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# Regulated Splicing of the *Drosophila Sex-lethal* Male Exon Involves a Blockage Mechanism

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**In *Drosophila melanogaster*, sex determination in somatic cells is controlled by a cascade of genes whose expression is regulated by alternative splicing [B. S. Baker, Nature (London) 340:521–524, 1989; J. Hodgkin, Cell 56:905–906, 1989]. The master switch gene in this hierarchy is *Sex-lethal*. *Sex-lethal* is turned on only in females, and an autoregulatory feedback loop which controls alternative splicing maintains this state (L. R. Bell, J. I. Horabin, P. Schedl, and T. W. Cline, Cell 65:229–239, 1991; L. N. Keyes, T. W. Cline, and P. Schedl, Cell 68:933–943, 1992). *Sex-lethal* also promotes female differentiation by controlling the splicing of RNA from the next gene in the hierarchy, *transformer*. Sosnowski et al. (B. A. Sosnowski, J. M. Belote, and M. McKeown, Cell 58:449–459, 1989) have shown that the mechanism for generating female *transformer* transcripts is not through the activation of the alternative splice site but by the blockage of the default splice site. We have tested whether an activation or a blockage mechanism is involved in *Sex-lethal* autoregulation. The male exon of *Sex-lethal* with flanking splice sites was placed into the introns of heterologous genes. Our results support the blockage mechanism. The poly(U) run at the male exon 3′ splice site is required for sex-specific splicing. However, unlike *transformer*, default splicing to the male exon is sensitive to the sequence context within which the exon resides. This and the observation that the splice signals at the exon are suboptimal are discussed with regard to alternate splicing.**

Not only does alternate splicing provide a mechanism for generating different protein products from a single transcription unit, it is also used to control gene expression and to orchestrate complex developmental pathways (2, 12, 31). In *Drosophila melanogaster*, sex determination in somatic cells is controlled by a cascade of genes whose expression is regulated by alternative splicing (1, 15). At the top of this hierarchy is the *Sex-lethal* (*Sxl*) gene. The activity state of this master switch gene is set early in development in response to the primary sex determination signal, the X chromosome-to-autosome ratio (9, 11, 19, 30). *Sxl* is turned on in females by setting in motion an autoregulatory feedback loop in which female *Sxl* proteins promote their own synthesis by directing the female-specific splicing of *Sxl* primary transcripts (3, 19). This autoregulatory feedback loop functions to maintain the female determined state during the remainder of development. In males the gene is off and no functional *Sxl* proteins are produced. *Sxl* primary transcripts are spliced in the male mode, and the male determined state is maintained by default. The critical difference between the processed mRNAs in the two sexes is exon 3, which contains in-frame translation stop signals that prematurely truncate the open reading frame (4). Inclusion of this exon by the default splicing machinery in males produces mRNAs which do not encode functional proteins (7). Conversely, in females, *Sxl* proteins mediate the skipping of this exon (3), producing mRNAs with full-length open reading frames.

*Sxl* controls the sexual differentiation of somatic cells by determining the splicing pattern of *transformer* (*tra*) RNA (5). When *Sxl* is on it directs the female-specific splicing of *tra* RNAs to generate a long open reading frame. The resulting *tra* protein, together with the protein product from the constitutively active *tra-2* gene, then specifies the female-specific splicing of *doublesex* (*dsx*) (8, 13, 16, 27). In males, where *Sxl* is off, the default splicing of *tra* transcripts produces mRNAs with a truncated open reading frame and, in the absence of functional *tra* protein, *dsx* is spliced in the male mode.

A good deal is known about the alternate splicing of *tra* transcripts (32). In the regulated splice, a common 5′ splice site is joined to one of two competing 3′ splice sites. In the absence of *Sxl* protein, the 3′ splice site closest to the 5′ splice site, the default site, is invariably used. However, in the presence of *Sxl* protein, approximately half the *tra* transcripts are spliced to the second, female-specific 3′ splice site. Sosnowski et al. (32) demonstrated that use of this female-specific 3′ splice site is not due to its activation by *Sxl* protein. Rather, *Sxl* protein blocks the use of the default 3′ splice site and forces the splicing machinery to choose the downstream female-specific site. Critical to this blockage model was the demonstration that a poly(U) sequence run in the polypyrimidine tract of the default 3′ splice site was required for *Sxl* splicing regulation. Inouye et al. (17) subsequently demonstrated that mutations which disrupt or remove this poly(U) sequence in the polypyrimidine tract are no longer recognized by *Sxl* protein in vitro. Interestingly, a comparison of the sequences around the splice junctions of *tra* and *Sxl* reveals that the only significant stretch of homology lies at the *tra* default and *Sxl* male 3′ splice sites (see Fig. 1). The presence of a poly(U) run at the male 3′ splice site of *Sxl* suggested that a blockage mechanism, like that used in *tra*, might account for *Sxl* autoregulation. In this study we have tested whether a blockage mechanism is involved in autoregulation and have identified sequences that may be important for regulated splicing.

## MATERIALS AND METHODS

**Plasmid construction and *Drosophila* transformation.** The *ftz-Sxl* construct was made by placing a 2.0-kb blunt-ended *Bgl*II-*Pst*I fragment of genomic *Sxl* which contains the male

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exon into the *Acc*I site in the intron of the *fushi tarazu* (*ftz*) gene. The *Bgl*II site is about 1,170 bp upstream of the male exon, and the *Pst*I site is 650 bp downstream of the male exon. *lacZ* sequences were fused to the second exon of *ftz* at the *Bgl*II site. The *ftz*-male exon-*lacZ* fragment was placed downstream of the hs83 promoter in a pCaSpeR transformation vector (24) to generate *ftz-Sxl*.

The *white-Sxl* construct was made by placing a 1.27-kb blunt-ended *Hin*cII-*Pst*I fragment of genomic *Sxl* which contains the male exon into the filled-in *Xho* sites of the *white*-Carn3 (26) transformation vector. The *Hin*cII site is 440 bp upstream of the male exon, and the *Pst*I site is 650 bp downstream of the male exon.

To insert the minimal male exon with splice sites into the *Xho* sites of *white*-Carn3 (26), a polymerase chain reaction (PCR) fragment was generated using *Sxl* genomic DNA that included the male exon and primers that extend only beyond the splice signals at the male exon. The resulting fragment was placed directly into *white*-Carn3 either after filling in the *Xho* overhangs or after placing the PCR fragment into Bluescript at the *Sma* site and generating a second *Xho* site at the *Sac*I site by using *Xho* linkers. To mutate the splice sites to match the *Drosophila* consensus, primers with altered sequences were used in the PCR. For all constructs the sequence of the PCR product was determined to ensure that no additional changes were introduced by the PCR.

Germ line transformations were done by injecting the plasmids into a $w^1$; delta 2-3 (25) background or by injecting $w^1$ embryos with plasmid DNA and the helper vector, pTurbo (33). Gene mutations are listed in the work by Lindsley and Zimm (20).

**Analysis of splicing products.** Reverse transcription was done as described by Frohman et al. (10). The *lacZ* primer used was located immediately 3′ to the one shown in Fig. 2, while a primer located in exon 4 of *white* was used for the *white* constructs. Approximately 4% of the cDNA mixture was then amplified by PCR. Each amplification employed one cycle of 95°C for 3 min, 62°C for 2 min, and 72°C for 40 min followed by 26 repeats of the cycle 95°C for 45 s, 62°C for 2 min, and 72°C for 1.5 min. Detection of products was done by Southern analysis with either the male-specific exon as the probe or *ftz* or *white* sequences as the probe.

## RESULTS

**The male exon contains information for *Sxl* splicing regulation.** If a blockage mechanism is employed in autoregulation, *Sxl* protein would force female splicing—the joining of exons 2 to 4—by preventing the splicing machinery from utilizing the male exon (Fig. 1). In this case, the signals required for the female-specific splicing of *Sxl* transcripts should be associated with the male exon, exon 3, while the sequences around exons 2 and 4 should be dispensable (Fig. 1). Hence, a prediction of this model is that it should be possible to confer sex-specific alternate splicing on a heterologous gene by placing the *Sxl* male exon with its surrounding splice signals into one of the introns of that gene. To test this prediction, a 2-kb fragment containing the ~190-bp *Sxl* male exon plus 1,170 bp of 5′ and 650 bp of 3′ intron sequence was placed into the middle of the 150-bp intron of the *Drosophila ftz* gene. This fragment should contain all standard *cis*-acting elements required for splicing (e.g., the branch point and polypyrimidine tract). As indicated in Fig. 2, *lacZ* sequences were fused to the second exon of *ftz* so that we could examine RNA specific to the construct by reverse transcription and PCR amplification. The hs83 promoter, which is

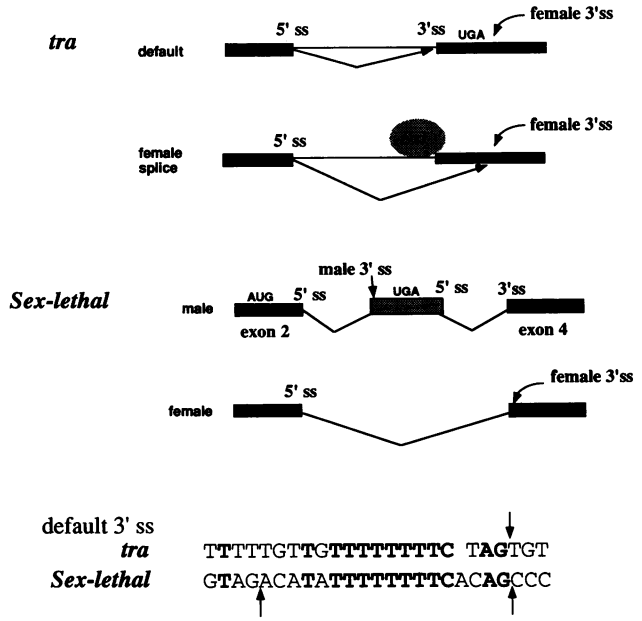


FIG. 1. Comparison of the alternative spliced regions of *tra* (top) and *Sxl* (bottom). The blockage model proposes that *Sxl* protein binds to the first 3′ splice site (ss) of *tra* to block its use so that the second 3′ splice site is used. The sequence of homology at the default splice sites in *tra* and *Sxl* is also shown. Identical bases are in boldface type. Bars represent exons, lines connecting the bars represent introns, and arrows represent splicing events.

constitutively active at most developmental stages (34), was used to drive expression of this chimeric gene. We decided to study the splicing of transcripts from this *ftz-Sxl-lacZ* chimera in transgenic flies as we were concerned that a tissue culture transient assay system might not faithfully duplicate the actual in vivo splicing condition. In particular, previous experiments by Inoue et al. (17) using Kc tissue culture cells showed that an exogenous source of *Sxl* protein is required for female splicing of transiently expressed *tra* RNAs despite the fact that this cell line is female and expresses *Sxl* protein (6). Additionally, the effect of gene dose, evident in the experiments of Inoue et al. (17), would make it difficult to interpret results, particularly in cases in which the splicing products from a chimeric gene were of mixed sex.

Figure 2A shows the splicing pattern of transcripts from the *ftz-Sxl* male exon construct in adult male and female flies. Two findings are of interest. First, the splicing of transcripts from this chimeric gene in adult males does not reproduce the default splicing pattern observed for *Sxl*. In the case of *Sxl*, all processed RNAs in males appear to include the male exon. In contrast, only ~20% of the RNAs from the chimeric gene are spliced to include the male-specific exon. Most RNAs in males show the splicing pattern predicted for the female mode of splicing, i.e., the first *ftz* exon is directly joined to the second. Second, the splicing of transcripts from this chimeric gene in adult females does reproduce the regulated splicing of *Sxl*; all the transcripts from the chimeric gene are spliced in the female mode. Hence, this small male exon fragment appears to contain sequences which can specify *Sxl*-regulated splicing to a heterologous gene.

We were somewhat surprised that our chimeric *ftz-Sxl* construct did not faithfully reproduce the default splicing

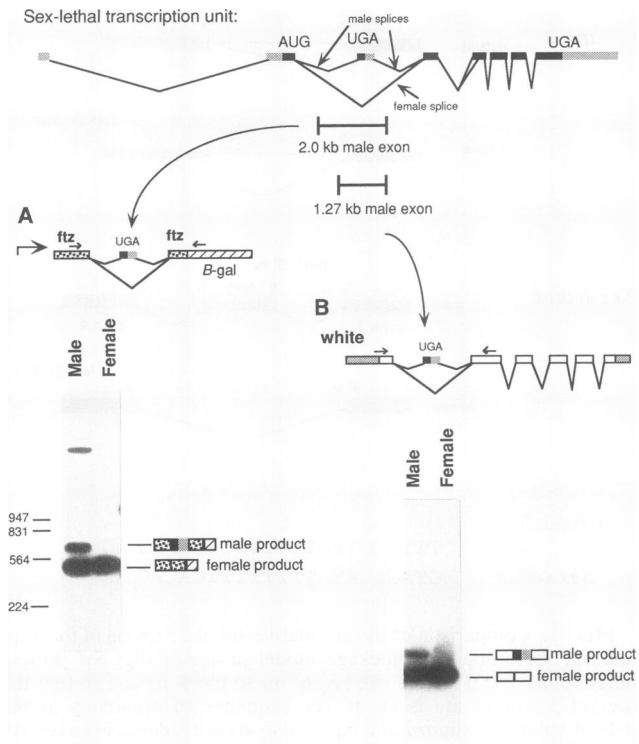


FIG. 2. Splicing pattern of chimeric *ftz-Sxl* (A) and *white-Sxl* (B) genes in adult males and females. Top, *Sxl* transcription unit with the exons shown as bars. Lines joining the bars indicate splicing; the female versus male splicing choice is indicated around the male exon, exon 3 (the exon which contains a UGA codon immediately downstream of the exon with the AUG codon). Genomic fragments containing the male exon placed into *ftz* or *white* are also shown. The fragment placed in *ftz* is about 2 kb and includes 1,170 bp of upstream and 650 bp of downstream sequences. In *white*, the fragment is 1.27 kb and includes 440 bp of upstream and 650 bp of downstream sequences. *lacZ* sequences are indicated by *B*-gal. The arrow in front of the *ftz-Sxl* chimera represents the hs83 promoter. The small arrows above the genes depict the position of primers used for the PCR. Alongside the Southern blots are the positions of migration of products which include the male exon ("male product") or products which spliced around the male exon ("female product") and the exon structures of the products. In the male lane in panel A, the band at the top is from contaminating genomic DNA. Note that in panel B, the endogenous *white* gene RNA contributes a product of the same size as the female product in both males and females. The dark red eye color observed in males indicates that a substantial amount of the RNA is spliced around the male exon. Additionally, in a deletion strain ($w^{1118}$) the product ratio appears to be similar, suggesting that the contribution to the female-sized band from the $w^{1}$ background is low.

pattern. Mistakes in default splicing of *Sxl* in males could have disastrous consequences; *Sxl* protein produced from transcripts spliced in the female mode could set the autoregulatory feedback loop in motion and switch the sexual development pathway from male to female. Given these demands for fidelity in default splicing, we had anticipated that the *Sxl* male exon would be an excellent substrate for the splicing machinery and would function efficiently in a heterologous context. One plausible explanation is that some unusual features of the *ftz* transcript prevent utilization of the male exon. To test this possibility we placed the male exon (a slightly smaller fragment than that used for *ftz*, 1.3 kb) into the 3-kb first intron of the *Drosophila white* gene (Fig. 2B). We then transformed this *white-Sxl* gene into *white* mutant flies. If the male exon is included in *white* transcripts with the same efficiency as in *Sxl* transcripts, the eye color of male flies should be same as the starting mutant, i.e., white. Females, on the other hand, should have red eyes by virtue of the exclusion of the male exon from the *white-Sxl* transcripts.

Contrary to expectations, both male and female transgenic flies had dark red eyes (not shown), indicating that the splicing machinery was excluding the male exon from at least some of the *white-Sxl* transcripts in both sexes. Figure 2B shows that the splicing pattern observed with the *white-Sxl* construct is essentially the same as that observed for the *ftz-Sxl* construct. In males, less than one-third of the *white* RNA includes the male-specific exon. Hence, even in this context, the male exon appears to function poorly as a splicing substrate. Once again, though default splicing is defective, regulated splicing appears to be quite efficient and all *white-Sxl* transcripts in females are spliced in the female mode. Taken together with the splicing of the *ftz-Sxl* transcripts in females, this finding indicates that the signals required for regulated splicing are associated with the male exon and supports the suggestion that a blockage mechanism accounts for *Sxl* autoregulation.

**Suboptimal splice sites at the male-specific exon.** While the results described above suggest that a blockage mechanism is used for *Sxl* autoregulation, the fragments containing the ~190-bp male exon in both the *ftz* and the *white* experiments were over 1 kb in length (2 and 1.3 kb, respectively). It could be argued that the extensive intron sequences in these *Sxl* fragments might have structural features that somehow facilitate the joining of the flanking splice sites in the *ftz* or *white* genes. Hence, it was important to determine whether a much smaller fragment containing only the intron sequences intimately associated with the male exon is sufficient to confer *Sxl*-mediated regulation. For this purpose we inserted a minimal male exon into the first intron of the *white* gene. This minimal exon is 228 bp in length and extends from just upstream of the putative branch site to just downstream of the 5′ splice site. Transgenic flies were isolated for this construct, and RNA from males and females was analyzed. In this form, the male exon is not even recognized by the splicing machinery in males (or females) (Fig. 3) and all transcripts are spliced in the female mode, that is, the first and second exons of *white* are joined.

This finding, together with the results described above, suggests that the male exon may be an inherently poor substrate for the (default) splicing machinery. An examination of the splice signals at the male exon reveals suboptimal features (Fig. 4A). There are two 3′ splice sites, two AG dinucleotides, which are used at nearly equal frequencies as judged from RNase protection data and cDNA analysis (4, 29). The proximal AG is located upstream instead of downstream of the polypyrimidine tract and is only 10 nucleotides from the putative branch point (which is a reasonably good fit to the *Drosophila* branch point consensus [18, 21]). The distal AG is downstream of the polypyrimidine tract and is at the more conventional distance of 28 nucleotides from the branch point. Conceivably, these two closely spaced splice sites might compete for the assembly of splicing complexes, each destabilizing a complex that is beginning to form at the other site. Moreover, there are four other AG dinucleotides in the first 15 nucleotides immediately downstream of the distal 3′ splice site (Fig. 4A) and two more in the next 26 nucleotides. These additional AG dinucleotides could also compete with the two bona fide splice sites for factors and

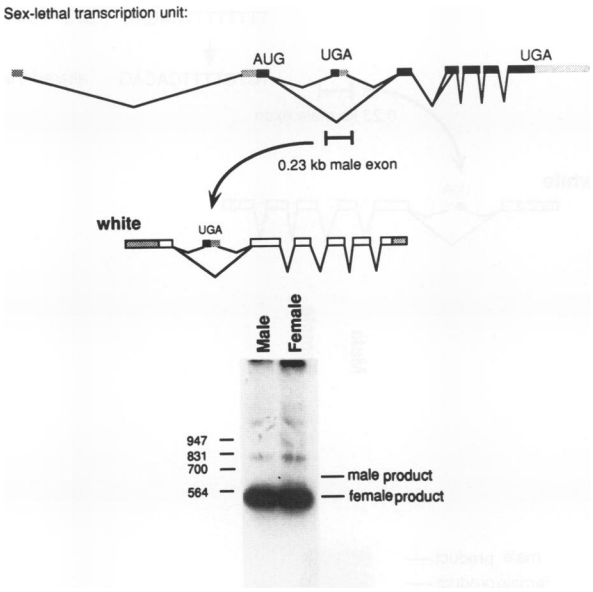


FIG. 3. Splicing of *white*-male exon gene. A fragment with the male exon and splice sites only was placed in the same position of *white* as the larger genomic fragment shown in Fig. 2B. Neither males nor females include the male exon in their RNAs. The result is the same in a $w^{1118}$ background. Symbols and markers are as described in the legend to Fig. 2.

interfere with utilization of the male exon. The 5′ splice site of the exon also has an unusual, and potentially suboptimal, sequence. Instead of the highly invariant AG/GT at the exon-intron boundary, the male 5′ site is CT/GT (Fig. 4A).

We decided to determine whether optimizing the splice signals of this minimal male exon might increase its utilization by the default splicing machinery. Either the putative branch site was optimized to the yeast UACUAAC (for which the *Drosophila* consensus is a subset), or the UAC UAAC signal was introduced and the first (unconventional) AG at the male exon 3′ splice site was removed. These two improved 3′ splice sites were paired either with the canonical male exon 5′ splice site or with a 5′ splice site that had the CT/GT converted to the consensus AG/GT sequence. Transgenic flies for the different constructs were isolated, and we examined the splicing pattern in males. Figure 5 shows that the minimal male exon was not recognized as an exon by the default splicing machinery (in males) except when both the 3′ splice site and the 5′ splice site were altered to fit the *Drosophila* consensus splice site sequence. These results indicate that utilization of the male exon can be improved by altering the splice signals to more closely match the consensus sequences. However, the degree of recognition of the improved minimal male exon in the context of the *white* gene is still poor, and as can be seen in the figure, a majority of the transcripts in male flies are spliced in the female mode.

Since the optimized minimal male exon is recognized (though inefficiently) by the default splicing machinery, we can ask whether it contains sequences able to confer *Sxl*-mediated regulation. As can be seen in Fig. 5, most of the male-spliced products are spliced in the female mode in female flies. This finding indicates that the minimal male exon contains sequences capable of mediating *Sxl*-regulated splicing and would support the blockage model. Interestingly, in contrast to the results obtained with the larger male exon fragments (see above), a low level of male-spliced RNA can be detected in females carrying the *white* gene with the optimized minimal male exon. The small amounts of male-spliced RNA produced by this construct could indicate that improving the splice sites of the male exon to match the


FIG. 4. (A) Comparison of *Drosophila* consensus 3′ and 5′ splice sites with the splice sites at the male exon. Bases at highly conserved positions that deviate from the consensus are shown in boldface type. AG dinucleotides within the male exon are also underlined, although splicing occurs only at the positions marked by arrows. Changes made to improve the splice sites at the male exon are shown below the wild-type sequence. (B) Comparison of *Sxl* splice sites flanking the male exon with the *Drosophila* consensus. Bases at highly conserved positions that deviate from the consensus are shown in boldface type.

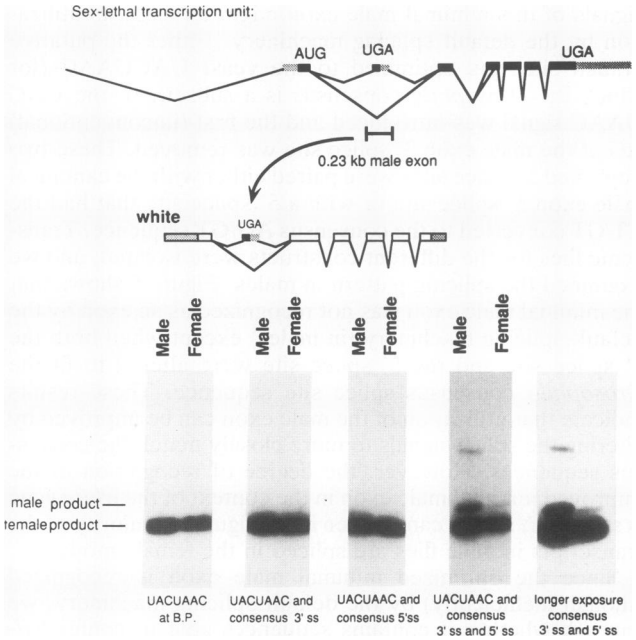


FIG. 5. Splicing pattern of *white*-male exon genes with an altered 3′ or 5′ splice site (ss) or both. Symbols and markers are as described in the legend to Fig. 2. No recognition of the male exon occurs when either splice site alone is altered. The male exon is included in males when both splice sites are optimized. Regulation of these transcripts occurs fairly well in females—only low levels of RNA that include the male exon are detected, as shown in the longer exposure. In the deletion strain ($w^{1118}$) the product ratio was similar, suggesting that the contribution to the female-sized band from the endogenous $w^{1}$ allele is low. B.P., branch point.

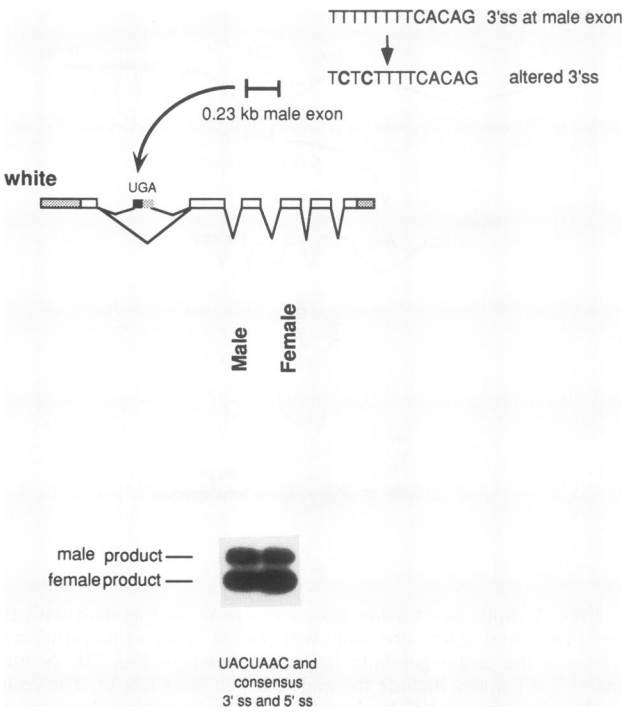


FIG. 6. Altering the polypyrimidine tract at the male exon disrupts female splicing regulation. Changes made at the polypyrimidine tract are shown at the top. Symbols and markers are as described in the legend to Fig. 2. ss, splice site.

consensus may not be compatible with efficient female-specific regulation. Also, there may be additional elements important for *Sxl* regulation in the two introns flanking the male exon that were not included in our minimal exon fragment.

**The run of Us at the polypyrimidine tract is required for female-specific regulation.** The studies of Sosnowski et al. (32) showed that the poly(U) run in the polypyrimidine tract just upstream of the default splice site was essential for *Sxl* regulation of *tra* splicing. Since the 3′ splice site of the *Sxl* male exon also contains a poly(U) run, it was of interest to determine whether it plays a similar role in *Sxl* splicing regulation. In *tra*, changing the third, fifth, and seventh U residues in the poly(U) run makes the default 3′ splice site insensitive to *Sxl*. As indicated in Fig. 6, we altered the poly(U) run of the optimized minimal male exon and then inserted this exon into the *white* gene. As is evident from a comparison of the RNAs in transgenic males and females, this change in the poly(U) run of the optimized minimal male exon disrupts *Sxl* regulation. In fact, *Sxl* appears to have little or no effect on the splicing of RNA from this construct, and the ratio of male-spliced to female-spliced products is essentially the same in the two sexes.

## DISCUSSION

Two general mechanisms can be considered for how *Sxl* might control the alternate splicing of the male exon. In the first, the function of *Sxl* protein would be positive; it would direct the splicing machinery to join the flanking exons 2 and 4 by somehow activating the splice sites of one or both of these exons. In the second, *Sxl* protein would have a negative or inhibitory function; by blocking the utilization of the male exon it would promote the joining of the two flanking exons. In a mechanism of the first type, the key *cis*-acting targets for *Sxl* action are likely to be associated with one or both flanking exons, while the male exon itself should be irrelevant for regulated splicing. In a mechanism of the second type, in which *Sxl* blocks the utilization of the male exon, the key *cis*-acting targets are likely to be associated with the male exon itself. In this case it should be possible to replace the flanking exons with entirely heterologous exon sequences.

In the work reported here we have tested the blockage mechanism by asking whether the male exon is able to confer regulated alternate splicing on a heterologous gene. Our results support the blockage mechanism. We have shown that the male exon is subject to *Sxl* regulation when a fragment containing the exon plus flanking intron sequences is placed in the introns of two different genes, *ftz* and *white*. Extensive intron sequences do not appear to be required for regulation in a heterologous context; a very small fragment containing only the male exon plus 3′ and 5′ splice signals optimized to fit the consensus is sufficient. These experiments also point to a probable target for *Sxl* action. This minimal male exon contains the only homologous sequence between the regulated splice sites of *tra* and *Sxl*, the bulk of which is a long poly(U) run in the pyrimidine tract of the male (or default) 3′ splice site. Sosnowski et al. (32) have shown that in *tra*, this poly(U) run is required for sex-specific splicing regulation. The results reported here indicate that the poly(U) run in the *Sxl* 3′ splice site is also required for the sex-specific splicing of the optimized mini-

mal male exon in a heterologous context. From in vitro studies on the sequence specificity of *Sxl* protein (17, 28), it would be reasonable to suppose that the protein binds to the poly(U) run at the 3′ splice site of the male exon and prevents the splicing machinery from using this site.

Although the experiments reported here indicate that a blockage mechanism is probably used in *Sxl* autoregulation, two disquieting observations suggest that directly applying the *tra* model to autoregulation may be an oversimplification. The first is that splicing of the optimized minimal male exon is not completely regulated in females and a small amount of male spliced product is observed. Though several hypotheses could be advanced to explain this result, we suspect that the most likely possibility is that this minimal male exon fragment does not contain all the *cis*-acting targets for *Sxl* regulation. A priori this is not an unreasonable suggestion. The male exon differs from *tra* in that it contains both a 3′ and a 5′ splice site. To completely prevent utilization of the male exon it may not be sufficient to block only the 3′ splice site, and there could be other *Sxl* targets in the flanking introns that are positioned to interfere with the use of the 5′ splice site.

That there are additional levels of complexity involved in the splicing regulation of *Sxl* is also suggested by the second and altogether unexpected observation that default splicing is quite inefficient when the male exon is placed in a heterologous context. As pointed out above, the autoregulatory potential of *Sxl* protein products makes fidelity in default splicing essential for normal male development and viability. Although we do not know to what extent males can tolerate mistakes in default splicing without stably activating the autoregulatory feedback loop, default splicing must nevertheless be extremely efficient, as we have never been able to detect female-spliced *Sxl* transcripts in male flies. These considerations had mistakenly led us to expect that the male exon would be an excellent substrate for the splicing machinery. Instead, the male exon appears to be an inherently poor substrate, raising the question of why it is able to function so well in *Sxl*. A number of factors may be responsible.

First, the sequence context in *Sxl* may favor efficient inclusion of the male exon. This possibility is suggested by the finding that inclusion of the male exon in the *white* gene decreased to nothing when most of the flanking intron sequences were removed. This result would indicate that the introns flanking the male exon contain information that contributes to its utilization by the splicing machinery. Such information could be specific sequences which facilitate the recognition or use of the suboptimal splice sites at the male exon. Alternatively, structural features in the *Sxl* transcript might promote inclusion of the male exon. For example, the intron separating exon 2 and the male exon (exon 3) may assume a secondary structure that is particularly favorable for joining these two exons. When the exon is placed in a heterologous context, formation of this structure might be disrupted, resulting in the skipping of the male exon.

Second, efficient utilization of the male exon within the context of *Sxl* may also depend on closely matching the strengths of the splice sites of the flanking exons with the suboptimal competing sites at the male exon. Indeed, the splice sites of the flanking exons in *Sxl* have features which suggest that they may also be suboptimal (Fig. 4B). The 5′ splice site of exon 2 has an A residue at position +5 in the intron instead of the conserved G, a difference that might perturb the base pairing of U1 small nuclear ribonucleoprotein particles to this site. The 3′ splice site of exon 4 has two differences from the consensus which might make it suboptimal; there is a T instead of an A or G at position +1 of the exon, and the predicted branch point adenosine is located further than normal (48 as opposed to 18 to 35 nucleotides [21]) from the 3′ splice site AG. These features might weaken the 5′ and 3′ splice sites of *Sxl* exons 2 and 4, respectively, and allow efficient inclusion of the male exon in *Sxl* transcripts.

Of course, it is possible that the presence of weak splice sites in the alternately spliced region of *Sxl* is necessary for efficient regulation. Alternate splicing is likely to be a kinetic phenomenon, dictated by factors such as the time of appearance of splice sites (position on the transcript), the rate of assembly of splicing complexes at competing sites, and the rate of utilization of these complexes. In this view, the features of the male exon 3′ splice site which might perturb efficient assembly of splicing complexes (two 3′ splice site AG dinucleotides that are used at nearly equal frequencies as well as six additional AG dinucleotides further downstream in the male exon) might also enable *Sxl* protein to more readily block the formation of complexes and so influence splicing choice. Fidelity of default splicing in males, on the other hand, would require that the strengths of the flanking *Sxl* splice sites (at exons 2 and 4) be weakened to match the suboptimal ones of the male exon. Suboptimal splice sites have also been implicated in the alternate splicing of other transcripts (14, 22, 23), suggesting that this may be a common theme in regulated splicing.

## ACKNOWLEDGMENTS

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