis (1991) reported the results of binding studies indicating that *tra-2* protein might bind specifically to the 13-nucleotide repeat sequence, as an RNA containing multimers of a 33-nucleotide sequence overlapping the sixth

dsx repeat appears to interact in a gel-shift assay with *tra-2* protein produced in bacteria.

Consistent with these results implicating the repeat sequence in the *dsx* female exon in regulation are the findings with regard to two other genes, *tra-2* itself (Mattox and Baker 1991) and *exu* (T. Hazelrigg, unpubl.), which are also regulated at the level of RNA processing by *tra-2*. In particular, there are good matches to a 10-nucleotide core of the 13-bp *dsx* repeat near the 5' SS of the regulated intron in *tra-2* (Mattox and Baker 1991) and the polyadenylation site whose usage is regulated in *exu* (T. Hazelrigg, unpubl.).

While the above experiments establish that sequences necessary for the activation of the female-specific pattern of *dsx* processing are located in the region containing the repeats and strongly implicate the repeat sequences themselves, none of these data preclude the existence of other sequences outside of this region necessary for female-specific processing of *dsx* pre-mRNA. Our finding that a 540-nucleotide region that contains all six repeats, when placed next to a 3' SS that is not normally activated by *tra* and *tra-2* (the *dsx* male-specific 3' SS), leads to the heterologous 3' SS being used in a *tra*- and *tra-2*-dependent manner shows that this region contains sequences that are not only necessary, but also sufficient, for regulation of processing by *tra* and *tra-2*. Whether the repeated sequence alone is sufficient for regulation is presently being tested.

Because regulation of *dsx* pre-mRNA processing involves the choice between sex-specific 3'-terminal exons, the activation of the female-specific exon of *dsx* could be the result of regulated splicing, polyadenylation, or both. Our results from the minigene in which the female-specific polyadenylation site has been deleted (*pdsxΔPA*) clearly show that *tra* and *tra-2* can regulate 3' SS use in the absence of the female polyadenylation site. To test whether polyadenylation could be regulated independently of splicing, we examined the polyadenylation efficiency of a number of different minigene constructs in which splicing at the female 3' SS had been eliminated. We found that polyadenylation either did not respond to *tra* and *tra-2*, as a result of efficient use of the site; or in the case where polyadenylation was activated, this was concomitant with activation of cryptic 3' SS's. Although these data do not rule out the possibility that the female-specific polyadenylation site can be regulated independently, they are most compatible with a model in which the commitment to splicing at the 3' SS dictates use of the polyadenylation site. Consistent with this model is our finding of a positive correlation between how efficiently the upstream intron is spliced and use of the polyadenylation site. This correlation is most evident with the truncated minigenes in which the female-specific polyadenylation site is the only site available, and yet it is not used efficiently unless the upstream intron is spliced efficiently. However, it was also the case that when an upstream intron was not present, the female polyadenylation site was used all the time. Taken together, these results suggest the possibility that the presence of an unspliced intron inhibits polyadeny-

lation. We are presently examining this possibility, because such a linkage between splicing and polyadenylation could be part of an important control mechanism where alternative 3'-terminal exons are involved and may also play a role in unregulated 3'-exon processing (Niwa et al. 1990).

Hedley and Maniatis (1991) reported that use of the *dsx* female-specific polyadenylation site can be regulated independently of splicing by using a transfection assay similar to ours. Their claim was based on the observation that in a construct similar to our minigene, *pdsxΔInt*, in which the potential to splice has been eliminated, the efficiency of polyadenylation at the female-specific site was sensitive to the presence of the region containing the *dsx* repeats. However, they did not show that use of the polyadenylation site in these mutant minigenes was dependent on the presence of *tra* and *tra-2* nor did they address the possibility that cryptic splice sites were used in constructs lacking the *dsx* splice sites. Therefore, the decrease in polyadenylation observed with their construct lacking the 13-nucleotide repeats does not necessarily indicate either that polyadenylation is regulated by *tra* and *tra-2* or that polyadenylation can be regulated independently of splicing.

Studies of processing of other pre-mRNAs also support a model in which polyadenylation is dependent on splice-site choice. The cell-specific alternative processing pathway of rat calcitonin/CGRP pre-mRNA (which has a structural organization essentially the same as *dsx*) is consistent with such a splice-commitment model, as cell-specific use of the polyadenylation sites in the absence of splice sites does not occur and *cis*-acting sequences required for cell-specific processing are located near the regulated 3'SS, making it more likely that splice-site selection is the regulated event (Leff et al. 1987). Moreover, in a study of histone 3'-end formation, Pandey et al. (1990) found that the presence of an intron interfered with normal histone 3'-end formation and activated the use of cryptic polyadenylation sites, supporting the notion that splicing can direct polyadenylation. In addition, in a study by Huang and Gorman (1990), the presence of an intron in an expression vector increased the levels of poly(A)⁺ transcripts, again implying that splicing can influence polyadenylation. Recent *in vitro* data also suggest this (Niwa et al. 1990).

A model in which splicing directs polyadenylation requires that recognition of splice sites occurs before polyadenylation. Evidence consistent with this order comes from two different studies. The analysis of nascent transcripts by electron microscopy of Beyer and Osheim (1988) indicates that spliceosomes are probably assembled at splice junctions prior to the completion of transcription and that transcripts near the end of transcription units have shortened, indicating that intron removal can precede completion of transcription. In addition, LeMaire and Thummel (1990) have shown by a direct analysis of nascent transcripts from a 60-kb transcription unit, that splicing occurred before transcription termination and polyadenylation.

The finding that regulation of *dsx* RNA processing oc-

curs by activation of the female-specific 3'SS allows one to begin to address the mechanism by which activation takes place. We can envisage two basic classes of models for activation of splice-site usage.

The first of these is based on cases of negative regulation where it is easy to imagine how a *trans*-acting factor might work to prevent RNA-processing reactions from occurring. Most simply, by binding to a particular sequence such a regulatory factor could displace or prevent the binding of general processing factors. With respect to a *trans*-acting factor that regulates processing positively, an equivalent model would be that it blocks or interferes with an element that normally represses processing. Such inhibitory elements could be either *cis*-acting structural features that prevent processing or *trans*-acting inhibitory factors that interact with *cis*-acting sequences. To activate processing the regulatory factor could disrupt the secondary structure or displace/prevent the binding of the inhibitory factor. Such "derepression" mechanisms could be identified by the isolation of mutations of *cis*-acting sequences that cause constitutive use of processing sites. In our dissection of *dsx* pre-mRNA processing we did not find a sequence that, when eliminated, caused constitutive female-specific splicing. However, the analysis of this region is still at a relatively gross level.

The known properties of the *dsx* female 3'SS appear to be sufficient to account for regulation without invoking a derepression mechanism. The female 3'SS has a suboptimal 3' acceptor. Specifically, the polypyrimidine tract has a low pyrimidine content, containing 8 purines of 20 nucleotides upstream of the NCAG/3'SS consensus sequence. The finding that the suboptimal nature of this acceptor is evolutionarily conserved led to the suggestion that this property of the acceptor was important for regulated processing of *dsx* pre-mRNA (Burtis and Baker 1989). This conclusion is strengthened by the recent results of Hoshijima et al. (1991), which show that the pyrimidine content of this acceptor does effect the efficiency with which this acceptor is used. Our finding that the female-specific exon is processed very inefficiently even in the absence of a competing male-specific exon establishes that this 3'SS is a biologically poor acceptor. In addition, our finding that activation of splicing by *tra* and *tra-2* works on heterologous acceptors (the *dsx* male-specific acceptor and two cryptic acceptors) when the *cis*-acting regulatory region is present downstream indicate that it is not the particular sequence of the acceptor that is important for regulation, but the inability of the acceptor to be spliced efficiently that enables regulation to occur. Taken together, all of these results suggest that the presence of a weak female-specific 3' acceptor may be sufficient to account for the failure of this acceptor to be used in the absence of *tra* and *tra-2* products.

A second class of positive regulatory mechanisms includes models that involve *trans*-acting factors that increase processing efficiency directly. One subclass of such models invokes a *trans*-acting regulatory factor, which is a variant of one of the general processing factors, that can facilitate recognition of a nonconsensus

processing site. The poor female-specific 3' acceptor could be such a processing site. However, this type of mechanism does not seem to be at work in the case of *dsx*, because we were able to identify a 540-nucleotide region that is sufficient for regulation by *tra* and *tra-2* that does not overlap the recognition sequences of the female-specific 3'SS or polyadenylation site. Another subclass of these models includes those in which a *trans*-acting regulatory factor interacts with *cis*-acting sequence elements to help recruit general processing factors or participates in a process such as exon definition (Robberson et al. 1990) to activate processing. These types of activation mechanisms seem to best fit the characteristics of *dsx* RNA processing regulation, primarily because regulation occurs through the functioning of exonic sequences that are not immediately adjacent to the regulated processing sites and the regulation is involved in promoting usage of an otherwise weak 3'SS.

The results described here focus our attention on the mechanism by which the *tra* and *tra-2* proteins function through sequences in the female exon of *dsx* to activate the use of the weak female-specific 3'SS. At present, it seems most likely that these components interact so as to confer most favored status on this processing site. Among the major questions that now need to be addressed are the following. In the regulatory region of the *dsx* female exon are the repeat sequences the only elements important for regulation, and if there are other elements, what are their respective roles? How do *tra* and *tra-2* proteins activate splicing? Do they interact with the *dsx* pre-mRNA separately or as a complex? How does their action serve to increase the utilization of this processing site by general processing factors? We hope that understanding these and related questions with respect to the mechanism of activation in this system will not only deepen our understanding of regulated splicing but also teach us something about one of the most poorly understood aspects of constitutive splicing—how are authentic splice sites selected?

Materials and methods

Northern blot analysis

Total RNA from untransfected S-L2 cells and male and female Canton-S flies was fractionated on oligo d(T) columns (Sambrook et al. 1989). Poly(A)⁺ RNA (1–5 µg) was electrophoresed in a formaldehyde gel, blotted to Nytran (Schleicher & Schuell), and hybridized with gene-specific probes as described in Sambrook et al. (1989). Single-stranded gene-specific probes were synthesized as described in Nagoshi et al. (1988) for *dsx* and *tra*, and in Mattox et al. (1990) for *tra-2*. The *Sxl* probe was made from a double-stranded DNA fragment isolated from plasmid pSxlT41 (kindly provided by H. Salz, Case Western Reserve University, Cleveland, Oh) labeled as described in Feinberg and Vogelstein (1983).

Expression plasmids

All expression vectors for transfection into S-L2 cells were con-

structed in pAct which consists of a fragment of ~2.5-kb containing the *Drosophila* actin 5C distal promoter (Bond-Matthews and Davidson 1988) inserted into pSK(+) (Stratagene). The protein expression plasmid pAct-*tra* was constructed by inserting into pAct the 158-bp *EcoRI*–*NcoI* fragment from a female *tra* cDNA (+31 to –127, relative to the transcriptional start site) and the ~1180-bp *NcoI*–*BamHI* fragment of *tra* genomic sequence starting at the same *NcoI* site as the *tra* cDNA fragment and extending past the *tra* polyadenylation site. Both the *tra* cDNA and genomic DNA were kindly provided by M. McKeown (Salk Institute, San Diego, CA). The protein expression plasmid pAct-*tra-2* was made by inserting into pAct a 2585-bp *Clal*–*XhoI* fragment of *tra-2* genomic DNA (kindly provided by W. Mattox, Stanford University), which contains the potential to express all three of the possible *tra-2* polypeptides. The *dsx* minigene, pdsx, was constructed by inserting into pAct *dsx* sequence including ~250 bp of exon 2 in a cDNA configuration with exon 3, extending intact as genomic DNA to a *NotI* site near the 3' end of the first male exon. A *PstI* fragment containing the *tra-2* polyadenylation site was placed immediately downstream of the *dsx* sequence within the polylinker of pAct. pdsxΔ*Avi* has the same structure as pdsx except that the 540-bp *AviII* fragment was deleted from the female-specific exon and removes the sequence from 69 to 609 bp downstream of the female-specific 3'SS. pdsxIn*Avi* has the same structure as pdsxΔ*Avi* except that the 540-bp *AviII* fragment was reinserted at the same site but in the opposite orientation. pdsxΔ*S* was made by replacing the *Scal* fragment from within the female-specific exon of pdsx with the same fragment isolated from genomic DNA of the *dsx*^S mutant. The truncated versions of the *dsx* minigenes diagrammed in Figure 4B were made by inserting into pSK(+) *XbaI*–*EcoRI* fragments (from the minigenes described above) that contain the actin 5C promoter and 5' *dsx* minigene sequences to an *EcoRI* site ~430 bp downstream of the female-specific polyadenylation site. pdsxΔ3'SS has the same structure as pdsx except that *dsx* minigene sequences from the *BamHI* site of exon 2 to the *AviII* site 66 bp downstream of the female-specific 3'SS have been replaced with the 316-bp *BclI* fragment that contains 301 bp of the *dsx* exons 2 and 3 cDNA sequence plus 15 bp of intron including the common 5'SS. pdsxΔInt has the same structure as pdsxΔ3'SS except that the 316-bp *BclI* fragment was inserted in the opposite orientation. pdsxΔPA has the same structure as pdsx except that the sequence has been deleted from the *Clal* site, ~160 bp upstream of the female-specific polyadenylation site, to a *StyI* site ~590 bp upstream of the male-specific 3'SS. The pdsxΔSTp*Avi* and pdsxΔSTpIn*Avi* were made by inserting the 540-bp *AviII* fragment into the *BglII* site 35 bp downstream of the male 3'SS in pdsxΔ*S*, in both orientations.

The wild-type T3 RNA polymerase riboprobe expression plasmid was made by inserting two *dsx* fragments into pSK(+). One is the 503-bp *DdeI* fragment that spans the female-specific intron including 179 bp of exon 2 and 3 cDNA and 209 bp of female-specific exon sequence. The other is a 198-bp *SfaNI* fragment that spans the male 3'SS including 83 bp of male intron and 115 bp of male exon sequence. The fragments were oriented such that a T3 RNA polymerase antisense riboprobe could be synthesized containing sequences covering both fragments on a single transcript. Additional riboprobe expression constructs were made from the above wild-type construct by inserting the 5' *AviII*–*MluI* and the 3' *MluI*–*AviII* fragment from the female exon into the *BglII* site of the male exon, in the sense and antisense orientations, respectively.

The chloramphenicol acetyltransferase (CAT) expression plasmid, pActCAT, was a gift from K. Han. (Columbia University, NY).

All plasmid DNAs for transfections were purified by CsCl gradient centrifugation.

Cell culture and transfections

S-L2 cells (Schneider 1972) were grown in Schneider's *Drosophila* media (GIBCO) plus 12% fetal bovine serum that had been heat inactivated at 60°C for 30 min. Cells (29×10^6) were plated on 150-mm cell culture dishes 1 day before transfection and transfected by the calcium phosphate coprecipitation method as described by Di Nocera and Dawid (1983). Each transfection contained a total of 130 µg DNA, which included 20 µg of *dsx* minigene plasmid, 1 µg of pAct-*tra* and/or pAct-*tra-2*, where indicated, 5 µg of pActCAT, and 105 µg of pSK(+) as carrier. Mock transfections included the same except that pSK(+) DNA was substituted for the *dsx* minigene plasmids. (Note that analysis of RNA from mock-transfected S-L2 cells is not shown in the figures, because it did not generate detectable levels of protection products.) After 48 hr cells were harvested and washed once with $1 \times$ PBS. A small aliquot of cells from each transfection was assayed for CAT activity to test for transfection efficiency by using the method of Neumann et al. (1987). Because transfection efficiencies did not vary significantly within a given experiment we did not adjust the amount of RNA used in protection assays to reflect such differences. However, in the case of the RNase protection assays (see below) the signal detected by the probe protecting the common region of the *dsx* pre-mRNA can serve as an internal standard for judging the absolute quantities of *dsx* RNA present at the time of RNA isolation. Total RNA was prepared by the guanidinium/cesium chloride centrifugation method described by MacDonald et al. (1987).

RNA protection assays

For RNase protection assays we used the method of Zinn et al. (1983). 32 P-Radiolabeled riboprobes were gel purified by the method used by Maxam and Gilbert (1980) to purify DNA fragments. Excess riboprobe was hybridized with 20 µg of total S-L2 cell RNA 8–12 hr at 46°C. For RNase treatment we used 13 µg/ml of pancreatic RNase A and no RNase T1.

S1 nuclease analysis was performed by the method of Berk and Sharp (1978) with end-labeled single-stranded probes. The DNA fragments used as probes are indicated in the figure legends. Hybridizations included 30 µg of S-L2 total RNA and an excess of end-labeled probe in 50% formamide, 40 mM PIPES (pH 6.4), 400 mM NaCl, and 1 mM EDTA and were incubated at 37°C for 8–12 hr. S1 nuclease digestions were performed at room temperature and included 500 U/ml of S1 nuclease (Sigma).

Protection products were fractionated in 5% polyacrylamide/urea sequencing gels (Maxam and Gilbert 1980). Autoradiograms appropriate for quantification were scanned by using a Quick Scan R&D (Helena Laboratories) densitometer.

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