Nucleic Acids Research, Vol. 20, No. 21 5533–5540

Control of *Drosophila Sex-lethal* pre-mRNA splicing by its own female-specific product

Hiroshi Sakamoto+, Kunio Inoue, Ikuko Higuchi§, Yuichi Ono and Yoshiro Shimura*
Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606, Japan

Received September 11, 1992; Revised and Accepted October 13, 1992

ABSTRACT

***Drosophila melanogaster* somatic sexual differentiation is accomplished by serial function of the products of sex-determination genes. *Sex-lethal* (*Sxl*), is one such gene. It is functionally expressed only in female flies. The sex-specific expression of this gene is regulated by alternative mRNA splicing which results in either the inclusion or exclusion of the translation stop codon containing third exon. Although previous genetic and molecular analyses suggest that functional *Sxl* expression is maintained by a positive feedback loop, where the female-specific *Sxl* product promotes the synthesis of its own female-specific mRNA, the mechanistic details of such regulation have remained unclear. We have developed a cotransfection system using *Drosophila* cultured (Kc) cells in which *Sxl* primary transcripts are expressed with or without the female specific *Sxl* product. Here we show that the female-specific *Sxl* product induces the synthesis of its own female-specific mRNA by negative control of male-specific splicing. Deletion, substitution, and binding experiments have demonstrated that multiple uridine-rich sequences in the introns around the male-specific third exon are involved in the splicing regulation of *Sxl* pre-mRNA.**

INTRODUCTION

The *Drosophila* gene *Sex-lethal* (*Sxl*) plays key roles in the sexual differentiation of both somatic and germline cells and in dosage compensation (see references 1–3 for reviews). During somatic sexual differentiation, there is a genetic hierarchy of sex-determination genes in which *Sxl* functions as a binary switch depending on the X/A ratio, the ratio of the number of X chromosomes relative to the sets of autosomes. In chromosomally female embryos, where the X/A ratio is 1, *Sxl* is turned 'on' and determines the female mode of expression of the downstream sex-determination genes, *transformer* (*tra*) and *doublesex* (*dsx*) (4, 5). In male embryos, where the X/A ratio is 0.5, *Sxl* remains in the 'off' state, leading to the male mode of expression of downstream genes. Genetic analyses have suggested that female-specific expression of *Sxl* consists of two stages: namely its establishment and maintenance (6–10). Several numerator genes are thought to count the X/A ratio in early embryogenesis and to transmit an activating signal to *Sxl* if the ratio is 1. In response to this signal, *Sxl* is transiently expressed and commits the cells to the female fate (establishment). Once the state of *Sxl* activity is determined in early embryogenesis, *Sxl* no longer responds to the X/A signal and its activity remains fixed by an autoregulatory mechanism (maintenance).

Previous molecular analyses have revealed that the *Sxl* locus produces several distinct mRNAs and that sex-specific alternative splicing controls *Sxl* expression (11–13). The most obvious difference between male-specific and female-specific mRNAs is the existence of a third exon sequence that contains two translation stop codons. Female-specific mRNAs do not contain this third exon sequence, thereby having a long open reading frame that encodes a protein product with two potential RNA binding domains (11). Because male-specific mRNAs contain the third exon, they encode a shorter, non-functional, protein (15), reflecting the 'off' state of *Sxl* expression. Thus, the 'on' and 'off' state of *Sxl* expression corresponds to the absence or presence of the third exon sequence in *Sxl* mRNA, respectively. Recently, Bell *et al.* have clearly demonstrated, through transgenic experiments, that female-specific splicing of *Sxl* is promoted in the presence of the female *Sxl* product and that a continuous supply of female *Sxl* product is required for maintenance of such female-specific splicing (14).

Although these transgenic analyses showed that the female-specific *Sxl* product autoregulates *Sxl* pre-mRNA splicing, the underlying mechanism still remains to be elucidated. We have addressed this issue by cotransfection of *Sxl* mini-genes, with or without the female-specific *Sxl* cDNA, into *Drosophila* Kc cells. Here we report that the female *Sxl* product represses the inclusion of the third exon into *Sxl* mRNA. We also show that the uridine-rich sequences around the male-specific third exon represent the *cis*-acting elements required for splicing regulation and that partially purified female *Sxl* gene product binds to these uridine-rich sequences.

* To whom correspondence should be addressed

Present addresses: + Department of Biology, Faculty of Science, Kobe University, Nada-ku, Rokkodaicho 1-1, Kobe 657 and § Sumitomo Pharmaceutical Co., Ltd Research Laboratories, Takatsukasa, Takarazuka 665, Hyogo, Japan

MATERIALS AND METHODS

Chemicals and enzymes

^{32}P-labeled nucleotides were obtained from Amersham. Other nucleotides were from Pharmacia. RNase A and RNase T1 were from Sigma and Sankyo, respectively. Other enzymes were from Takara Shuzo or Toyobo. RNA-PCR kit was from Perkin Elmer Cetus.

Primers

The synthetic oligonucleotides used for *in vitro* mutagenesis are as follows. The sequences underlined are specific for *Sxl* genomic sequences.

PS2U2 (for copia-SxlE23): GGGAATTCATGTACGGCAACAATAATCC
PS3D2 (for copia-SxlE23): GGGAATTCGATTCAAAAACATTGCGTTT
P3S (for copia-SxlE34): GGTCGACCCCAGAAAGAAGCAGCC
P4A (for copia-SxlE34): AAAGCTTGCCGGATGGCAGA
T1: GCGTCGACGACACTTGCTCAACAAGCGCCAAAACAATTGAAGAG
P1: GCGGTACCCAGCTCATAAAAATGTCACG
P2: GCGGTACCGGGGTCGTATAATTTATGGC
P3: GCGGTACCACTGTGCAAAATGAAATCAAGCTC
P4: GCGGTACCCATGGGACTATACTAGTAACCC
P5: GCGGTACCCGGTGATTCGAAAGTACAAAAAA
P6: GAGAGGGTACCATCACTTTTGGCACTTTTTCATC
P7: GAGAGGGTACCCAAAATCCACTAATGGTTTTCTC
P8: GAGAGGGATCCTTAGGTAAACACTGTATCATC
P9: GAGAGGGATCCGAATAGTTTGTACGAAGAAG
T2: GGAAGCTTGCATCGAATCAGTAGCCGGATGGCAGAGAATGGGAC
PT1: GTCGACGACACTTGCTCAAC
PT2: AAGCTTGCATCGAATCAGTAG
UU16: GAGAGGGTACCTTTTTTTTTTTTTTTTACTGTGCAAAATGAAATCAAGCTC
DU16: GAGAGGGTACCAAAAAAAAAAAAAAAATCACTTTTGGCACTTTTTCATC

Plasmid construction

The plasmid copia-SxlE234 was constructed by cloning the *Apa* LI-*Acc* III *Sxl* genomic fragment (approximately 4400 nt), which contains exon 2 (369 nt), the second intron (2909 nt), exon 3 (190 nt), the third intron (919 nt), and exon 4 (42 nt) of the *Sxl* gene into pBScopia-cat vector (15). In this plasmid, the *Bgl* II site in the second intron was disrupted by blunting with Klenow fragment followed by re-ligation. Plasmid copia-SxlE23 was constructed by cloning the PCR amplified fragment containing the region from exon 2 (77 nt) to exon 3 (170 nt from M1 acceptor site). Plasmid copia-SxlE34 was constructed by cloning the PCR amplified fragment containing the region from exon 3 (172 nt) to exon 4 (42 nt). Plasmid SP-SxlHcP was obtained by cloning of the *Hinc* II-*Pst* I fragment containing exon 3 surrounded by portions of intron sequences into a *Sma* I-*Pst* I cut vector SP73. Plasmid SP-SxlBgX was constructed by cloning the *Bgl* II-*Xho* I fragment containing exon 2 surrounded by portions of introns into the vector SP73. Plasmid SP-SxlPvSsp was constructed by cloning the *Pvu* II-*Ssp* I fragment containing exon 3 and portions of the surrounding introns into the *Sma* I site of SP73 . Plasmid SP-SxlPvSspU8M was obtained by an *in vitro* mutagenesis step (15), in which three T-to-C mutations were introduced into SP-SxlPvSsp. Plasmid SP-SxlURS2/3 was constructed by cloning the *Dde* I-*Alu* I fragment (172 nt) from copia-SxlE23 into the *Eco* RV site of SP73. Plasmid SP-SxlURS4 was constructed by cloning the *Taq* I-*Nsp* 7524V fragment (195 nt) from copia-SxlE34 into the *Cla* I site of SP73. Plasmid hsp-SxlF1cDNA was described previously (15).

PCR was performed using copia-SxlE234 as a template and oligonucleotides T1 and T2 as primers. The amplified fragment with tag sequences spanning from exon 2 (241 nt) to exon 4 (42 nt) was cloned into a *Sal* I-*Hind* III-cut pBScopia-cat vector. The resultant plasmid was designated copia-SxlTE234. *In vitro* mutagenesis based on PCR was used to construct deletion plasmids (template: copia-SxlTE234; primers: P1 & P2 for ΔA; P1 & P3 for ΔB; P4 & P5 for ΔC; P6 & P7 for ΔD; P8 & P9 for ΔE). All PCR reactions used in plasmid construction were performed as described previously (19) not only to minimize the number of unexpected mutations but also to obtain long amplified products. Deleted regions are as follows: ΔA, 1925−1950 nt from the donor site of exon 2; ΔB, 1925−2668 nt from the donor site of exon 2; ΔC, 335−356 nt from the donor site of exon 3; ΔD, 257−568 nt from the donor site of exon 3; ΔE, 148−227 nt from the donor site of exon 2. The copia-SxlTE234U8M plasmid was constructed by *in vitro* mutagenesis (15). Plasmids with both U8M and deletion mutations were constructed by recombination of two parental plasmids at appropriate restriction sites. The plasmid ΔBDE-URS (U16-inserted at the ΔB and ΔD regions) was constructed from the ΔBDE construct by PCR method using the primers UU16 and DU16.

Transfection

Transfection was performed as described previously (15). Usually, 4 μg of each test plasmid was transfected into Kc cells with 1 μg of the appropriate effector plasmids (pBShsp-cat as a control or hsp-SxlF1cDNA). 40 hours after transfection, cells were harvested and total RNA was extracted by the hot phenol method (15).

RNase protection

Linearized template plasmids were transcribed *in vitro* in the presence of α-^{32}P-GTP by SP6 or T7 RNA polymerase to obtain anti-sense RNA probes. *Bgl* II-cut SP-SxlBgX was used for the exon 2-specific probe (P-E2), whilst for the exon 3-specific probe (P-E3), *Bgl* II-cut SP-SxlHcP was used. Each RNase protection experiment was performed as described previously using 30 μg total RNA (15, 16). The protected fragments were fractionated by denaturing polyacrylamide gel electrophoresis.

Reverse transcription-PCR

Total RNA from each transfection (1 μg) was used for RT-PCR analysis using an RNA-PCR kit (Perkin Elmer Cetus) and specific primers (PT1 and PT2). After the reverse transcription reaction, PCR (100 μl) was performed under the following conditions: [94 (2 min)], 1 cycle; [94 (1 min), 60 (1 min), 72 (1 min)], 25 cycles, [72 (5 min)], 1 cycle. Other conditions were in accordance with the manufacturer's procedures. 10 μl of each reaction mixture was electrophoresed on a 1.5% agarose gel to assay the reactions.

Purification of the female Sxl protein

250 ml culture of *E.coli* carrying the plasmid pET-Sxl (15) was harvested following induction and washed with PBS. The cell pellet was frozen at −80, thawed, and resuspended with 5 ml of buffer A [50 mM Tris-HCl pH 8.0, 2 mM EDTA, 0.1 mM PAMSF, 0.1 mM DTT, 5%(v/v) glycerol]. Lysozyme (final concentration: 0.2 mg/ml) was added and the suspension was placed on ice for 30 min. After vigorous shaking had been repeated 6 times, the suspension was centrifuged at 12,500 rpm (Beckman JA20) for 30 min and the pellet was resuspended in 5 ml of buffer A. The sample was sonicated, Triton X-100 was added to a final concentration of 1%, and it was placed on ice for 15 min. After subsequent centrifugation at 12,500 rpm for 45 min, the supernatant was dialyzed against the buffer B [20 mM Hepes-NaOH pH 7.9, 100 mM KCl, 0.1 mM PAMSF, 1 mM DTT, 10%(v/v) glycerol] for 16 hours. The dialyzed sample was loaded onto a DEAE-cellulose column, and the 0.1−0.3 M KCl eluate was collected and used as partially purified *Sxl* protein.

RNA binding analysis by UV-crosslinking

Probe RNAs containing uridine-rich sequences were synthesized by *in vitro* transcription in the presence of α-^{32}P-UTP using SP6 or T7 RNA polymerase. The relevant template plasmids were as follows: *Bam* HI-cut SP73 for control polylinker RNA; *Bam* HI-cut SP-SxlPvSsp for the RNA with a U-octamer sequence near the male-specific acceptor site; *Bam* HI-cut SP-SxlPvSspU8M for the RNA with U-to-C substitutions in the U-octamer sequence; *Bgl* II-cut SP-SxlURS2/3 for the RNA with URS2 and URS3; *Bgl* II-cut SP-SxlURS4 for the RNA with URS4 and two additional U7 sequences. The standard reaction mixture contained 2 μl of purified *Sxl* protein, 0.7 μl of 10× NET-2 [500 mM Tris-HCl pH 7.4, 1.5 M NaCl, 0.5% NP40], 1 μl of probe RNA (15,000 cpm), 1 μl of yeast RNA (10 μg/ml), and 5.3 μl of distilled water. In the competition experiments, 1 μl of poly(U) or poly(C) RNA (10 μg/ml) was added in the reaction mixture. The reaction mixtures were incubated at 20°C for 20 min, and then irradiated by UV light (0.6 joule, Stratalinker, Stratagene) on ice. After the addition of 1 μl of RNase A (10 mg/ml), the irradiated samples were incubated at 37 for 20 min. The RNase-treated samples were electrophoresed on a 15% SDS-polyacrylamide gel.

RESULTS

Exclusion of the third exon from mature mRNA by the female-specific Sxl product

We have isolated several genomic clones containing the *Sxl* gene by screening a *Drosophila* genomic library (originating from *D. melanogaster* Oregon R) and determined the entire nucleotide sequence of the region spanning from the second exon (exon 2) to the fourth exon (exon 4). Since the most prominent difference between the female-specific and male-specific mRNAs is the absence of the third exon (exon 3), we assumed that the *cis*-acting information for the alternative splicing could be confined within the region between exons 2 and 4 (see Materials and Methods, Fig. 1A). To examine this possibility, several minigene constructs containing portions of *Sxl* gene were constructed as test plasmids and their splicing patterns were examined in the presence or absence of the effector plasmid which supplies the female *Sxl* protein product. One of the test plasmids, copia-SxlE234, contains the region from exon 2 to exon 4 under the control of the *Drosophila* copia LTR promoter that is known to show strong activity in *Drosophila* cultured cells. As shown in Fig. 1, when only the copia-SxlE234 plasmid was transfected into Kc cells, we observed efficient inclusion of the male-specific exon 3 into *Sxl* mRNA as indicated by the two protected bands of 190 nt and 172 nt (lane 5). These two bands correspond to two male-specific mRNAs (M1 and M2), which differ by the alternative use of two male-specific acceptor sites (11). The result is consistent with the previous observations that Kc cells reflect the male state when other sex-determination genes are overproduced (15, 16).

To determine whether the female-specific *Sxl* product affects the splicing of *Sxl* pre-mRNA, we used hsp-SxlF1cDNA as an effector plasmid construct. In this construct, a female-specific F1 cDNA (11) is under the control of the *Drosophila* heat shock protein 70 promoter. It has been confirmed that the female-specific *Sxl* product is expressed from this construct when introduced into Kc cells (15). When copia-SxlE234 was cotransfected with hsp-SxlF1cDNA, the intensity of the protected bands corresponding to the third exon was considerably reduced (lane 6), whilst the levels of the protected band corresponding to exon 2 were not grossly different (lanes 3 and 4). The expression of the *Sxl* F1 cDNA was confirmed by the presence of two protected bands (lanes 2 and 4; the smaller one seemingly arising from an additional cleavage at an A/U-rich region by RNase). The level of protection of the band corresponding to exon 4 was also almost the same as that of exon 2 in the presence or absence of female *Sxl* product (data not shown). These results show that the female-specific *Sxl* product specifically causes the exclusion of exon 3 from mature mRNA.

Inhibition of splicing between exon 2 and exon 3 by Sxl product

To address the question of how the female *Sxl* product causes the exclusion of exon 3, two constructs (copia-SxlE23 and copia-SxlE34), which contain the region from exon 2 to exon 3 and the region from exon 3 to exon 4, respectively (see Materials and Methods), were constructed. As shown in Fig. 2, when these constructs were transfected into Kc cells, we observed a protection pattern indicative of splicing at the splice sites of exon 3 in both cases (lanes 1 and 3). The splicing between exon 3 and exon 4 occurred efficiently even when copia-SxlE34 was cotransfected with hsp-SxlF1cDNA (lane 2). In contrast, when

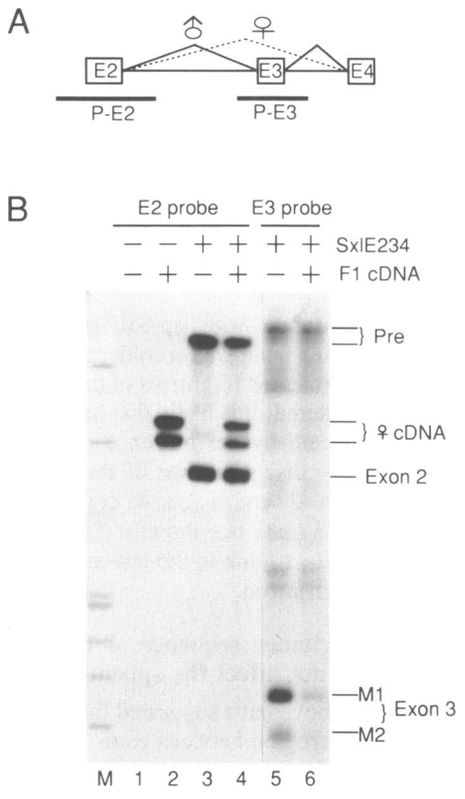

Figure 1. The effect of the female *Sxl* product on the splicing pattern of the *Sxl* pre-mRNA in Kc cells. (A) The sex-specific splicing of *Sxl* pre-mRNA is schematically represented. Boxes and the lines between them indicate exons and introns, respectively. Thick bars below the boxes show the anti-sense RNA probes used in RNase protection analysis (P-E2 for exon 2 and P-E3 for exon 3). (B) RNase protection analysis of total RNAs from transfected cells: copia vector plasmid (lane 1), hsp-SxlF1cDNA (lane 2), copia-SxlE234 (lanes 3 and 5), copia-SxlE234 with hsp-SxlF1cDNA (lanes 4 and 6). Anti-sense RNA probes specific for exon 2 (lanes 1−4) and exon 3 (lanes 5 and 6) were used. The structure of each protected band is shown at the right hand side of the figure. Pre: precursor RNAs. M: *Hpa* II-digested pBR322 DNAs as size markers.

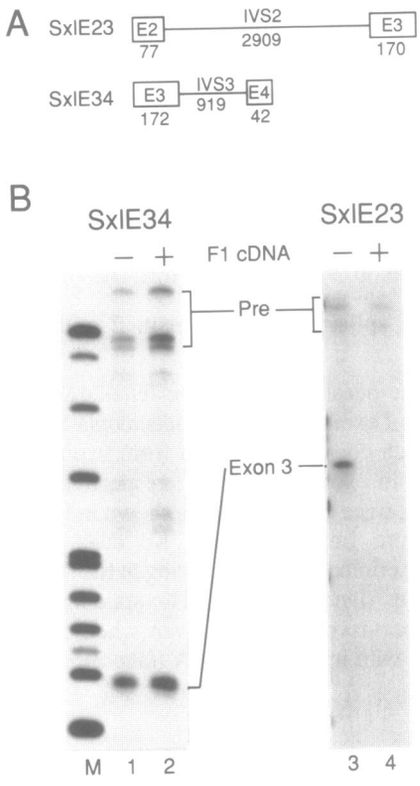

Figure 2. The female *Sxl* product inhibits the splicing of the second, but not the third intron. copia-SxlE34 and copia-SxlE23 were transfected without (lanes 1 and 3) or with (lanes 2 and 4) hsp-SxlF1cDNA into Kc cells. Total RNA from each transfection was analyzed by RNase protection using the P-E3 RNA probe. Several additional protected bands for precursor RNAs were produced by cleavage at the AU-rich regions by RNase treatment. Pre: precursor RNAs. M: *Hpa* II-digested pBR322 DNAs as size markers.

copia-SxlE23 was cotransfected with hsp-SxlF1cDNA, the degree of protection of the exon 3 band was considerably reduced (lane 4). Since the levels of protected fragments of the precursor RNA were not significantly altered, it is likely that the stability of each pre-mRNA is not affected by the presence of the *Sxl* female product. Appearance or disappearance of the protected bands corresponding to exon 2 and exon 4 correlated to those corresponding to exon 3 (data not shown). These results show that the female *Sxl* product inhibits the splicing of the second, but not that of the third intron.

Mutation in the U-octamer sequence of the male-specific acceptor region does not affect the splicing regulation

The results described above have suggested that some *cis*-acting element(s) exists in the region between exon 2 and exon 3. An uridine-rich sequence (U-octamer sequence) is present very close to the male-specific acceptor sites of the second intron of *Sxl* pre-mRNA. This U-octamer sequence was proposed to be the *cis*-acting element for splicing regulation, because the same sequence exists near the non-sex-specific acceptor site of *tra* pre-mRNA, whose splicing is also regulated by the female *Sxl* product (17). In the case of *tra*, a mutation at the U-octamer sequence completely abolished *Sxl* product-mediated splicing regulation (15, 17). To examine the possibility that the same mechanism operates in the control of *Sxl* splicing regulation, we introduced the same mutation (U8M) into copia-SxlTE234, a modified construct derived from copia-SxlE234 (see Materials and Methods; three U-to-C substitutions, AG/ACATAUUUUUUUCACAG/CC to AG/ACATAUUCUCUCUCACAG/CC; the former and the latter slashes indicate the M1 and M2 acceptor sites of exon 3, respectively). The copia-SxlTE234 construct contains two distinct tag sequences (PT1 and PT2) outside the *Sxl* genomic fragment in order to easily assess the splicing pattern of this construct by reverse transcription combined with polymerase chain reaction (RT-PCR). The resultant construct was designated copia-SxlTE234U8M. These constructs were transfected into Kc cells with or without hsp-SxlF1cDNA and the splicing pattern of each was analyzed by RT-PCR using primers PT1 and PT2 (Fig. 3). In this assay, we expected three kinds of PCR-amplified fragments: 515 bp (for M1 acceptor) and 497 bp (for M2 acceptor) bands for the male-specific mRNA, and 325 bp band for the female-specific mRNA.

When only the copia vector plasmid was transfected with or without hsp-SxlF1cDNA, neither male- nor female-specific bands were observed (lanes 1 and 2). When copia-SxlTE234 was transfected alone, bands corresponding to the male-specific mRNAs were exclusively observed (lane 3, two bands for the male-specific mRNAs could not be resolved under the electrophoretic conditions employed). In contrast, the band corresponding to the female-specific mRNA was almost exclusively observed when copia-SxlTE234 was cotransfected with hsp-SxlF1cDNA (lane 4). These results have demonstrated that PCR primers (PT1 and PT2) are specific to the constructs containing the tag sequences, and that the female *Sxl* product causes a complete change in the splicing pattern from the male-specific to female-specific mode. Unexpectedly, when copia-SxlTE234U8M was cotransfected with hsp-SxlF1cDNA, the band corresponding to the female-specific mRNA was almost exclusively observed as is the case with its wild type counterpart, copia-SxlTE234 (lane 6). Thus, the regulation of *Sxl* pre-mRNA splicing could not be disrupted by mutation of the U-octamer sequence near the male-specific acceptor sites.

Multiple uridine-rich sequences in introns are involved in the splicing regulation

The result described above raised the possibility that sequences other than the U-octamer sequence are involved in the splicing regulation. We focused on the uridine-rich sequences as candidates for being responsible for splicing regulation, since such sequences, as is the case with *tra* pre-mRNA, seem to be the targets of *Sxl* protein (15). Four sequences containing 8 or more consecutive uridine residues were found by searching for such sequences in the *Sxl* region of copia-SxlTE234. We designated these sequences as URSs (Uridine-Rich Sequences). Three of them, URS1 (U9; about 960 nt upstream of the acceptor sites of exon 3), URS2 (U11CU8; about 360 nt upstream of the acceptor sites of exon 3), and URS3 (U11GCAUAU7; about 250 nt upstream of the acceptor sites of exon 3) are in the second intron. One of them, URS4 (U9AU8; about 340 nt downstream of the donor site of exon 3), is in the third intron. These uridine-rich sequences were deleted from the copia-SxlTE234 or copia-SxlTE234U8M constructs. The splicing pattern with each deletion construct was then analyzed by RT-PCR. It should be noted that these deletion mutations did not affect the stability of the RNA products from each mutant construct as the total amount of PCR-amplified products was virtually constant in each RT-PCR analysis.

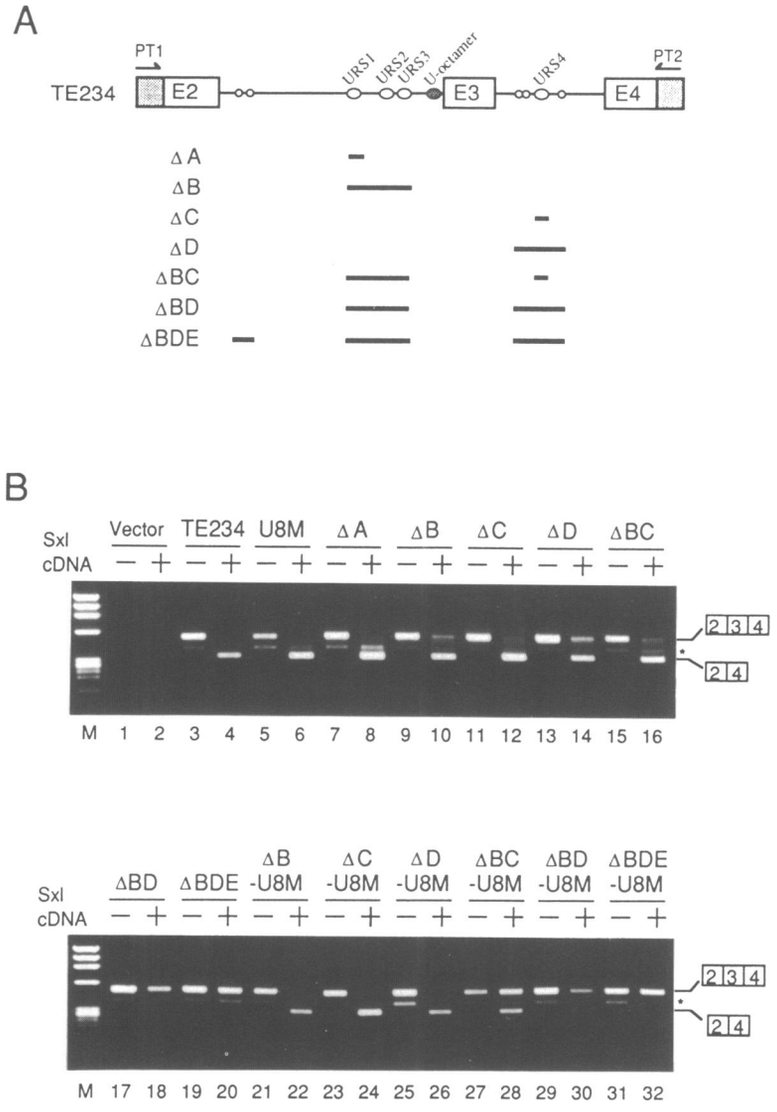

Figure 3. The effect of deletion and/or base-substitution in the intron sequences on the sex-specific splicing of *Sxl* pre-mRNA. (A) Schematic representation of copia-SxlTE234. The open boxes and the lines between them show exons and introns, respectively. Shaded boxes indicate the regions specific for the PCR primers (PT1 and PT2). Open and shaded ovals show the uridine-rich sequences (URS1−4) and the U-octamer sequences near the male-specific acceptor site, respectively. Small circles show the sequences of 7 consecutive uridine residues. Thick bars show deleted regions of derivatives from copia-SxlTE234. (B) Analysis of the splicing patterns of copia-SxlTE234 and its derivatives by the RT-PCR method (see Materials and Methods). Each construct was transfected with (even number lanes) or without (odd number lanes) hsp-SxlF1cDNA. PCR-amplified products were electrophoresed on a 1.5% agarose gel. The structures of PCR-amplified products are shown on the right hand side of the figure. Asterisks indicate non-specific products which were observed even when cells were transfected with the vector control plasmid. M: *Hae* III-digested ΠfX174 DNAs as size markers.

Very little effect on splicing regulation was observed when URS1 or URS4 was deleted from copia-SxlTE234 (compare copia-SxlTE234 with ΔA and ΔC, lanes 7, 8, 11 and 12). For constructs in which three or four URSs were deleted (ΔB and ΔBC, lanes 10 and 16), the male-specific splicing products increased slightly in the presence of the female *Sxl* cDNA. Furthermore, when ΔBC mutation was combined with the U8M mutation (ΔBC-U8M, lanes 27 and 28), approximately half of the splicing products in the presence of the female *Sxl* cDNA represent the male-specific splicing product. Considering the result of the ΔBC-U8M mutation, we suspected that *cis*-acting element(s) other than URSs could still remain intact and direct the partial production of the female-specific mRNA. We found two U7 sequences about 150 and 220 nt downstream of the donor site of exon 2, and three U7 sequences around URS4, two of which reside about 260 nt downstream of the donor site of exon 3, whilst the other resides about 350 nt upstream of the acceptor site of exon 4. When three U7 sequences were deleted together with URS4, a slight increase in the levels of the male specific mRNA was observed in the presence of the female *Sxl* product (ΔD, lanes 13 and 14). In the case of a combination of the ΔD with the ΔB deletion, we observed considerable changes in the splicing pattern, where most of the splicing product, even in the presence of the female *Sxl* cDNA, was the male-specific mRNA (ΔBD, lanes 17 and 18). When the deletion of two U7 sequences near exon 2 (ΔE) was combined with ΔBD deletion (ΔBDE), the change in splicing pattern was almost identical to that observed with ΔBD alone (ΔBDE, lanes 19 and 20). It is unlikely that

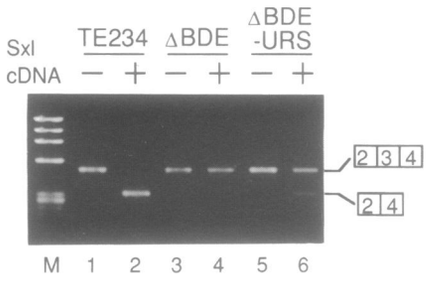

Figure 4. Restoration of splicing regulation by introduction of U16 sequences into the ΔBDE construct. Each construct was transfected with (even number lanes) or without (odd number lanes) hsp-SxlF1cDNA. The splicing pattern of each construct was analyzed by RT-PCR. The structure of PCR-amplified products is shown on the right hand side of the figure. Lanes: 1 and 2, copia-SxlTE234; 3 and 4, ΔBDE; 5 and 6, ΔBDE-URS. M: *Hae* III-digested ΦX174 DNAs as size markers.

large deletions within intron sequences simply enhance the splicing efficiency of the second and the third introns, as when the regions deleted in ΔBD were re-introduced in an antisense orientation to restore the intron length, the splicing regulation remained disrupted (data not shown). In addition, the ΔBC construct, which contains the same deletion as ΔBC-U8M, did not show as drastic a splicing pattern change as ΔBC-U8M.

ΔBDE-U8M construct showed almost the same splicing pattern as the ΔBDE construct. This indicates that ΔBDE deletion is sufficient for virtually complete disruption of the splicing regulation by the female *Sxl* product, and that the U-octamer sequence could not function in this deletion construct. Although a very small amount of the band corresponding to the female-specific mRNA was still observed with the ΔBDE-U8M construct, this may be due to a little enhancement of the basal level of the female-specific splicing by the deletion and/or substitution mutations, since an equivalent amount of the same band was also observed in the absence of the female *Sxl* product (lanes 31 and 32). These results have shown that virtually all of the uridine-rich sequences including U7 sequences in the second and the third introns are involved in the splicing regulation of *Sxl*.

Restoration of splicing regulation by introduction of uridine-rich sequence

To confirm the involvement of uridine-rich sequences in *Sxl* splicing regulation, synthetic uridine-rich sequences (sixteen consecutive uridine residues, U16) were introduced into two deletion points (ΔB and ΔD regions) of the ΔBDE construct which showed almost complete disruption of splicing regulation. When the resultant construct ΔBDE-URS was transfected alone, the band corresponding to the male-specific mRNAs was almost exclusively detected (Fig. 4, lane 5). By contrast, the ΔBDE-URS construct was cotransfected with *Sxl* cDNA, partial production of the female-specific mRNA was detected (lane 6). Thus, the disruption of the splicing regulation with the ΔBDE construct was partially suppressed by introduction of U16 sequences. The result clearly demonstrates that uridine-rich sequences play a direct role in female *Sxl* product-mediated splicing regulation of *Sxl* pre-mRNA, although it seems that more copies of uridine-rich sequences are needed for the full restoration of the splicing regulation.

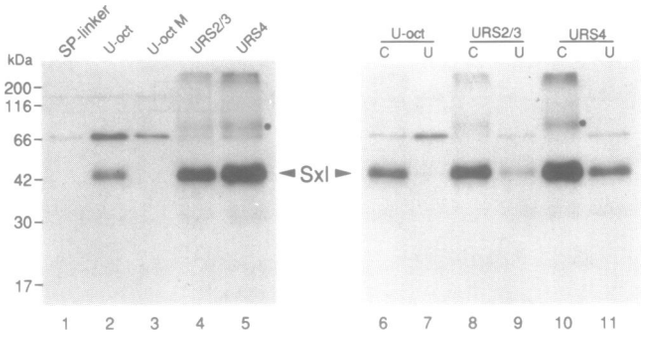

Figure 5. The female *Sxl* protein binds to the uridine-rich sequences *in vitro*. Partially purified *Sxl* protein was incubated with ^{32}P-labeled RNAs containing SP6 polylinker sequence (lane 1), the U-octamer sequence near the male-specific acceptor site (lane 2, 6 and 7), U8M mutation (lane 3), and the other uridine-rich sequences (URS2/3: lanes 4, 8, and 9; URS4: lanes 5, 10 and 11). Poly(C) RNA (lanes 6, 8 and 10) and poly(U) RNA (lanes 7, 9 and 11) were added in the reaction mixtures. After incubation, the reaction mixtures were UV-irradiated, RNase-treated, and electrophoresed on a 15% SDS-polyacrylamide gel. The position of the female *Sxl* protein is shown by arrowheads. Points indicate an additional crosslinked product of 86 kDa. The 66 kDa band possibly corresponds to a non-specific RNA binding protein from *E.coli*, since the band was observed in the control lane (lane 1). The relative positions of molecular weight markers (kilodalton, kDa) are shown to the left of the figure.

Binding of the female Sxl protein to the RNAs containing the uridine-rich sequences

To examine whether the female *Sxl* protein was able to bind to the URSs including the U-octamer sequence, *in vitro* binding experiments utilizing a UV-crosslinking method were performed. We synthesized RNAs containing the uridine-rich sequences *in vitro* and examined the binding of partially purified female *Sxl* protein produced in *E.coli* to these RNAs. As shown in Fig. 5, the female *Sxl* protein (indicated by arrowheads) bound to the RNA containing a U-octamer sequence near the male-specific acceptor site (lane 2), but not to the mutant RNA containing the U8M mutation or the RNA containing SP73 polylinker sequence (lanes 1 and 3). The *Sxl* protein also bound to the URS2/3 RNA containing URS2 and URS3 from the second intron and the URS4 RNA containing URS4 and two additional U7 sequences from the third intron (lanes 4 and 5). Based on the intensity of each *Sxl* protein band, it appears to bind more efficiently to URS2/3 RNA and URS4 RNA than to the U-octamer RNA by approximately 2- and 4-fold, respectively. The binding of *Sxl* protein to each RNA was strongly inhibited by the addition of poly(U) but not poly(C) RNA (lanes 6−11). These results clearly demonstrate that the female *Sxl* protein binds specifically to the uridine-rich sequences (URSs and U-octamer sequence) of the *Sxl* pre-mRNA.

In these crosslinking experiments, we observed another product crosslinked with uridine-rich sequences of approximately 86 kilodalton (indicated by points in lanes 5 and 10). This molecular weight corresponds to the sum of two molecules of the female *Sxl* proteins. Moreover, the appearance of this band was also inhibited by addition of poly(U) RNA and its intensity seemed to correlate with that of the *Sxl* protein band. From these observations, it seems likely that the 86 kilodalton protein band is a dimer molecule of the female *Sxl* protein which arose from protein-protein crosslinking during the binding reaction.

DISCUSSION

By using cotransfection experiments into Kc cells, we have shown here that the female *Sxl* product promotes the female-specific splicing of *Sxl* pre-mRNA. This finding is totally consistent with the results from transgenic analyses *in vivo* reported by Bell *et al* (14). Thus, the female *Sxl* product regulates its own pre-mRNA splicing in either a positive (enhancement of the female-specific splicing between exon 2 and exon 4) or a negative manner (repression of the use of exon 3). In this respect, we have shown, from the analyses of mini-gene constructs with a single intron, that the splicing of the second but not the third intron is inhibited by the female *Sxl* product. Moreover, analyses of deletion constructs with two introns (derivatives from copia-SxlTE234) indicate that, in addition to the similar uridine-rich sequences in the second intron, several uridine-rich sequences in the third intron are also important for the splicing regulation. Furthermore, introduction of U16 sequences into a deletion construct restored splicing regulation by the female *Sxl* product. Taken together, these results strongly suggest that the splicing regulation of *Sxl* pre-mRNA by its own female-specific product is primarily based on the negative control of splicing, which is mediated by several uridine-rich sequences within the second and the third introns.

Mechanistically, there are extensive similarities between the negative control of splicing of *Sxl* and *tra* pre-mRNA splicing. In both cases, the female *Sxl* product acts as a splicing regulator that blocks the default splicing pathway, and the same U-octamer sequence is found very close to the default acceptor sites (15, 17). In the case of *tra* pre-mRNA splicing, the basic mechanism of splicing regulation is possibly based on competition between two acceptor sites. The female *Sxl* product binds to the U-octamer sequence near the non-sex-specific acceptor site of the *tra* pre-mRNA. In addition to being the binding site of the *Sxl* product, the U-octamer sequence of *tra* also represents the polypyrimidine stretch essential for splicing. Therefore, the *Sxl* product may block the interaction of some basic splicing factors such as U2AF (18) which recognize the polypyrimidine stretch, thereby leading to splicing at another female-specific acceptor site.

Such a simple regulatory mechanism by splice site blockage could not explain the regulation of *Sxl* pre-mRNA splicing. We have shown that, in addition to the U-octamer sequence near the male-specific acceptor site of *Sxl* pre-mRNA, the female *Sxl* product binds to several uridine-rich sequences in two introns around exon 3. If the binding of the *Sxl* product alone to the U-octamer sequence is necessary and sufficient for exclusion of the male-specific exon 3, the U8M mutation should disrupt splicing regulation since the mutation has been shown to cause a loss of *Sxl* product binding. The result that the mutation alone did not affect splicing regulation shows that this is not the case. Only in the case of a combination of the U-octamer mutation with deletion of other uridine-rich sequences (U8M-ΔBC), was splicing regulation partially disrupted. Moreover, ΔBD and ΔBDE constructs, in which the U-octamer sequence remains intact, showed almost complete disruption of splicing regulation. These results indicate that most of the *cis*-acting elements required for the splicing regulation of *Sxl* pre-mRNA have been lost in these constructs. Therefore, the four URSs and the three U7 sequences located around URS4 seem to represent the primary *cis*-acting elements, although the U-octamer sequence near the male-specific acceptor site does seem to be partially involved in splicing regulation. Consistent with this notion, introduction of U16 sequences into the ΔBDE construct partially restored the splicing regulation. Furthermore, UV-crosslinking experiments showed that the female *Sxl* protein bound to URS2/3 and URS4 RNAs more efficiently than to the RNA containing the U-octamer sequence.

Although we are yet to achieve complete understanding of the mechanism underlying repression of the inclusion of exon 3 by the female *Sxl* product, our results strongly suggest that several uridine-rich sequences work in an additive manner as *cis*-acting elements for *Sxl* pre-mRNA splicing regulation. In addition, *in vitro* binding experiments suggest that there may be some protein-protein interaction between two female *Sxl* protein molecules. Thus interaction at multiple binding sites would lead to conformational changes of the *Sxl* pre-mRNA and impair recognition of the male-specific exon by the basic splicing machinery. Alternatively, the female *Sxl* protein may affect the ASF/SF2 activity which promotes selection of the proximal 5′ splice site (20, 21). Recently, a heterogeneous ribonucleoprotein (hnRNP) A1 was shown to antagonize SF2 activity and to cause selection of the distal 5′ splice site *in vitro* (22). The hnRNP A1 protein also contains two RNA binding domains (23) as is the case with the female *Sxl* protein. From these findings, it is speculated that the female *Sxl* protein functions as a gene-specific hnRNP A1 homolog and switches the splicing of *Sxl* pre-mRNA to the female mode. In any case, the uridine-rich sequences within the introns play a key role in the control of *Sxl* pre-mRNA splicing. It remains to be elucidated how many uridine-rich sequences are required and how critical their location in the *Sxl* pre-mRNA is for splicing regulation. Multiple binding sites for the female *Sxl* protein in the introns around the male-specific exon 3 would guarantee the strict *in vivo* autoregulation of *Sxl* pre-mRNA splicing.

ACKNOWLEDGMENTS

We thank Leslie R.Bell for kind gift of *Sxl* F1 cDNA and Iain Hagan for reviewing the manuscript. This work was supported by research grants from the Ministry of Education, Science and Culture of Japan and from Mitsubishi Foundation.

REFERENCES

1. Baker, B. S. (1989) *Nature* **340**, 521–524.
2. Cline, T. W. (1989) *Cell* **59**, 231–234.
3. Steinmann-Zwicky, M., Amrein, H., and Nöthiger, R. (1990) *Adv. Genet.* **27**, 189–237.
4. Nagoshi, R. N., McKeown, M., Burtis, K. C., Belote, J. M., and Baker, B. S. (1988) *Cell* **53**, 229–236.
5. McKeown, M., Belote, J. M., and Boggs, R. T. (1988) *Cell* **53**, 887–895.
6. Sanchez, L. and Nöthiger, R. (1983) *EMBO J.* **2**, 211–214.
7. Cline, T. W. (1984) *Genetics* **107**, 231–277.
8. Parkhurst, S. M., Bopp, D., and Ish-Horowicz, D. (1990) *Cell* **63**, 1179–1191.
9. Erickson, J. W. and Cline, T. W. (1991) *Science* **251**, 1071–1074.
10. Keyes, L. N., Cline, T. W., and Schedl, P. (1992) *Cell* **68**, 933–943.
11. Bell, L. R., Maine, E. M., Schedl, P., and Cline, T. W. (1988) *Cell* **55**, 1037–1046.
12. Salz, H. K., Maine, E. M., Keyes, L. N., Samuels, M. E., Cline, T. W., and Schedl, P. (1989) *Genes Dev.* **3**, 708–719.
13. Samuels, M. E., Schedl, P., and Cline, T. W. (1991) *Mol. Cell. Biol.* **11**, 3584–3602.
14. Bell, L. R., Horabin, J. I., Schedl, P., and Cline, T. W. (1991) *Cell* **65**, 229–239.
15. Inoue, K., Hoshijima, K., Sakamoto, H., and Shimura, Y. (1990) *Nature* **344**, 461–463.

16. Hoshijima, K., Inoue, K., Higuchi, I., Sakamoto, H., and Shimura, Y. (1991) *Science* **252**, 833–836.
17. Sosnowski, B. A., Belote, J. M., and McKeown, M. (1989) *Cell* **58**, 449–459.
18. Zamore, P. D., Patton, J. G., and Green, M. R. (1992) *Nature* **355**, 609–614.
19. Ponce, M. R. and Micol, J. L. (1992) *Nucleic Acids Res.* **20**, 623.
20. Ge, H. and Manley, J. L. (1990) *Cell* **62**, 25–34.
21. Krainer, A. R., Conway, G. C. and Kozak, D. (1990) *Cell* **62**, 35–42.
22. Mayeda, A. and Krainer, A. R. (1992) *Cell* **68**, 365–375.
23. Bandziulis, R. J., Swanson, M. S. and Dreyfuss, G. (1989) *Genes Dev.* **3**, 431–437.