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; Zhuang et al. 1987; Peterson and Perry 1989; Lear et al. 1990; Kuo et al. 1991] and branch point/polypurimidine 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-]]; 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
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-
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
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 1,9,10] 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 pdsxAS (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 pdsxAS 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 pdsxAS 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, pdsxAVi, 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 pdsxAVi 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 pdsxAVi at the EcoRI site downstream of the female-specific exon [pdsxAViR], enables the detection of some female-specific splicing, but this low level of...
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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 diagrametically 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), *pdsxAS* (AS); (lanes 7,8), *pdsxAAvi* (AAvi); (lanes 9,10), *pdsxInAvi* (InAvi). (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-nucleotide region deleted in *pdsxAvi* are essential for regulation we tested a construct, *pdsxAvi*II, containing the 540-nucleotide AviII 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 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 (*pdsxAvi*II) 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) pdsxAAviRI (AAviRI); (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.

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 regulation, 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
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Figure 5. Analysis of RNA-processing products from \( \text{dsx} \) 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 \( \text{pdsxASTpAvi} \) and \( \text{pdsxASTpInAvi} \) as indicated at top. Both of the 5'-end-labeled single-stranded probes used for S1 nuclease analysis covered \( \text{dsx} \) sequence from 83 nucleotides upstream of the male 3'SS at an \( \text{SfiI} \) restriction site to the \( \text{MluI} \) site of the chimeric exons (for the exact structure of these minigenes, see Materials and methods). The \( \text{dsx} \) minigenes transfected are indicated at top. (Lanes 1-4) \( \text{pdsx~STpAvi (ASTpAvi)} \); (lanes 5,6), \( \text{pdsxASTpInAvi (ASTpInAvi)} \). (B) The structures of the \( \text{dsx} \) minigenes are diagramed as in Fig. 3B. The orientation of the 540-nucleotide AviII fragment inserted into the male exon is indicated by the arrow.

The experiments presented above demonstrate that requiring sequences that have been displaced by the insert. This suggestion is supported by our analysis of an additional construct that contains all sequences of \( \text{pdsx} \) 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 results show that the chimeric exon is activated by \( \text{tra} \) and \( \text{tra-2} \) 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 \( \text{tra} \) and \( \text{tra-2} \) 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 \( \text{tra} \) and \( \text{tra-2} \).

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 requiring sequences that have been displaced by the insert.
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 dsx (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 dsx—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 predominantly). 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 (pdsxΔAviIRI), 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,
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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) pdsxAInt (AInt); (lanes 5,6) pdsxAPA (APA); (lanes 7,8), pdsxA3' SS (A3' SS). (B) The structure of the dsx minigenes are diagramed 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.

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 processed 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 pdsxAPA, 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 pdsxAPA. Re-
Regardless 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, dsx4, 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 (dsx5) 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 (Burris 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-
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, dsxAInt, 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 cis-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 (Jeff 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 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 occurs 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 poly pyrimidine 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 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 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 untransformed S-L2 cells and male and female Canton-S flies was fractionated on oligo (dT) 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 SxI 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 constructed 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. pdsxA3′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. pdsxA3′Avi has the same structure as pdsxA3′Avi except that the 540-bp AviII fragment was reinserted at the same site but in the opposite orientation. pdsxΔSS was made by replacing the Scol fragment from within the female-specific exon of pdsx with the same fragment isolated from genomic DNA of the dsx5 mutant. The truncated versions of the dsx minigenes diagramed 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. pdsxA3′Avi 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. pdsxA3′Avi was made the same structure as pdsxA3′Avi except that the 316-bp BclI fragment was inserted in the opposite orientation. pdsxΔAPA 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 Styl site ~590 bp upstream of the male-specific 3′SS. The pdsxΔStpAvi and pdsxΔStpInAvi were made by inserting the 540-bp AviII fragment into the BglII site 35 bp downstream of the male 3′SS in pdsxΔSS, 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 Ddel 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 x 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 dix minigene plasmid, 1 μg of pActCAT and/or pAct-tra-2, where indicated, 5 μg of pActr, and 105 μg of pSkl as carrier. Mock transfections included the same except that pSkl DNA was substituted for the dix 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 x 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 dix pre-mRNA can serve as an internal standard for judging the absolute quantities of dix 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]. 32P-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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References

Aebi, M., H. Hornyk, and C. Weissmann. 1987. 5’ cleavage site in eukaryotic pre-mRNA splicing is determined by the overall 5’ splice region, not by the conserved 5’ GU. Cell 50: 237-246.


Feinberg, A.P. and B. Vogelstein. 1983. A technique for radiola-
beling DNA restriction endonuclease fragments to high spe-


Fu, X.-Y., H. Ge, and J.L. Manley. 1988. The role of the polypy-
rimidine stretch at the SV40 early pre-mRNA 3’ splice site in

Ge, H. and J.L. Manley. 1990. A protein factor, ASF, controls
cell-specific alternative splicing of SV40 early pre-mRNA in

Ge, H., P. Zuo, and J.L. Manley. 1991. Primary structure of the
human splicing factor ASF reveals similarities with *Drosos-

Goralski, T.J., J.-E. Edstrom, and B.S. Baker. 1989. The sex de-
termination locus transformer-2 of *Drosophila* encodes a
polypeptide with similarity to RNA binding proteins. *Cell*
56: 1011–1018.

Goux-Pelletan, M., D. Libri, Y. d’Aubenton-Carafa, M. Fiszman,
E. Brody, and J. Marie. 1990. In vitro splicing of mutually
exclusive exons from the chicken β-tropomyosin gene: Role of
the branch point location very long pyrimidine stretch. *EMBO J.*

Hedge, M.L. and T. Maniatis. 1991. Sex-specific splicing and
polyadenylation of *dsx* pre-mRNA requires a sequence that

alternative splicing of tropomyosin pre-mRNA. *Nucleic Acids
Res.* 17: 5633–5650.

Hoshijima, K., K. Inoue, I. Higuchi, H. Sakamoto, and Y.
Shimura. 1991. Control of doublesex alternative splicing by
transformer and transformer-2 in *Drosophila*. *Science*

Huang, M.T.F. and C.M. Gorman. 1990. Intervening sequences
increase efficiency of RNA 3’ processing and accumulation of

Binding of the *Drosophila* Sex-lethal gene product to the
alternative splice site of transformer primary transcript. *Na-
ture* 344: 461–463.

pre-mRNA splicing factor SF2 influences 5’ splice site selec-
tion by acting at alternatively spliced sites. *Cell* 62: 35–42.

tional expression of cloned human splicing factor SF2: Ho-
omology to RNA-binding proteins, U1 70K, and
RNA-binding proteins, U1 70K, and

Kuo, H.-C., F.-U. Nasim, and P. Grabowski. 1991. Control of
alternative splicing by the differential binding of U1 small

Hierarchy for 5’ splice site preference determined in vivo. *J.

Leff, S.E., R.M. Evans, and M.G. Rosenfeld. 1987. Splice com-
mittance dictates neuron-specific alternative RNA processing

LeMaire, M.F. and C.S. Thummel. 1990. Splicing precedes poly-

Maniatis, T. 1991. Mechanisms of alternative pre-mRNA splic-

MacDonald, R.J., G.H. Swift, A.E. Przybyla, and J.M. Chirgwin.

from the transformer-2 gene of *Drosophila*. *Genes & Dev.*
5: 786–796.

Mattoo, W., J.M. Palmer, and B.S. Baker. 1990. Alternative splic-
ing of the sex determination gene transformer-2 is sex-spe-
cific in the germ line but not in the soma. *Genes & Dev.*
4: 789–805.

dNA with base-specific chemical cleavages. *Methods Enzy-


McKeown, M., J.M. Belote, and R.T. Boggs. 1988. Ectopic ex-
pression of the female transformer gene product leads to
female differentiation of chromosomally male *Drosophila*. *Cell*
53: 887–895.

1991. α-Tropomyosin mutually exclusive selection: Compet-
tition between branchpoint/polypyrimidine tracts deter-

Nagoshi, R.N. and B.S. Baker. 1990. Regulation of sex-specific
RNA splicing at the *Drosophila* doublesex gene: cis-acting
mutations in exon sequences alter sex-specific RNA splicing

Nagoshi, R.N., M. McKeown, K.C. Burris, J.M. Belote, and B.S.
Baker. 1988. The control of alternative splicing at genes regu-
lating sexual differentiation in *D. melanogaster*. *Cell*
53: 229–236.

a sequence-specific RNA-binding activity. *Genes & Dev.*
3: 1562–1571.

rapid assay for chloramphenicol acetyltransferase gene ex-

Niwa, M., S.C. Rose, and S.M. Berget. 1990. In vitro polyadeny-
lation is stimulated by the presence of an upstream intron.

Noble, J.C.S., C. Prives, and J.L. Manley. 1988. Alternative splic-
ing of SV40 early pre-mRNA is determined by branch site

Noble, J.C.S., H. Ge, M. Choudhuri, and J.L. Manley. 1989. Factor
interactions with the Simian Virus 40 early pre-mRNA

Introns in histone genes alter the distribution of 3’ ends.

Petersen, M.L. and R.P. Perry. 1989. The regulated production of
μS and μA mRNA is dependent on the relative efficiencies of
Biol.* 9: 726–738.

Reed, R. and T. Maniatis. 1988. The role of the mammalian
branch point sequence in pre-mRNA splicing. *Genes & Dev.*
2: 1268–1276.

Robberson, B.L., G.J. Cote, and S.M. Berget. 1990. Exon defini-
tion may facilitate splice site selection in RNAs with mul-


Schneider, I. 1972. Cell lines derived from late embryonic stages

Siebel, C.W. and D.C. Rio. 1990. Regulated splicing of the
*Drosophila* P transposable element third intron in vitro: So-

splicing of α-tropomyosin exons enforced by an unusual lar-

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Regulation of dsx pre-mRNA processing


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

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