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Sex-lethal Autoregulation Requires Multiple *cis*-Acting Elements Upstream and Downstream of the Male Exon and Appears To Depend Largely on Controlling the Use of the Male Exon 5′ Splice Site

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The on/off state of the binary switch gene *Sex-lethal* (*Sxl*), which controls somatic sexual development in *Drosophila melanogaster*, is regulated at the level of alternative splicing. In males, in which the gene is off, the default splicing machinery produces nonfunctional mRNAs; in females, in which the gene is on, the autoregulatory activity of the Sxl proteins directs the splicing machinery to produce functional mRNAs. We have used germ line transformation to analyze the mechanism of default and regulated splicing. Our results demonstrate that a blockage mechanism is employed in *Sxl* autoregulation. However, in contrast to *transformer*, in which *Sxl* appears to function by preventing the interaction of splicing factors with the default 3′ splice site, a different strategy is used in autoregulation. (i) Multiple *cis*-acting elements, both upstream and downstream of the male exon, are required. (ii) These *cis*-acting elements are distant from the splice sites they regulate, suggesting that the Sxl protein cannot function in autoregulation by directly competing with splicing factors for interaction with the regulated splice sites. (iii) The 5′ splice site of the male exon appears to be dominant in regulation while the 3′ splice site plays a subordinate role.

Posttranscriptional regulatory mechanisms play a key role in cell fate decisions in many developmental pathways. In the somatic sexual-development pathway of the fruit fly, *Drosophila melanogaster*, both the maintenance and elaboration of pathway choice are controlled at the level of alternative pre-mRNA splicing (2, 21). The binary switch gene *Sex-lethal* (*Sxl*) sits at the top of the somatic sexual-development pathway and functions in both determination and differentiation. The activity state of the *Sxl* gene is chosen during early embryogenesis in response to the primary sex determination signal, the ratio of X chromosomes to autosomes (9, 14, 27, 47). *Sxl* is turned on in females, while it remains off in males. Activation in females triggers an autoregulatory feedback loop in which female Sxl proteins promote their own synthesis by directing the female-specific splicing of *Sxl* primary transcripts (3, 4). This autoregulatory feedback loop then functions to maintain the female-determined state during the remainder of development. In males, in which the gene is not activated, *Sxl* primary transcripts are spliced in the nonproductive male mode and the male-determined state is maintained by default. The critical difference between the processed *Sxl* mRNAs in the two sexes is exon 3, which contains in-frame translation stop signals that prematurely truncate the open reading frame (4, 6). In females, *Sxl* proteins mediate the skipping of this exon (3), generating mRNAs which are predicted to encode protein species containing two RNA recognition motif (RRM) RNA-binding domains. In contrast, inclusion of this exon by the default splicing machinery in males produces mRNAs which encode only short, presumably nonfunctional polypeptides.

Sxl controls differentiation by regulating several gene cascades that specify different aspects of somatic sexual development. In the best understood of these pathways, *Sxl* regulates the splicing of *transformer* (*tra*) RNA (5). When *Sxl* is on, it directs the female-specific splicing of *tra* primary transcripts, producing mRNAs which have an intact open reading frame. The resulting tra protein, together with the constitutively expressed tra-2 protein, then activates the female-specific splice site of *doublesex* (*dsx*) to generate female *dsx* RNA (7, 18, 24, 43). When *Sxl* is off, the default splicing of *tra* gives mRNAs with a truncated open reading frame, and in the absence of functional *tra* protein, *dsx* is spliced in the male mode.

The mechanism for *Sxl* regulation of *tra* splicing is now reasonably well understood. As diagrammed in Fig. 1, a common 5′ splice site is joined to one of two 3′ splice sites. In the absence of Sxl protein, the splicing machinery uses the first or default 3′ splice site. When Sxl protein is present, it promotes female splicing by blocking the utilization of the default 3′ splice site, and approximately half of the *tra* transcripts are spliced to the second or female 3′ splice site. Sosnowski et al. (49) have shown that a poly(U) run in the polypyrimidine tract of the default 3′ splice site is required for *Sxl* splicing regulation; the introduction of C residues into this poly(U) run eliminates sex-specific regulation in vivo. That this sequence is the actual target for Sxl protein action is suggested by the finding that *tra* transcripts carrying mutations in the poly(U) run are not recognized by Sxl protein in vitro (25, 45, 54). These observations have lead to a model in which Sxl protein prevents utilization of the default 3′ splice site by binding to the poly(U) run in the *tra* polypyrimidine tract and competing with the binding of splicing factors that interact with the polypyrimidine tract such as U2AF (see references 33 and 54).

While much less is known about the regulated splicing of *Sxl* transcripts, several lines of evidence have suggested that an analogous blockage mechanism, involving the male exon 3′ splice site, might be used in autoregulation. The first comes from a sequence comparison of the regulated splice

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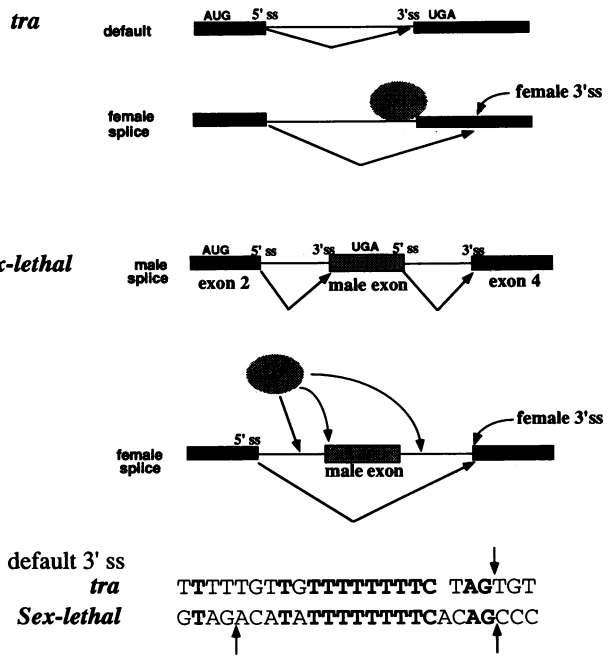

FIG. 1. Splicing regulation of *tra* and *Sxl*. The *tra* gene is diagrammed at the top, and *Sxl* is diagrammed below. Splicing regulation of *tra* by the blockage model is represented. Sxl protein binds to the first 3′ splice site of *tra* and blocks its use so that the second female 3′ splice site is used. In the regulation of *Sxl*, Sxl protein is shown interacting not only with the male exon 3′ splice site but also with intron sequences upstream and downstream of the male exon. The region of sequence homology at the default splice site in *tra* and *Sxl* is shown at the bottom. Identical bases are in boldface, and arrows depict splicing events. Boxes represent exons. Lines connecting the boxes represent introns, and arrows below the exons represent splicing events. ss, splice site.

sites of *tra* and *Sxl*. Like *tra*, the polypyrimidine tract of the male exon has a large poly(U) run which would be expected to bind Sxl protein (4). The second comes from recent experiments on the splicing properties of the male exon in a heterologous context. In the blockage model, Sxl protein would direct female splicing—the joining of exons 2 to 4 (Fig. 1)—by interfering with the utilization of the male exon splice signals. In this case, the targets for *Sxl* action should be associated with the male exon, exon 3, while sequences around exons 2 and 4 should not be required for regulation. Consistent with this prediction, the *Sxl* male exon plus flanking 5′ and 3′ intron sequences is capable of conferring *Sxl*-dependent regulation when placed in a heterologous context (23). The third comes from an analysis of sequences required for *Sxl*-dependent splicing of the male exon. We were able to generate an "optimized minimal male exon" flanked by 3′ and 5′ splice signals (optimized to more closely match the consensus) which was subject to *Sxl* regulation when placed in a heterologous intron. As in *tra*, the regulated splicing of this optimized minimal male exon was dependent on the integrity of the poly(U) run in the *Sxl* polypyrimidine tract. When C residues were introduced into the *Sxl* poly(U) run, *Sxl*-dependent splicing regulation was abolished.

Although our results would seem to support a simple blockage mechanism for autoregulation—that is, Sxl protein interferes with a polypyrimidine tract-specific splicing factor by binding to the poly(U) run at the male exon 3′ splice—there were two unsettling observations. First, we never observed faithful default splicing, and the bulk of RNAs in males were spliced in the female mode. In the *Drosophila* sexual development pathway, fidelity in the default splicing of *Sxl* is essential, since even infrequent mistakes in males could potentially induce a permanent switch in sexual identity by activating the autoregulatory feedback loop. This failure in default splicing suggested that important aspects of splicing regulation might have been missed when we analyzed the processing of the male exon in a heterologous context. The second observation was the splicing pattern of the optimized minimal male exon in females. We found that the optimized minimal male exon was not completely regulated in females and that a small amount of male spliced product was observed. The lack of complete regulation was somewhat surprising, considering that the splicing system in males, in which Sxl protein is absent, is already poised to skip the male exon most of the time. This finding would suggest that efficient female regulation requires sequences in addition to the poly(U) run at the 3′ splice site that may have been deleted from our minimal male exon fragment.

These two observations prompted us to reinvestigate the requirements for faithful default and regulated splicing in the context of the *Sxl* exon and intron sequences. The studies reported here indicate that the splicing regulation of *Sxl* does not fit the simple picture that has been developed from in vivo and in vitro studies of the splicing of *tra* transcripts. The conclusion that a more complex mechanism may be involved in *Sxl* autoregulation is also supported by recent work of Sakamoto et al. (44) using a transient tissue culture assay system.

MATERIALS AND METHODS

Plasmid construction and *Drosophila* transformation. The *Sxl* miniconstruct diagrammed in Fig. 2 was made by first isolating a 310-bp *Pst*I-*Hpa*II fragment of genomic *Sxl* which contains exon 4 and upstream intron sequences. This fragment was placed into the vector BlueScript (Promega), and *lacZ* sequences were then cloned, in frame, into the downstream *Bam*HI site. A *Pst*I fragment from a phage containing the *Sxl* region from exon 2 to exon 4 was then ligated upstream of the exon 4-*lacZ* sequences to reconstruct the regulated region of *Sxl*. The *Sxl* miniconstruct also served as the substrate for site-directed mutagenesis (29) of the polypyrimidine tract at the male exon 3′ splice site (see Fig. 5) and for the deletion constructs described below.

The unique *Nco*I site in the male exon was used to delete most of the male exon sequence (by exonuclease III) for the construct diagrammed in Fig. 3. The ends were made blunt with Klenow fragment digested with *Pst*I, and the fragment from exon 2 to the shortened male exon was cloned upstream of *lacZ* sequences to create the single-intron construct. The male exon-*lacZ* junction was determined by sequencing with a primer in *lacZ* sequences. The construct diagrammed in Fig. 3 had 11 bp of the exon from the second AG splice site of the male exon.

To generate the *tra*-like constructs, two approaches were used. In the first, the shortened male exon fragment for generating the single-intron construct shown in Fig. 3 was placed upstream of the 310-bp exon 4-*lacZ* fusion plasmid (used to make the construct described above and diagrammed in Fig. 2). This produced the construct shown in Fig. 3A. The construct shown in Fig. 3B was made in the same way, except that the exon 2-to-male exon fragment was

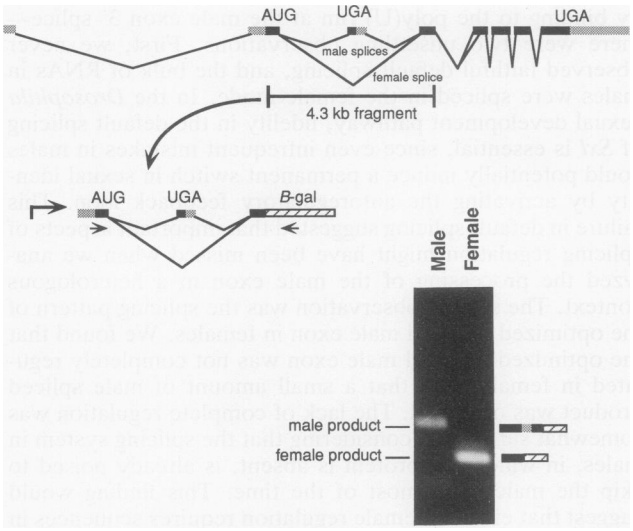

FIG. 2. The *Sxl* minigene reproduces sex-specific splicing. The top of the figure diagrams the *Sxl* transcription unit with the exons shown as boxes. Lines joining the boxes indicate splicing; female versus male splicing choice is indicated around the male exon-exon 3 (exon which contains the UGA codon immediately downstream of the exon with the AUG). A 4.3-kb genomic fragment spanning the three exons and two introns around the regulated splices was cloned upstream of *lacZ* sequences and downstream of the *hs83* promoter (represented by a bent arrow). *lacZ* sequences are indicated by *B*-gal. The small arrows below the gene depict the positions of primers used for the PCRs. RNA from males or females is indicated at the top of the gel lanes. Alongside the gel are marked the expected positions of migration of products which include the male exon (male product) or products which splice around the male exon (female product). The exon structures of the products are also shown.

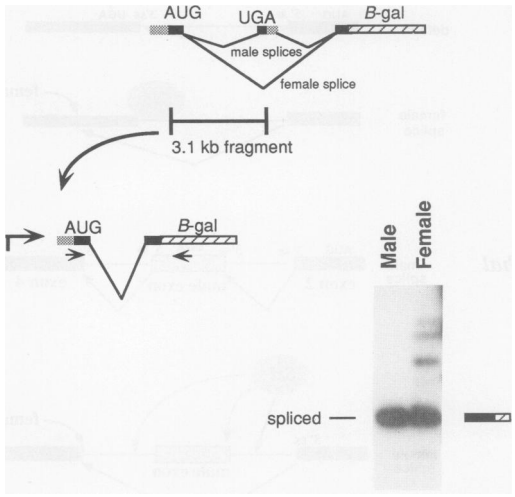

FIG. 3. Single intron construct of *Sxl*. A 3.1-kb genomic fragment from exon 2 to the male exon was cloned upstream of *lacZ* sequences and downstream of the *hs83* promoter. Symbols and markers are as described in the legend to Fig. 2. The expected position of migration of product when the intron is spliced is shown alongside the Southern blot.

generated by polymerase chain reaction (PCR) with a primer upstream of exon 2 and a primer that included all but the last two bases of the male exon. The male exon junction of the construct was confirmed by sequencing.

To delete the 3′ splice site at the male exon, an *Acc*I partial digest was done on the DNA of the *Sxl* miniconstruct. The singly cut fragment was isolated, digested to completion with *Nco*I (unique site in the male exon), and ligated. Transformants of the correct size gave the product shown in Fig. 6.

The upstream intron deletion was made by replacing a genomic *Bgl*II-*Pst*I male exon fragment from the *Sxl* minigene (Fig. 2) by a *Pvu*II-*Pst*I fragment of the male exon. This had the effect of deleting 1,150 bases upstream of the putative branch point of the male exon (see the construct diagrammed in Fig. 7A), since the *Pvu*II site resides downstream of the *Bgl*II site but upstream of the male exon (see reference 46 for restriction map of *Sxl*). To introduce the T-to-C changes in the polypyrimidine tract of the male exon 3′ splice site, the *Pvu*II-*Pst*I male exon fragment was isolated from the mutagenized construct shown in Fig. 5 instead of the wild-type fragment to generate the construct shown in Fig. 7B.

The downstream intron deletions shown in Fig. 8A and B were created in the same way as the *tra*-like construct shown in Fig. 4B, except that the PCR primer used to generate the exon 2-male exon fragment extended into the intron downstream of the male exon to include the 5′ splice site sequence and 6 or 250 additional intron bases, respectively. To replace the deleted intron sequences with the pp19 fragment, the pp19 fragment was first cloned, in both orientations, upstream of the exon 4-*lacZ* clone used in generating the *Sxl* miniconstruct (see above). The exon 2-male exon fragment shown in Fig. 8A was then cloned upstream of the pp19 fragment.

All constructs were placed into a CasPeR vector (39) that had been modified to include the *Drosophila hs83* promoter in the polylinker cloning site so that transcription from the *hs83* promoter would drive expression of the *Sxl-lacZ* fusion construct. Germ line transformations (50) were done by injecting the plasmids into a w^1; Δ2-3 (42) background or by injecting w^1 embryos with plasmid DNA and the helper vector, pTurbo (52). Gene mutations are listed by Lindsley and Zimm (31).

Analysis of splicing products. RNAs from male and female flies were isolated as described previously (3). Reverse transcription was done as described by Frohman et al. (13). The *lacZ* primer used was located as shown in Fig. 2. Approximately 4% of the cDNA mixture was amplified by PCR with the *lacZ* primer used for reverse transcription and a primer located in exon 2 of *Sxl*. In the first amplification, there was one cycle of 95°C for 3 min, 62°C for 2 min, and 72°C for 40 min and then 26 repeats of a cycle consisting of 95°C for 45 s, 61°C for 2 min, and 72°C for 1.5 min. A 1 to 2% aliquot of the first amplification mixture was then reamplified by the exon 2 *Sxl* primer and a *lacZ* primer internal to the one used in the first amplification. Twenty rounds of the second cycle described above were used. In Fig. 3 and 4A, samples were analyzed after the first amplification; the remaining samples were analyzed after reamplification. Products were detected by Southern analysis with either the male-specific exon or exon 4-*lacZ* sequences as the probe. The sequences are as follows: *lacZ* primer used for reverse transcription and first amplification, 5′ CGCATCGTAAC CGTGCATCTGC 3′; *lacZ* primer for second amplification, 5′ CGCCATTCAGGCTGCGCAACTG 3′; *Sxl* exon 2 primer, 5′ GTGGTTATCCCCCATATGGC 3′.

The products shown in Fig. 7 and 8 were also probed by

fragments from the two introns in the *Sxl* miniconstruct. Fragments in the central regions of the intron were generated by using available restriction sites. PCR amplification with primers close to the exon-intron boundaries were used to generate probes in cases in which convenient restriction sites were not available.

Sequencing and cloning of PCR products. Splicing products from the male exon 3′ splice site deletion construct (see Fig. 6) were amplified as described above and were digested with *Bam*HI to cleave the *lacZ* portion away from the *Sxl* portion, and the resulting products were cloned into Bluescript digested with *Bam*HI and *Eco*RV. White bacterial colonies plated on isopropyl-β-D-thiogalactopyranoside (IPTG)–5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates were sequenced by standard single-stranded DNA techniques.

Quantifying relative ratios of splice products. Blots probed with exon 4-*lacZ* sequences (the region common to both sexes) were imaged on a Molecular Dynamics PhosphorImager system. Counts in the male and female bands minus background values were quantified to give estimates of the ratios of splicing products.

RESULTS

The three exons and two introns around the regulated splice sites are sufficient for sex-specific splicing. Previously, we showed that an *Sxl* minigene which extends from exons 2 to 5 could reproduce the sex-specific splicing pattern of the endogenous gene (3). To determine whether sex-specific splicing is faithfully reproduced by just the exons and introns involved in alternate splicing, we constructed an *Sxl* minigene containing a 4.3-kb genomic fragment that spans *Sxl* exons 2-3-4 (Fig. 2). This fragment was placed downstream of a *Drosophila hs83* promoter. The *hs83* promoter is constitutively expressed at 25°C (56), and transcripts from the construct can be analyzed without subjecting the flies to heat shock. To distinguish transcripts expressed from the minigene construct from endogenous *Sxl* RNAs, we fused *lacZ* to exon 4. We could then analyze the splicing patterns of the minigene transcripts by reverse transcription and PCR amplification using a 3′ primer complementary to *lacZ* sequences and a 5′ primer derived from exon 2 of *Sxl*. Since we were concerned that a transient tissue culture assay system would not faithfully reproduce in vivo *Sxl* splicing regulation (see reference 23 and below), we examined the splicing of transcripts expressed from the minigene construct in transgenic flies. From the sizes of the PCR products (Fig. 2) and the presence of the male exon only in the RNAs from males (data not shown), the 4.3-kb fragment has all the information to faithfully reproduce the splicing pattern of the endogenous gene. In males, all transcripts from this minigene are spliced exons 2-3-4 (to include the male-specific exon), while in females all transcripts are spliced exons 2-4 (to exclude the male-specific exon). In the experiments whose descriptions follow, only this region of *Sxl* is analyzed.

The male exon 3′ splice site is not the primary target for *Sxl* autoregulation. (i) Sxl protein cannot prevent use of the male exon 3′ splice site when an alternate 3′ splice site is not available. In the simple blockage model, Sxl protein binds to the poly(U) run in the male exon polypyrimidine tract and interferes with the binding of ubiquitous splicing factors to prevent the splicing machinery from using the 3′ splice site. This model predicts that Sxl protein would also be able to prevent utilization of the male 3′ splice site when no alternative 3′ splice site is available. In a transient tissue culture assay, Sakamoto et al. (44) found that Sxl protein completely blocked the splicing of a minigene construct which extends from exon 2 to the beginning of exon 3 (diagram in Fig. 3). Consequently, we expected to observe little or no male spliced RNA in female flies carrying this construct.

In the experiment shown in Fig. 3, we analyzed the spliced products from males and females using a low number of PCR cycles so that the amplification was still in the linear range (products were not visible by ethidium bromide staining and required detection by Southern analysis). In contrast to our expectations and the results of Sakamoto et al. (44), high levels of male spliced products (exons 2-3) are observed in females (Fig. 3). Thus, in the absence of an alternative 3′ splice site, Sxl protein in female flies is unable to efficiently prevent the utilization of the male exon 3′ splice site. Although blockage in females is clearly ineffective in the absence of an alternative 3′ splice site, some regulation is observed. We detect a low level of spliced products which are larger than the male-spliced RNA but smaller than the full-length intron. Presumably, these splice products arise from the use of cryptic sites in the intron (see below) when the male exon 3′ splice site is successfully blocked.

(ii) *Sxl* structure cannot be altered to make splicing resemble *tra*. Alternative splicing in *tra* pivots on the competition between two 3′ splice sites. In the absence of Sxl protein, the default 3′ splice site outcompetes the distal splice site, and all transcripts are spliced to the default 3′ splice site. When Sxl protein is present, the default splice becomes less competitive, and the female splice site is used 40 to 50% of the time. If a similar strategy is employed in *Sxl* alternate splicing, then the 3′ splice sites of exon 3 (the male exon) and exon 4 should be in direct competition. In males, the stronger 3′ splice site of exon 3 should outcompete the weaker 3′ splice site of exon 4. The situation would be reversed in females, in which Sxl protein would "weaken" the 3′ splice site of the male exon by interfering with the assembly of splicing complexes.

To test whether *Sxl* autoregulation also pivots on a competition between the 3′ splice sites of the male exon and exon 4, we generated *tra*-like versions of *Sxl* by deleting the 5′ splice site of the male exon. In the first experiment, we deleted a DNA segment extending from 11 bp downstream of the male exon 3′ splice site (counting from the second of the two AG dinucleotides used at this 3′ splice site) to a position 270 bp upstream of exon 4. As shown in Fig. 4A, the splicing products from this construct do not fit the predictions of the 3′ splice site competition model. Instead of the expected default splice products (exon 2-3-intron-4) being observed in males, the male exon 3′ splice site is skipped altogether, and all transcripts are spliced exon 2-4. Of course, the same is true in females.

The failure of the male exon 3′ splice site to compete effectively with the 3′ splice site of exon 4 could be due to the fact that only 11 bases of male exon sequences are present in this construct. Indeed, a number of studies have shown that exon sequences can affect the use of splice sites (32, 40, 55 and references therein). To explore this possibility, a second *tra*-like construction of *Sxl* which includes all but the last two bases of the male exon was made (Fig. 4). However, as shown in Fig. 4B, these additional male exon sequences (which include purine-rich elements that might constitute the exon recognition elements described by Watakabe et al. [55]) do not improve the functioning of the male exon 3′ splice site, and the splicing pattern of transcripts from this second *tra*-like construct was identical to that observed with the first. Since the 3′ splice site of the male

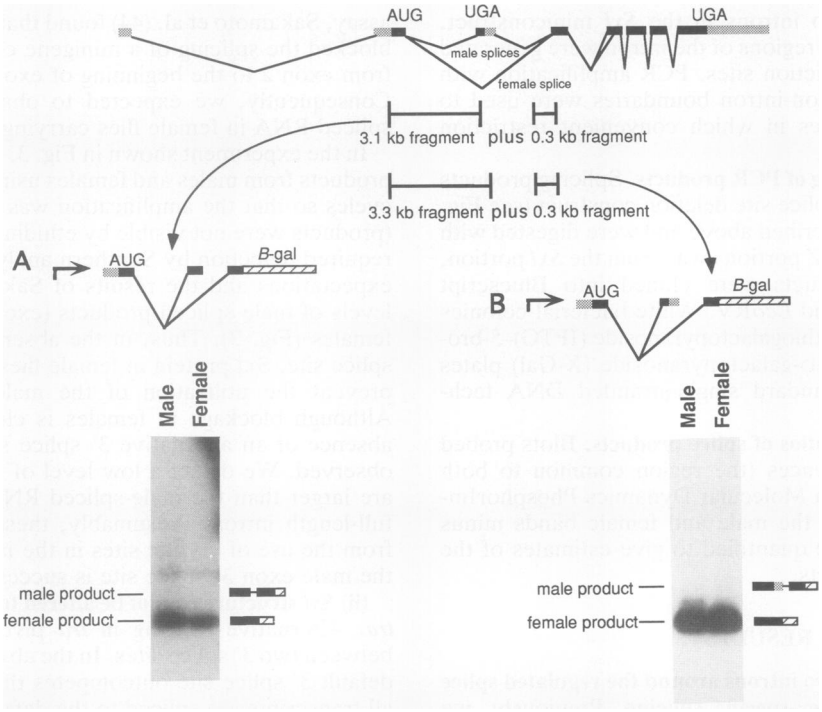

FIG. 4. Alteration of *Sxl* structure to resemble *tra* organization. The *Sxl* transcription unit is diagrammed at the top. Beneath this, the regions of *Sxl* taken to generate the *tra*-like constructs are depicted. (A) A 3.1-kb fragment containing exon 2 and part of exon 3 (11 bp downstream of the second 3′ splice site AG dinucleotide) placed with a 0.3-kb fragment containing exon 4; (B) 3.3-kb fragment containing exon 2 and all but the last two bases of the male exon fused to the same 0.3-kb exon 4-containing fragment. Splice products are shown at the bottom, and the expected positions of migration and exon structures are depicted. Symbols are as described in the legend to Fig. 2.

exon is not used at all in either construct, one interpretation is that the male exon has a 3′ splice site that is weaker rather than stronger than that of exon 4. If this is correct, then the strategy used for *Sxl* autoregulation cannot be based simply on a competition between alternate 3′ splice sites as in *tra*.

(iii) The poly(U) run at the male exon 3′ splice site is not essential for female splicing regulation. In *tra*, the poly(U) run in the polypyrimidine tract of the default 3′ splice site is essential for regulation. A poly(U) run is also found at the 3′ splice site of the *Sxl* male exon, and, in the simple blockage model, the binding of Sxl protein to this sequence should prevent the splicing machinery from incorporating the male exon. Consistent with this model, we found that this poly(U) run is essential for regulated splicing when the male exon is placed in a heterologous context (23). To test whether this sequence is also required for regulated splicing of the male exon in its normal context, we introduced two T-to-C changes in the male exon poly(U) run of the *Sxl* minigene construct (see Fig. 5). These same T-to-C changes in the optimized minimal male exon eliminate *Sxl* regulation when the exon is placed in a heterologous context. Similarly, equivalent changes in the poly(U) run at the default 3′ splice site of *tra* also eliminate regulation by *Sxl* (49).

The splicing patterns of transcripts from this mutant T-to-C *Sxl* minigene construct are shown in Fig. 5. As can be seen, the T-to-C changes in the poly(U) run of the male exon 3′ splice site have no apparent effect on default splicing, and in males, all of the transcripts are spliced exons 2-3-4. Significantly, the T-to-C changes also have no effect on regulated splicing; all transcripts in females appear to be spliced exons 2-4, just as is observed for the wild-type *Sxl* minigene or for *Sxl* itself. Hence, in spite of the fact that the poly(U) run in the male exon 3′ splice site plays an essential role in the splicing regulation of the (optimized minimal) male exon when it is placed in a heterologous context, this sequence does not appear to be required when the male exon is in its normal context. Some of the reasons for this apparent contradiction will become clear below.

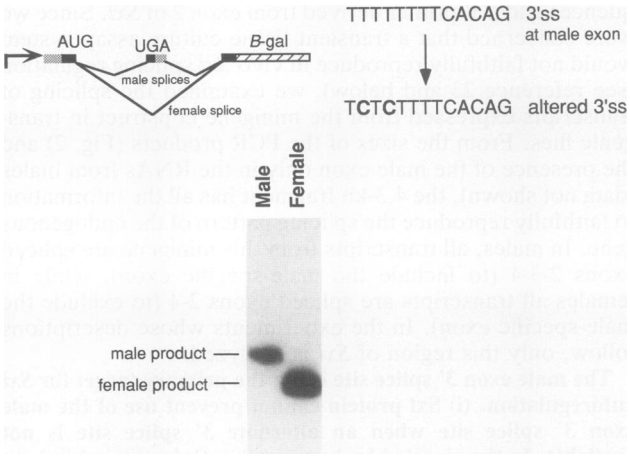

FIG. 5. Disruption of the poly(U) tract at the male exon and its effect on female splicing regulation. The altered bases are shown to the right of the *Sxl* minigene construct (Fig. 2). These changes are the same as those used to disrupt regulation of the isolated male exon (23). Splice products from male and female RNAs are shown in the lower half of the figure, and the expected positions of migration of male and female products are marked. ss, splice site.

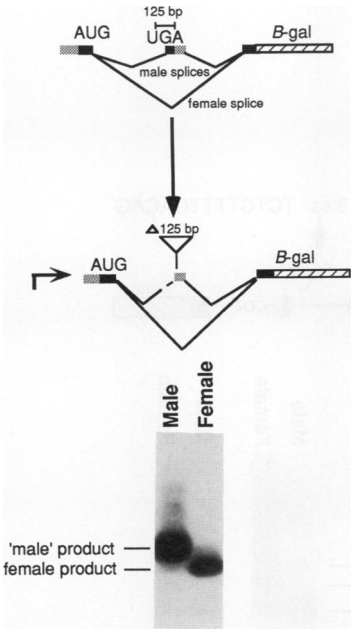

FIG. 6. The male exon 3′ splice site is not required for female splicing regulation. A 125-bp deletion that eliminated the region of homology between *Sxl* and *tra* and extended through the male exon was made. This 125-bp deletion removes the polypyrimidine tract, the second AG dinucleotide, and about two-thirds of the male exon. Spliced products are shown at the bottom, and the expected positions of migration of male and female products are marked. The male products arise from the use of closely spaced cryptic 3′ splice sites immediately upstream of the male exon (cDNAs were cloned and sequenced). Symbols are as described in the legend to Fig. 2.

(iv) The male exon 3′ splice site is not required for autoregulation. The results described above suggest that the 3′ splice site of the male exon may not be the primary target for autoregulation. To further explore this possibility, we deleted the male exon 3′ splice site. Analogously to the *tra*-like constructs described above, this deletion places the 5′ splice sites of exon 2 and the male exon in direct competition for splicing to the single 3′ splice site of exon 4. In males, two scenarios for default splicing might be expected, depending on the relative strengths (in the normal *Sxl* intron-exon structure) of the competing 5′ splice sites. If the male exon 5′ splice functions poorly in this context, it might be skipped and all of the splice products should join exon 2 to 4. On the other hand, if the male exon 5′ splice site is stronger than the 5′ splice site of exon 2, the splicing machinery should join the male exon to exon 4. (In turn, this might activate a cryptic 3′ splice in the intron sequences upstream of the male exon [10, 53].) Of course, if the relative strengths of the competing 5′ splice sites are nearly equal, both types of products may be produced.

The default splicing pattern of transcripts from this 3′ splice site deletion mutant is shown in the male lane in Fig. 6. The size of the splice product in males is nearly the same as that observed with the wild-type minigene. Sequence analysis reveals that these RNAs have the characteristic male structure, exons 2-"3"-4. The RNAs use the normal 5′ splice site of the male exon in joining exons 3 to 4 while the exon 2-3 splice uses one of two cryptic 3′ splice sites located in the intron just upstream of the male exon. Since all of the RNAs have this structure and since none are observed in which exon 2 is spliced directly to exon 4, it appears that the 5′ splice site of the male exon is "stronger" than the 5′ splice site of exon 2.

The normal default splicing exhibited by this male exon 3′ splice site deletion mutant enabled us to ask whether the mutant transcripts are still subject to autoregulation in females. As evident from the female lane in Fig. 6, the deletion has little or no effect on splicing regulation in females, and all transcripts appear to be spliced exons 2-4. Hence, the normal 3′ splice site at the male exon does not appear to be required for either default or regulated splicing of the male exon.

Multiple upstream *cis*-acting elements are required for *Sxl* autoregulation. (i) Sequences in the intron upstream of the male exon affect female splicing regulation. In the 3′ splice site deletion mutant, the use of cryptic 3′ splice sites upstream of the male exon is regulated in females. One explanation for this finding is that there are *cis*-acting elements for autoregulation in the large intron upstream of these cryptic 3′ splice sites. As discussed in the introduction, a variety of lines of evidence indicate that Sxl is a poly(U)-binding protein which recognizes poly(U) runs like those found at the 3′ splice sites of *tra* and the male exon. Inspection of the sequence of the upstream intron (8) reveals that it contains five poly(U) runs of seven or more U residues in length that would be good binding targets for Sxl protein (45). To determine whether these U runs or any other sequences in the upstream intron play a role in splicing regulation, we deleted an 1,150-bp segment from the 2,920-bp intron, starting from near the middle of the intron and extending to a point just before the putative branch point of the male exon 3′ splice site. The Δ1150 deletion removes the five poly(U) runs in the intron but retains another good target for Sxl protein binding, the poly(U) run at the male exon 3′ splice site (Fig. 7A).

The splicing patterns of transcripts from the Δ1150 construct are shown in Fig. 7A. In the default mode, most of the RNAs are spliced in the male pattern, exons 2-3-4. Although they are less abundant than the male RNAs, there are also some abnormal splice products. These include a small amount of female-size RNAs in which the male exon is skipped altogether, as well as one major and several minor aberrant species. Hybridization experiments with fragments spanning the DNA segment of exons 2-3-4 suggest that the strongest of these aberrant RNAs (A1) is probably generated by the use of a cryptic 3′ splice site in the intron upstream of the male exon and the normal male exon 5′ splice site. The presence of these abnormal splice products suggests that the Δ1150 deletion somehow interferes with utilization of the male exon 3′ splice site. (The endpoint of the deletion is close to the putative male exon branch point and could conceivably affect its function.) In females, the bulk of the RNA (~70%) from Δ1150 is spliced in the female mode, exons 2-4. However, regulation is not complete, and about 10 to 15% of the RNA is spliced in the male mode, suggesting that the deleted intron sequences contain information required for efficient autoregulation. Two aberrant splice products are also observed in females. One, A1, is the same as that in males, while the other, A2, is specifically enhanced in females. Like A1, A2 appears to be generated from a cryptic 3′ splice site located in the intron upstream of the male exon; however, unlike A1, it does not contain male exon sequences and instead appears to use a cryptic 5′ splice site located in the intron upstream of the male exon.

(ii) Evidence for a role of the poly(U) run at the male exon 3′ splice site. The effects of the upstream deletion on female

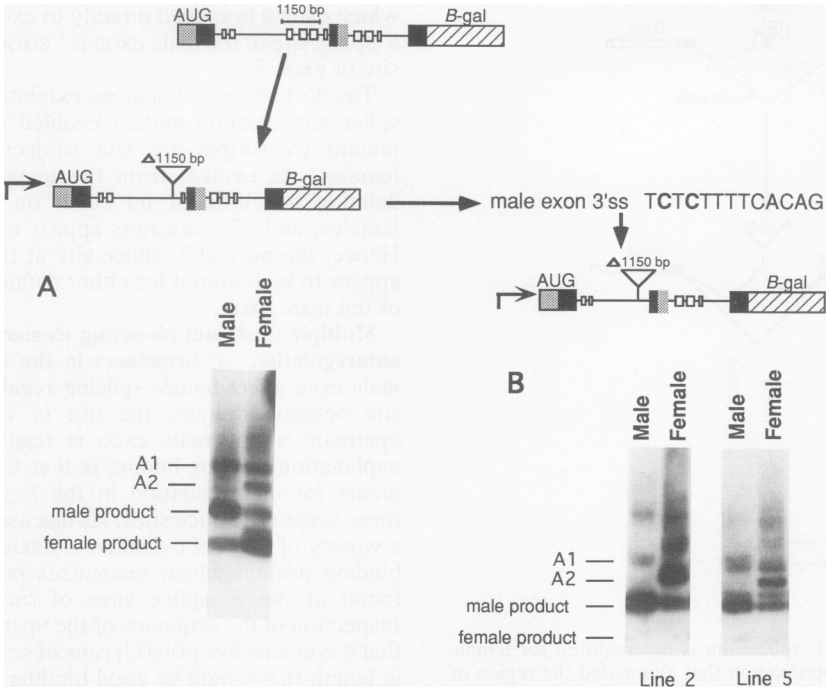

FIG. 7. Effect of deleting intron sequences immediately upstream of the male exon on splicing regulation. At the top is diagrammed the *Sxl* minigene, with the large filled boxes representing exons (male exon is in the middle) and the thin lines connecting them representing introns. Small unfilled boxes in the introns represent the poly(U) runs, with the longer or clustered poly(U) tracts shown as slightly larger boxes. The poly(U) tract at the male exon 3′ splice site is shown as the thin box immediately upstream of the male exon. Above the intron are shown the size and position of the deletion made. The resulting construct with poly(U) tracts eliminated is diagrammed below. (A) Splice products in males and females carrying the construct with the deletion alone; (B) splice products from two independent transgenic lines that have the Δ1150 deletion and the T-to-C changes at the male exon 3′ splice site. In addition to the male and female splice products, there are two other major products, A1 and A2. A1 contains male exon sequences and is present in RNA from both sexes. A2 does not contain the male exon and is enriched in the female lanes. ss, splice site.

splicing might explain why the poly(U) run at the male exon 3′ splice site is not required for *Sxl* autoregulation but is essential for splicing regulation of the (optimized) minimal male exon (23). In the latter case, the only target for Sxl protein is the poly(U) run at the male exon 3′ splice site. In contrast, there are several large poly(U) runs in the upstream intron of *Sxl* which could bind Sxl protein and function in splicing regulation. These additional targets would make the poly(U) run at the male exon 3′ splice site superfluous for autoregulation.

An expectation of this hypothesis is that we might be able to detect a requirement for the poly(U) run at the 3′ splice site when the upstream poly(U) runs are deleted. To test this, we combined Δ1150 with the T-to-C changes in the poly(U) run at the male exon 3′ splice site. Since the relative yield of different splice products (in both sexes) varied somewhat among transgenic lines, the products from two lines are shown in Fig. 7B. In males of both lines, the predominant RNA is the normal male spliced product. As was observed for the Δ1150 alone (see above), there are additional RNAs. These include a small amount of the female and A1 splice products; however, the yields of these two RNAs are reduced relative to those seen in the upstream deletion alone, suggesting that the T-to-C changes in the polypyrimidine tract enhance the utilization of the male exon 3′ splice site (compare male lanes in Fig. 7A and B).

The pattern of splice products in females from both transgenic lines differs in several respects from that observed in females carrying the Δ1150 upstream deletion alone. First, there is little or no female spliced RNA. Second, there is a substantial amount (~30%) of male spliced RNA. These results would suggest that efficient production of female spliced RNA requires, at the minimum, the presence of either the upstream poly(U) runs or the poly(U) run at the male exon 3′ splice site. Although production of female spliced RNA is severely reduced, there is a substantial enhancement in the yield of the A2 RNA species (Fig. 7B). Since the A2 RNA does not contain male exon sequences, it appears that the utilization of the male splice signals in this mutant construct can still be subject to regulation by *Sxl*.

The 5′ splice site of the male exon may be a key target for autoregulation. (i) A deletion in the downstream intron disrupts autoregulation. Two lines of evidence suggest that controlling the utilization of the male exon 5′ splice site may be pivotal to regulation. The first comes from the experiment in which we placed the 5′ splice sites of exon 2 and the male exon in direct competition for splicing to exon 4 (by elimination of the male exon 3′ splice site [Fig. 6]). In males, the 5′ splice site of the male exon outcompetes that of exon 2, indicating that in this context (in which the order of splice sites and the lengths of intron sequences are preserved) it is stronger than the 5′ splice site of exon 2. This result would argue that in order to block the inclusion of the male exon, *Sxl* must control the use of the male exon 5′ splice site. Consistent with this view, regulated splicing of transcripts from this 3′ splice site deletion mutant is normal in females. The second line of evidence comes from the female-specific

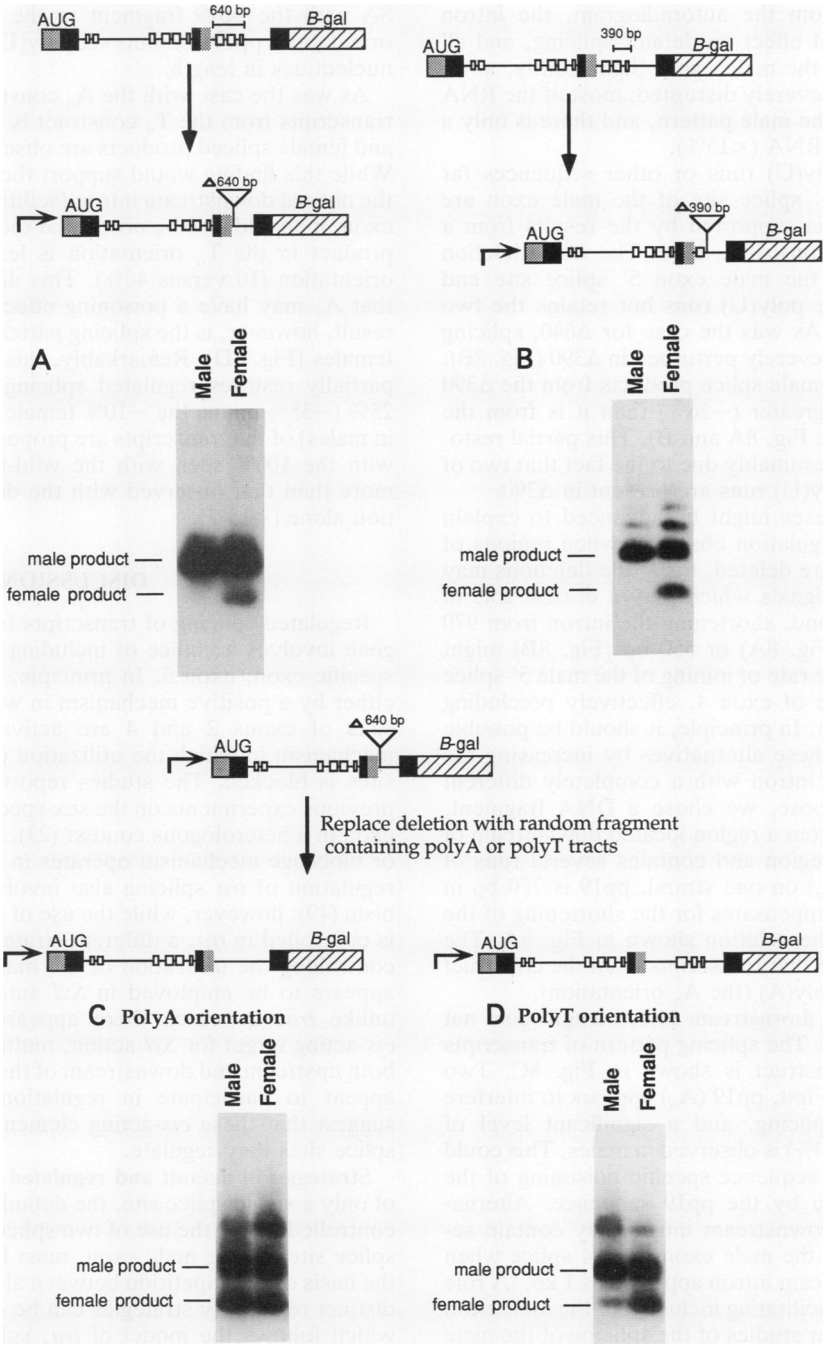

FIG. 8. Effect of deleting sequences downstream of the male exon and replacing them with a fragment containing poly(A) or poly(T) tracts. Symbols are as described in the legend to Fig. 7. At the top, the alterations made to the regulated region of *Sxl* are diagrammed, with the resulting constructs immediately below. (A and B) Splice products from the *Sxl* minigene with two different downstream intron deletions; (C) splice products when the deletion in panel A (Δ640) is replaced by a fragment that introduces poly(A) tracts; (D) splice products when the Δ640 deletion is replaced by the same fragment described for panel C but to introduce poly(T) tracts.

enhancement of the A2 RNA in the Δ1150 constructs [with or without the T-to-C changes in the poly(U) run at the male exon 3′ splice site (Fig. 7)]. One model to explain the production of the A2 RNA is that there are targets for *Sxl* near the male exon 5′ splice site which enable *Sxl* to regulate the use of this 5′ splice site, forcing the splicing machinery to use an upstream cryptic site instead.

The intron downstream of the male exon contains five poly(U) runs which could be targets for *Sxl* action (top of Fig. 8). To determine whether these poly(U) runs (or other sequences in the downstream intron) play an important role in autoregulation, we deleted a 640-bp sequence from the 920-bp downstream intron. This deletion begins 6 bp beyond the 5′ splice site of the male exon and ends 270 bp upstream of the 3′ splice site of exon 4, and it removes all five poly(U) runs. The splicing pattern of RNA from Δ640 is shown in

Fig. 8A. As evident from the autoradiogram, the intron deletion has no apparent effect on default splicing, and all RNAs in males include the male exon. Significantly, autoregulation in females is severely disrupted; most of the RNA in females is spliced in the male pattern, and there is only a small amount of female RNA (<15%).

The idea that the poly(U) runs or other sequences far downstream from the 5′ splice site of the male exon are required for regulation is supported by the results from a second intron deletion construct, Δ390. The Δ390 deletion begins 250 bp beyond the male exon 5′ splice site and removes the distal three poly(U) runs but retains the two proximal poly(U) runs. As was the case for Δ640, splicing regulation in females is severely perturbed in Δ390 (Fig. 8B). However, the yield of female splice products from the Δ390 construct is somewhat greater (~26%) than it is from the Δ640 construct (compare Fig. 8A and B). This partial restoration of regulation is presumably due to the fact that two of the five downstream poly(U) runs are present in Δ390.

Two different hypotheses might be advanced to explain the very poor female regulation observed when regions of the downstream intron are deleted. First, the deletions may remove key *cis*-acting signals which play a critical role in splicing regulation. Second, shortening the intron from 970 to fewer than 300 bp (Fig. 8A) or 550 bp (Fig. 8B) might substantially enhance the rate of joining of the male 5′ splice site to the 3′ splice site of exon 4, effectively precluding regulation by Sxl protein. In principle, it should be possible to distinguish between these alternatives by increasing the size of the downstream intron with a completely different sequence. For this purpose, we chose a DNA fragment, pp19, which is derived from a region located downstream of the *Sxl* mRNA coding region and contains several runs of deoxyribosylthymine (T_n) on one strand. pp19 is 770 bp in length and more than compensates for the shortening of the downstream intron by the deletion shown in Fig. 8A. The fragment was inserted so that transcripts from the construct would contain runs of poly(A) (the A_n orientation).

(ii) Reconstructing the downstream intron length does not restore female regulation. The splicing pattern of transcripts from the pp19 (A_n) construct is shown in Fig. 8C. Two findings are of interest. First, pp19 (A_n) appears to interfere with efficient default splicing, and a significant level of female spliced RNA (~40%) is observed in males. This could be due to some type of sequence-specific poisoning of the male exon-exon 4 splice by the pp19 sequence. Alternatively, the normal *Sxl* downstream intron may contain sequences which facilitate the male exon-exon 4 splice when the length of the downstream intron approaches 1 kb. (A role for intron sequences in facilitating inclusion of the male exon was also suggested by our studies of the splicing of the male exon in a heterologous context [23].) Second, autoregulation is still defective, and the amounts of female spliced RNA are almost the same in the two sexes. This finding would argue that female splicing regulation is impaired by the downstream deletion, not because the intron has been shortened, but rather because information critical to autoregulation has been removed.

(iii) Heterologous poly(T) sequences in the downstream intron partially restore splicing regulation. From what is known about the sequence specificity of Sxl protein (25, 44, 45, 54), it would be reasonable to suppose that the poly(U) runs eliminated by the two deletions correspond to (or at least are a component of) the critical *cis*-acting elements in the downstream intron. As a further test of this hypothesis, we replaced the 640-bp *Sxl* intron sequence deleted in Fig. 8A with the pp19 fragment in the T_n orientation. In this orientation, pp19 contains six poly(U) runs of seven or more nucleotides in length.

As was the case with the A_n construct, default splicing of transcripts from the T_n construct is not completely normal, and female spliced products are observed in males (Fig. 8D). While this finding would support the idea that sequences in the normal downstream intron facilitate inclusion of the male exon, it should also be noted that the level of female spliced product in the T_n orientation is less than it is in the A_n orientation (10 versus 40%). This difference would suggest that A_n may have a poisoning effect. The most interesting result, however, is the splicing pattern of the T_n construct in females (Fig. 8D). Remarkably, this unrelated T_n sequence partially restores regulated splicing. Although only about 25% (~35% minus the ~10% female spliced RNA observed in males) of the transcripts are properly regulated (compared with the 100% seen with the wild-type construct), this is more than that observed with the downstream intron deletion alone (<15%).

DISCUSSION

Regulated splicing of transcripts from the *Drosophila Sxl* gene involves a choice of including or excluding the male-specific exon, exon 3. In principle, this could be achieved either by a positive mechanism in which the flanking splice sites of exons 2 and 4 are activated or by a negative mechanism in which the utilization of the male exon splice sites is blocked. The studies reported here, together with previous experiments on the sex-specific splicing of the male exon in a heterologous context (23), indicate that a negative or blockage mechanism operates in *Sxl* autoregulation. *Sxl* regulation of *tra* splicing also involves a blockage mechanism (49); however, while the use of the default 3′ splice site is controlled in *tra*, a different strategy—one that hinges on controlling the utilization of the male exon 5′ splice site—appears to be employed in *Sxl* autoregulation. Moreover, unlike *tra*, in which there appears to be only a single *cis*-acting target for *Sxl* action, multiple *cis*-acting elements both upstream and downstream of the *Sxl* male-specific exon appear to participate in regulation. Finally, our results suggest that these *cis*-acting elements are distant from the splice sites they regulate.

Strategies in default and regulated splicing. While the use of only a single splice site, the default 3′ splice site, must be controlled in *tra*, the use of two splice sites, the 3′ and the 5′ splice sites of the male exon, must be regulated in *Sxl*. On the basis of a competition between alternate splice sites, two distinct regulatory strategies can be envisioned. In the first, which follows the model of *tra*, splicing regulation would pivot on a competition between 3′ splice sites. In the default state, the 3′ splice site of the male exon would be stronger and outcompete the 3′ splice site of exon 4. In females, *Sxl* protein would weaken the male exon 3′ splice, directing the splicing machinery to join the 5′ splice site of exon 2 to the 3′ splice site of exon 4. Since rapid splicing of the male exon 5′ splice site to the 3′ splice site of exon 4 would preclude regulation by *Sxl*, this strategy requires that the 5′ splice site of the male exon play a role subordinate to that of the 5′ splice site of exon 2. Only under this condition would it be possible to control utilization of the male exon by simply blocking the use of its 3′ splice site. In the second strategy, regulation would pivot on a competition between 5′ splice sites. In the default state, the male exon 5′ splice site would outcompete the 5′ splice site of exon 2, while in females,

utilization of the 5′ splice site of the male exon would be blocked, promoting the joining of exons 2 and 4. Since rapid splicing of exon 2 to the 3′ splice site of the male exon would preclude regulation by *Sxl*, this strategy requires that the 3′ splice site of the male exon play a role subordinate to that of the 3′ splice site of exon 4. Additionally, since splicing is coupled to transcription, splicing choice would have to be delayed until the 3′ splice site of exon 4 has been transcribed. Of course, these two models represent the extreme cases in which one splice site dominates the regulatory process. In reality, one might anticipate that competition among all of the relevant splice sites will, at least to some degree, come into play.

In this study, we have attempted to determine which of these regulatory strategies is used in *Sxl*. A number of lines of evidence suggest that the regulation of *Sxl* is based on the second strategy, a competition between the 5′ splice sites of exon 2 and the male exon for splicing to exon 4. The first comes from the analysis of the default splicing pattern of constructs which assess the relative strengths of the different splice sites. In one set of constructs, the 3′ splice sites of the male exon and exon 4 were placed in direct competition (Fig. 4). If the strategy for alternate splicing of *Sxl* were the same as that in *tra*, then the male exon 3′ splice site should outcompete the 3′ splice site of exon 4. This was not observed; default splicing of all transcripts is to exon 4 and not the male exon. While interpretation of this result is complicated by the question of exon definition (namely, whether removal of the male exon 5′ splice site interferes with definition of the male exon 3′ splice site [41]), it should be noted that the splicing configuration is identical to that in *tra*, in which the upstream default 3′ splice site outcompetes the downstream 3′ splice site. Moreover, similar *tra*-like constructs have been examined in other systems (12, 15, 28, 35). In these experiments, splicing is usually to the stronger site (better match to consensus), regardless of relative position in the transcript. We also placed the 5′ splice sites of exon 2 and the male exon in competition (Fig. 6). We found that the default splicing machinery did not skip the male exon 5′ splice site but exclusively joined the male exon to exon 4 and at the same time activated cryptic 3′ splice sites upstream of the deleted male exon 3′ splice site. Experiments which alter the splice site strengths of alternatively processed transcripts have demonstrated that the strength of a 5′ splice site in an internal exon influences the extent to which that internal exon is skipped or utilized by the splicing machinery (11, 16, 30). The ability of the male 5′ splice site to effect inclusion of the male exon in all transcripts would indicate that, in its normal context, it is a strong splice site, outcompeting the 5′ splice site of exon 2 for splicing to the 3′ splice site of exon 4. A comparison of the sequences of the 5′ splice sites would support this interpretation. Within the intron, the male exon 5′ splice site has a perfect match for base pairing to U1 small nuclear ribonucleoprotein (snRNP), while the 5′ splice site of exon 2 has a mismatch at the highly conserved G at the +5 position (for exact sequence, see reference 23). Both sets of competition experiments argue that, in default splicing, the "dominant splice" is between the 5′ splice site of the male exon and exon 4, while the "subordinate splice" is between exon 2 and the 3′ splice site of the male exon. Such a hierarchy in default splicing pattern is consistent with the expectations of the second regulatory strategy and would require that autoregulation focus primarily on controlling the 5′ rather than the 3′ splice site of the male exon.

The second line of evidence comes from analyzing the splicing of transcripts from several different constructs in females. Perhaps most telling is the finding that autoregulation is preserved even when the male exon 3′ splice site is inactivated by a deletion (Fig. 6). By contrast, when the male exon 3′ splice site is the only available splice site, *Sxl* does not efficiently block its utilization (Fig. 3). The effects of deleting putative *Sxl* sites upstream and downstream of the male exon are also consistent with the idea that the male exon 5′ splice site is dominant in autoregulation, while the 3′ splice site plays a subordinate role. Deletion of the putative *Sxl* targets in the intron upstream of the male exon had only a minor effect on regulation. Moreover, when this upstream intron deletion was combined with the T-to-C changes in the poly(U) run at the male exon, *Sxl* was still able to prevent inclusion of the male exon in the majority of transcripts, as evidenced by the marked decrease in the level of the male spliced product and the increase in the novel female-specific products (Fig. 7B). In contrast, deletions that remove the putative *Sxl* targets in the downstream intron have much more drastic effects on autoregulation (Fig. 8A and B). Taken together, these findings indicate that the critical targets for *Sxl* autoregulation are located in the downstream intron, while the targets in the upstream intron appear to play only a secondary role in autoregulation, influencing the efficiency of female splicing.

The idea that the 5′ splice site played any, let alone an important, role in autoregulation was not suggested by our previous studies on the splicing of the male exon in a heterologous context (23) (see introduction) or by the tissue culture experiments described by Sakamoto et al. (44). From their experiments, Sakamoto et al. (44) conclude that *Sxl* controls splicing from exon 2 to the 3′ splice site of the male exon, while the splice from the male exon 5′ splice site to exon 4 was not relevant. We found that in flies, Sxl protein was unable to efficiently block the splice from exon 2 to the male exon if an alternative 3′ splice site was not available (Fig. 3). We suspect that the difference between our results and those of Sakamoto et al. (44) arises from the very high levels of Sxl protein expressed in their transient tissue culture assay system. When Sxl protein is present in such a large excess, it might be able to saturate the *Sxl* transcript and block splicing altogether. Similarly, removal of sequences from the downstream intron which severely perturb autoregulation in transgenic flies had at most only modest effects in the transient tissue culture experiments (44). The lower sensitivity of the transient assay system to these deletions may also arise from the vast excess of Sxl protein. Alternatively, the difference may arise from the fact that Sxl protein interacts with the products of other genes to mediate splicing regulation (1, 17, 19, 37, 51). Since these were not coexpressed in the tissue culture cells, the ratio of Sxl protein to these other gene products (and general splicing factors) could be dramatically different from what it normally is in vivo.

Multiple targets for *Sxl* action. In vivo mutagenesis experiments on *tra* have implicated the poly(U) run at the default 3′ splice site as the likely target for *Sxl* action (49). This conclusion has been supported by in vitro experiments which demonstrate, first, that the binding of Sxl protein to *tra* RNA requires this poly(U) run and, second, that poly(U) but not poly(U · C) competes for binding (25, 44, 45). Several lines of evidence argue that the targets for *Sxl* action in autoregulation are the poly(U) runs. First, when a minimal male exon fragment is placed in a heterologous context, mutational analysis indicates that the poly(U) run at the male exon 3′ splice site is essential for splicing regulation (23).

While this poly(U) run is not essential for female-specific splicing of transcripts from the *Sxl* minigene construct described here, the fact that mutations in the poly(U) run enhance the effects of an upstream deletion suggests that this sequence can participate in regulation. Second, there are multiple poly(U) runs both upstream and downstream of the male exon. Deleting the upstream poly(U) runs has a small effect on regulation, while deleting the downstream poly(U) runs severely perturbs regulation. The third line of evidence comes from replacing the deleted region of the downstream intron with the heterologous sequence, pp19. When pp19 is oriented to introduce long U runs (T_n), regulated splicing is partially restored. In contrast, when pp19 is in the opposite (A_n) orientation, no regulation is observed. It is not entirely clear why the poly(U) runs in pp19 (T_n) did not restore full regulation. There could be other sequences in the downstream *Sxl* intron that are important for Sxl protein action, either for Sxl protein binding or for the binding of other cofactors. The configuration of the poly(U) runs in pp19 (T_n) may not be appropriate for optimal regulation. In *Sxl*, the first poly(U) runs begin about 250 nucleotides downstream of the male exon 5′ splice site. In pp19, in contrast, the first poly(U) run is about 400 nucleotides from the male exon 5′ splice site. Finally, Sakamoto et al. (44) also implicate multiple poly(U) runs in *Sxl* splicing regulation in transfected tissue culture cells. Under their experimental conditions, all of the major poly(U) runs in the upstream as well as downstream introns (but not the U run at the male exon 3′ splice site) must be deleted to disrupt splicing regulation. Moreover, they found that reintroducing poly(U) runs into such a deletion construct partially restores regulation.

If the poly(U) runs in the *Sxl* transcript are the key targets in autoregulation, the obvious question arises of how specificity is achieved with a recognition element that occurs frequently in the introns of *D. melanogaster* genes. Poly(U) runs are found even in the polypyrimidine tracts upstream of 3′ splice sites (34). From in vitro binding studies (25, 44, 45, 54), it seems likely that *Sxl* will interact with poly(U)-containing transcripts; however, in at least a number of instances (*Bicaudal-D*, *ninaC*, *U1 70K*, and *Notch*), the transcripts do not appear to be subject to sex-specific splicing regulation, at least in vivo. (It is quite possible, on the other hand, that when Sxl protein is present in vast excess as in the transfected tissue culture cells, these transcripts would be subject to some degree of regulation.) One plausible explanation for this apparent paradox is that Sxl protein is able to regulate the utilization of the male exon because the splicing signals in *Sxl* are suboptimal (cf. reference 23). In this case, specificity in regulation would not be dependent on the RNA-binding properties of the Sxl protein per se, but rather would be generated by features of the *Sxl* RNA that on the one hand slow down splicing (perhaps by reducing the rate of assembly of splicing complexes) and on the other provide alternative splice sites that can effectively compete for utilization. By contrast, if the splice signals are not suboptimal or if no competing splice sites are available, Sxl protein may be unable to modify the splicing pattern (cf. Fig. 2). It is interesting to note that the use of suboptimal splice sites appears to be a common theme in alternate splicing (20, 36, 38).

Mechanisms of regulation. In *tra*, Sxl protein is thought to prevent the binding of an essential splicing factor to the polypyrimidine tract, interfering with the assembly of a functional splicing complex at the default 3′ splice site. It seems unlikely that this mechanism could apply in *Sxl* autoregulation. First, since the 5′ splice site of the *Sxl* male exon appears to play a more central role in regulation, simply blocking the use of the 3′ splice site would be of little consequence. Rather, one would imagine that Sxl protein would have to interfere with U1 snRNP or with some other splicing factor that is associated with the male exon 5′ splice site. A second problem is the location of the likely binding sites for Sxl protein on the *Sxl* transcript. With the exception of the dispensable poly(U) run at the male exon 3′ splice site, the poly(U) runs both upstream and downstream of the exon are at a considerable distance (>240 bp) from the splice sites that they regulate. It is difficult to see how Sxl protein associated with these distant poly(U) runs could directly interfere with the binding of factors at either the 5′ or the 3′ splice sites of the male exon.

The ability of Sxl protein to function at a distance could be explained if the intron contained sequences required for efficient default splicing. Sxl protein bound to the poly(U) runs in the introns would prevent these sequences from promoting inclusion of the male exon. Consistent with this possibility, default splicing is perturbed by the upstream deletion and by substituting heterologous sequences for the downstream intron. On the other hand, while these mutations clearly reduce the efficiency of default splicing, the effects are rather modest, and this model could not account for the very efficient exclusion of the male exon that is normally observed in females.

A second model is that Sxl protein bound upstream and downstream of the male exon packages this region of the transcript into a complex that sequesters the splice sites from the splicing machinery. This packaging could be mediated by protein-protein interactions, either between Sxl proteins or through the interaction of Sxl protein with another cofactor(s). While this hypothesis could explain why female-spliced products (exons 2-4) are observed when Sxl sites in the upstream intron are deleted (since the site at the male exon still provides an upstream site for interaction), it does not account for the fact that *Sxl* is able to block the utilization of the male exon in the double mutant (upstream deletion mutant Δ1150 plus T-to-C changes [Fig. 7]). Additionally, the packaging model does not adequately explain the effects of the downstream intron deletions. In this model, providing two of the five downstream poly(U) runs would be expected to almost completely restore female splicing; however, it does not (Fig. 8B).

Since controlling utilization of the male exon 5′ splice site appears to be the critical step in autoregulation, a third possibility is that Sxl protein bound to the downstream intron somehow interferes with the functioning of U1 snRNP. This could be at the level of initial association of U1 snRNP with the 5′ splice site of the male exon; however, direct interference with this step is difficult to reconcile with the fact that sequences distant from the male exon 5′ splice site participate in regulation (Fig. 8B). Perhaps a more attractive possibility is that Sxl protein bound to target sites in the downstream intron blocks communication between U1 snRNP at the male exon 5′ splice site and the branch point and/or the 3′ splice site of exon 4. One of the earliest steps in spliceosome assembly, the formation of the commitment complex, involves interactions between U1 snRNP at the 5′ splice site and splice signals associated with the downstream exon (26, 48). In this model, Sxl would disrupt the formation of a commitment complex by blocking communication between splicing factors at the male exon 5′ splice site and the 3′ splice site of exon 4.

Other than the dispensable poly(U) tract at the male exon 3′ splice site, all *Sxl* sites in the upstream intron are also

distant from the regulated splice sites. The action of Sxl on these upstream sites could be analogous to that proposed above for the downstream sites, namely disruption of communication between U1 snRNP complexes at the 5′ splice site of exon 2 and the 3′ splice site of the male exon. By blocking the formation of commitment complexes to the male exon, the combined effect of Sxl protein on the sites both downstream and upstream of the male exon would thus promote formation of a female commitment complex between exons 2 and 4.

In addition to preventing the establishment of a commitment complex between the male exon 5′ splice site and the 3′ splice site of exon 4, Sxl protein might also destabilize the U1 snRNP bound to the male exon 5′ splice site. This destabilization could in turn affect the interactions of splicing factors with the male exon 3′ splice site. That the functioning of the weak male exon 3′ splice site may be dependent on events at the male exon 5′ site is suggested by the findings of Hoffman and Grabowski (22). They show that assembly of U1 snRNP complexes at a strong downstream 5′ splice site helps recruit splicing factors to a weaker upstream 3′ splice site. Such a two-step model could explain why Sxl protein (interacting with sites in the downstream intron) is able to block inclusion of the male exon in transcripts from the construct which has no *Sxl* targets upstream of the male exon 3′ splice site (Δ1150 plus T-to-C changes; Fig. 7B). Further studies will be required to determine how closely this two-step model applies to *Sxl* autoregulation and what specific elements within the downstream intron constitute the essential target(s) for Sxl protein action.

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