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Binding of the *Drosophila* transformer and transformer-2 proteins to the regulatory elements of doublesex primary transcript for sex-specific RNA processing

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ABSTRACT **Sex-specific alternative processing of doublesex (*dsx*) precursor messenger RNA (pre-mRNA) is one of the key steps that regulates somatic sexual differentiation in *Drosophila melanogaster*. By transfection analyses using *dsx* minigene constructs, we identified six copies of the 13-nucleotide sequences TC(T/A)(T/A)C(A/G)ATCAACA in the female-specific fourth exon that act as the cis elements for the female-specific splicing of *dsx* pre-mRNA. UV-crosslinking experiments revealed that both female-specific transformer (*tra*) and transformer-2 (*tra-2*) products bind to the 13-nucleotide sequences of *dsx* pre-mRNA. These results strongly suggest that the female-specific splicing of *dsx* pre-mRNA is activated by binding of these proteins to the 13-nucleotide sequences.**

Somatic sexual differentiation in *Drosophila* is accomplished by a hierarchy of regulatory genes (for reviews, see refs. 1–3). One of these genes, doublesex (*dsx*), is required for somatic sexual differentiation in both males and females (4). The *dsx* pre-mRNA undergoes sex-specific RNA processing (splicing and polyadenylylation reactions), which leads to the production of two distinct sex-specific mRNAs (Fig. 1) (5, 6). In female flies, the common third exon is spliced to the female-specific fourth exon and the cleavage/polyadenylylation reaction occurs immediately downstream of the fourth exon. In contrast, splicing between the common third exon and the male-specific fifth exon occurs in male flies.

Genetic studies have shown that the transformer (*tra*) and transformer-2 (*tra-2*) genes are necessary for the production of female-specific *dsx* mRNA (7). Functional *tra* mRNA is expressed only in females (8), whereas functional *tra-2* mRNA is expressed in both males and females (9, 10). Using a cotransfection system in which the *dsx* minigene was transfected into *Drosophila* Kc cells with the female-specific *tra* cDNA and the *tra-2* cDNA, we showed previously that the *tra* and *tra-2* products promoted the female-specific splicing of *dsx* pre-mRNA (11). Similar results consistent with our findings have been reported by other investigators (12, 13). The tra-2 protein (9, 10) contains a putative RNA-binding domain (RBD) (14, 15). This domain is found in many RNA-binding proteins, and in several cases this domain has been shown to be important for RNA-binding activity (14, 15). In addition to this domain, the tra-2 protein contains an arginine/serine-rich domain (R/S domain) (9, 10), the role of which is not well understood, although the domain is assumed to play a role in RNA processing. The female-specific tra protein does not contain RBD but does have an R/S domain (8). These facts suggest that the tra and tra-2 proteins may interact directly with *dsx* pre-mRNA to modulate splice site selection.

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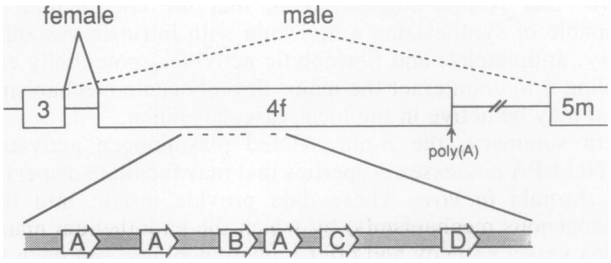

FIG. 1. The 13-nt sequences in the female-specific exon of *dsx*. Upper line shows the female- and male-specific splicing patterns. Open boxes and lines between boxes represent *dsx* exon and intron sequences, respectively. The female-specific poly(A) site is indicated by an arrow. Lower line shows the repeats of the 13-nt sequences (open thick arrows) of types A–D.

Analysis of flies with mutations in the *dsx* gene has suggested that tandemly interspersed six repeats of 13-nucleotide (nt) sequences, TC(T/A)(T/A)C(A/G)ATCAACA, in the female-specific fourth exon are necessary for the female-specific splicing of *dsx* pre-mRNA (16). It has been shown recently that deletion of the region containing the 13-nt sequences abolishes the female-specific splicing of *dsx* pre-mRNA (11–13). Also, the number of the 13-nt sequences has been shown to correlate with the efficiency of the female-specific splicing (11). These results strongly suggest that the 13-nt sequences represent the cis regulatory elements for the female-specific splicing, although the possibility that other sequences in *dsx* pre-mRNA act on the regulation of *dsx* alternative splicing was not completely excluded. If this is the case, it is likely that the 13-nt sequences in the *dsx* pre-mRNA are interaction sites of the tra and tra-2 proteins. The tra-2 protein produced in bacterial cells has been shown to bind RNAs containing the 13-nt sequences *in vitro* (12), but it remains to be elucidated whether the tra protein interacts with the 13-nt sequences.

In this study, we performed functional analysis of the 13-nt sequences and *in vitro* binding experiments of the tra and tra-2 proteins with *dsx* pre-mRNA. We have identified the 13-nt sequences as the cis regulatory elements for the female-specific splicing of *dsx* pre-mRNA and demonstrated that the female-specific tra protein and the tra-2 protein are able to bind the cis regulatory element. Moreover, immunoprecipitation data suggest that there exists protein–protein association between tra and tra-2 proteins.

MATERIALS AND METHODS

Chemicals and Enzymes. ^{32}P-labeled nucleotides were obtained from Amersham. RNases A and T_1 were from Sankyo. Other enzymes were purchased from Takara Shuzo (Kyoto).

Abbreviations: RBD, putative RNA-binding domain; R/S domain, arginine/serine-rich domain; nt, nucleotide(s).

Plasmid Construction. The R0 plasmid was constructed by deleting a portion of the female exon [190–599 base pairs (bp) downstream of the acceptor site] from the copia–dsx plasmid described previously (11). Synthetic oligonucleotides were inserted into the *Kpn* I site of R0. Sequences of the oligonucleotides were as follows (the 13-nt sequences are underlined).

(A) 5′-CGTCTTCAATCAACATACGCGTCTCTTCAATCAACACGGTAC-3′
(B) 5′-CGTCTACAATCAACATACGCGTCTCTACAATCAACACGGTAC-3′
(C) 5′-CGTCAACAATCAACATACGCGTCTCAACAATCAACACGGTAC-3′
(D) 5′-CGTCAACGATCAACATACGCGTCTCAACGATCAACACGGTAC-3′
(M1) 5′-CGGATTCAATCAACATACGCGTCGATTCAATCAACACGGTAC-3′

These oligonucleotides were also inserted into the *Kpn* I site of pSP73 (Promega) to synthesize RNA probes for UV-crosslinking experiments.

The *dsx* genomic fragment containing from the third exon to 1128 bp downstream of the female-specific acceptor site was subcloned into pSP73. The resulting plasmid was designated pSPdsxE34f.

A *Dde* I–*Eco*RI fragment containing the female-specific *tra* cDNA was inserted into the *Eco*RI site of pGEMEX (Promega). The *Nae* I–*Eco*RI fragment of the *tra-2* cDNA was ligated to the *Bam*HI site of pET3b (17).

***In Vitro* Synthesis of Labeled RNAs.** The SP plasmids in which the oligonucleotides had been inserted were linearized with *Bam*HI. pSPdsxE34f was digested with *Bst*BI 548 bp downstream of the female acceptor site. With these digests as templates, probe RNAs for UV-crosslinking experiments were synthesized with SP6 RNA polymerase in the presence of [α-^{32}P]UTP. The *dsx* antisense probe (Pf) for RNase protection was synthesized with T7 RNA polymerase in the presence of [α-^{32}P]GTP as described (11).

Transfections. Cultivation of Kc cells and transfections were performed as described (18). Forty micrograms of total cellular RNA was hybridized with the labeled Pf RNA (10^5 cpm) at 45°C and then digested with RNases A and T_1 (11). The digests were electrophoresed in a 6% polyacrylamide gel containing 8 M urea.

Preparation of Proteins Synthesized in Bacterial Cells. The T7 promoter expression system was used essentially as described (18). In the case of the gene 10 and gene 10–tra fusion proteins, inclusion bodies were purified and solubilized with buffer E (20 mM Hepes·NaOH, pH 7.9/0.1 mM EDTA/1 mM dithiothreitol/10% glycerol) (19) containing 6 M and 8 M urea, respectively. The gene 10 protein was further purified by DEAE-column chromatography in the presence of 5 M urea and then dialyzed in buffer E containing 0.1 M KCl. The gene 10–tra fusion protein was gradually dialyzed in buffer E containing decreasing concentrations of urea (final concentration, 3 M). The tra-2 protein was solubilized with buffer E containing 1% Triton X-100, further purified by DEAE-column chromatography, and then dialyzed in buffer E containing 0.1 M KCl.

RNA Binding Assay. The partially purified proteins were incubated with probe RNAs in 10 mM Hepes·NaOH, pH 7.9/1 mM EDTA/50 mM KCl containing yeast tRNA (1.3 μg/ml) at 20°C for 20 min. The reaction mixtures were irradiated with UV light (254 nm) for 4 min (0.6 J/cm^2; Stratalinker, Stratagene) on ice and then treated with RNase A (1 mg/ml) at 37°C for 20 min. The mixtures were then electrophoresed in a 15% polyacrylamide gel containing 0.1% SDS.

Immunoprecipitation. Rabbit antiserum raised against the gene 10 protein was obtained from Seikagaku Kogyo (Tokyo). After incubation of tra and/or tra-2 protein with the labeled C-type RNA followed by RNase treatment, the reaction mixture was incubated for 20 min on ice with the rabbit anti-gene 10 antiserum. Protein A-Sepharose (Pharmacia) was added and the mixture was incubated for 15 min on ice. The protein A-Sepharose/antibody complex was washed with 50 mM Tris·HCl, pH 7.4/150 mM NaCl/0.05% Nonidet P-40/0.5 mM dithiothreitol three times and then electrophoresed in a 15% polyacrylamide gel containing 0.1% SDS.

RESULTS

Cis Regulatory Elements for Female-Specific Splicing of *dsx* Pre-mRNA. By cotransfection experiments using the *dsx* minigene constructs, we showed previously that the regulatory element for female-specific splicing of *dsx* pre-mRNA lies in the female-specific fourth exon (11). When the R0 construct, which contains the region from the third common exon to the fifth male exon but lacks a large region within the female-specific fourth exon, was transfected into Kc cells, the female-specific splicing products were not observed even in the presence of the *tra* and *tra-2* products (Fig. 2*b*, lanes 9 and 10; see also ref. 11). The deleted region in the R0 construct contains six copies of tandemly interspersed 13-nt sequences, TC(T/A)(T/A)C(A/G)ATCAACA, which were proposed to be the regulatory sequences for the female-specific splicing (Fig. 1) (11–13, 16). To examine whether this proposal was correct, we inserted the synthetic oligonucleotides that contained two copies of the 13-nt sequences into the deleted site of the R0 construct (Fig. 2*a*). The 13-nt sequences can be classified into four groups that differ from each other by a few nucleotide sequences (Table 1). First, we examined whether the insertion of the A-type 13-nt sequence into the R0 construct would restore female-specific splicing in Kc cells. When only the RA construct, which contains two copies of the A-type 13-nt sequence, was transfected, no female-specific splicing product was detected (Fig. 2*b*, lane 1). By contrast, when the RA construct was cotransfected with the female-specific *tra* cDNA and the *tra-2* cDNA, the female-specific splicing product was observed, as indicated

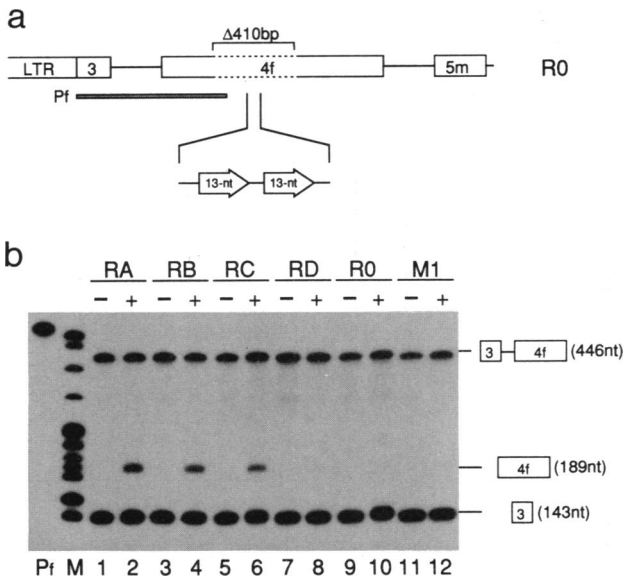

FIG. 2. Cotransfection analyses of splicing patterns of pre-mRNAs from *dsx* minigene constructs. (*a*) Schematic representation of the *dsx* minigene constructs and a probe (Pf) used for RNase protection analyses. Oligonucleotides containing two copies of each type of the 13-nt sequence were inserted into the deleted region (Δ410 bp) of the R0 construct. (*b*) RNase protection analyses of splicing products. Each minigene construct was transfected into Kc cells with (+) or without (−) the female-specific *tra* cDNA and the *tra-2* cDNA. RNA products were analyzed by RNase protection using Pf probe. Structure and size of each RNA molecule are illustrated at right. Lane Pf, probe RNA; lane M, the *Hpa* II digest of pBR322 used as size markers.

Table 1. Wild-type (A, B, C, and D) and mutant (M1) 13-nt sequences

Type	Sequence
A	TCTTCAATCAACA
B	TCTACAATCAACA
C	TCAACAATCAACA
D	TCAACgATCAACA
M1	gaTTCAATCAACA

Lowercase letters in D and M1 sequences indicate differences compared with the consensus 13-nt sequence.

by the RNase-protected band of 189 nt (lane 2). The male-specific splicing product decreased concomitantly with the increase of the female product (data not shown). Introduction of the A-type oligonucleotide in the antisense orientation did not restore female-specific splicing (data not shown). To confirm further that the 13-nt sequence was necessary for the female-specific splicing, we introduced base substitutions into the A-type sequence (Table 1, M1). When the RM1 plasmid, which contains two copies of the M1 13-nt sequence, was transfected, the female-specific splicing product was not observed even in the presence of the female-specific *tra* cDNA and the *tra-2* cDNA (Fig. 2*b*, lanes 11 and 12).

We examined whether other 13-nt sequences (B, C, and D types, Table 1) could restore female-specific splicing. With the RB and RC constructs, the same results were obtained as with the RA construct (Fig. 2*b*, lanes 3–6). When the RD construct was cotransfected with the *tra* and *tra-2* cDNAs, the female-specific splicing product was barely detectable (lanes 7 and 8), but a very small amount of the female-specific product was observed after long exposure of the gel. These results clearly show that these 13-nt sequences act as cis regulatory elements for the female-specific splicing of *dsx* pre-mRNA, although the D-type sequence is less active than the other sequences.

Female-Specific tra Protein Has RNA-Binding Activity. The female-specific acceptor site of *dsx* pre-mRNA is activated in the presence of the female-specific tra protein and the tra-2 protein (11). It is most likely that either or both of these proteins bind directly to *dsx* pre-mRNA. To determine whether the tra and tra-2 proteins directly associate with *dsx* pre-mRNA, we performed UV-crosslinking experiments. The female-specific *tra* cDNA was fused downstream of the T7 phage gene 10 open reading frame (Fig. 3*a*). The gene 10 protein, the gene 10–tra fusion protein, and the tra-2 protein were expressed in *E. coli* using the T7 phage promoter system and then partially purified as described in *Materials and Methods*. RNA-binding activity of each protein was tested with a *dsx* RNA probe (dsxE34f). This probe RNA includes the region from the third exon to the fourth exon with five copies of 13-nt sequences (A, B, and C). The gene 10–tra fusion protein bound to the probe, whereas the gene 10 protein did not (Fig. 3*b*, lanes 1 and 4). The tra-2 protein could also bind to the probe RNA (lane 2). When the gene 10–tra protein and the tra-2 protein were present in the same reaction mixture, binding of both proteins to the probe RNA was observed (lane 3). These proteins did not bind to the probe that contained the male-specific intron and the fifth exon sequence (data not shown). These results show that both the tra and tra-2 proteins bind to the RNA including the 13-nt sequences that are responsible for the female-specific splicing.

Binding of tra and tra-2 Proteins to the 13-nt Sequences. To clarify the relationship between the function of 13-nt cis regulatory elements and binding of the tra and tra-2 proteins to the *dsx* pre-mRNA, we synthesized short RNAs *in vitro* containing two copies of wild-type or mutant 13-nt sequences and examined the binding of these proteins to the RNAs by UV-crosslinking (Fig. 4). First, we analyzed binding of the gene 10–tra fusion protein to the probe RNAs (lanes 1–4). When A- or C-type RNA was used, efficient binding of the fusion protein to the probe RNA was observed (lanes 1 and 2). The fusion protein also bound to D-type RNA, but the binding was less efficient (lane 3). However, the gene 10–tra fusion protein could not bind to M1 RNA at all (lane 4). Next, we examined binding of the tra-2 protein to the same set of probe RNAs (lanes 5–8). The results obtained were essentially the same as those with the gene 10–tra protein. The tra-2 protein bound strongly to A and C RNAs (lanes 5 and 6), weakly to D-type RNA (lane 7), and not at all to M1 RNA (lane 8). Further, it seemed that the binding specificity was not changed when both proteins were present in the same reaction mixture (lanes 9–12). These two proteins also bound to the RNA containing B-type 13-nt sequence as efficiently as those containing A- and C-type sequences but did not bind to the RNA probe containing the antisense sequence of the A-type sequence (data not shown). These results indicate that both tra and tra-2 can bind specifically to the 13-nt sequences that function as the regulatory elements for the female-specific splicing of *dsx* pre-mRNA.

Coimmunoprecipitation of tra and tra-2 Proteins. To examine the possible interaction between the tra and tra-2 proteins, we performed an immunoprecipitation experiment combined with UV-crosslinking. The gene 10–tra protein was

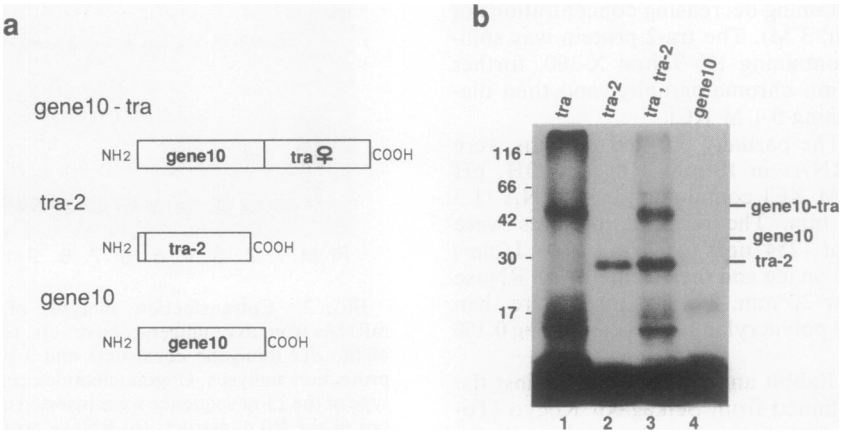

FIG. 3. Binding of the tra and tra-2 proteins to *dsx* pre-mRNA. (*a*) Structures of proteins produced in *E. coli* with the T7 phage expression system. Box labeled gene 10 represents the T7 phage gene 10 open reading frame. (*b*) UV crosslinking of the proteins produced in *E. coli* with a *dsx* probe RNA (dsxE34f) that contains the region from the third exon to the fourth exon. Crosslinked products were analyzed by SDS/PAGE. Lanes: 1, gene 10–tra fusion protein; 2, tra-2 protein; 3, gene 10–tra and tra-2 proteins; 4, gene 10 protein. Positions corresponding to each protein are indicated at right. Molecular size markers (kDa) are shown at left.

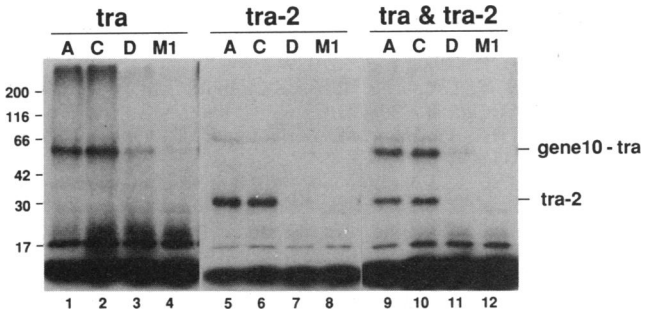

FIG. 4. Binding of tra and tra-2 proteins with RNAs containing the 13-nt motifs. The gene 10–tra and tra-2 proteins were crosslinked with probe RNAs containing two repeats of each type of the 13-nt sequences. Probe RNAs (A, C, D, and M1) are shown above the lanes. Crosslinked products were analyzed as in Fig. 3. Lanes: 1–4, gene 10–tra fusion protein; 5–8, tra-2 protein; 9–12, gene 10–tra and tra-2 proteins. Positions corresponding to each protein are indicated at right. Molecular size markers (kDa) are shown at left.

incubated with or without tra-2 protein in the presence of the labeled C-type RNA and the reaction mixture was UV-crosslinked. After RNase-treatment, the reaction mixture was immunoprecipitated by the antiserum to gene 10 protein. The gene 10–tra protein was precipitated by the antiserum, whereas tra-2 protein was not, as expected (Fig. 5, lanes 1 and 2). When both gene 10–tra and tra-2 proteins were present in the same reaction mixture, tra-2 protein was immunoprecipitated as well as gene 10–tra protein (lane 3). Immunoprecipitation of tra-2 protein by antiserum to the gene 10 protein was not observed when the gene 10 and tra-2 proteins were present in the same mixture (data not shown). These results suggest tra–tra-2 protein association.

DISCUSSION

By transfection experiments, we have shown directly that the 13-nt sequences are responsible for the female-specific splicing of *dsx* pre-mRNA. Moreover, we have demonstrated that the female-specific tra protein produced in *E. coli* is able to bind to the 13-nt sequences, like the tra-2 protein. Our results strongly suggest that the female-specific tra protein and the tra-2 protein bind to the 13-nt sequences in the female exon and activate the female-specific splicing.

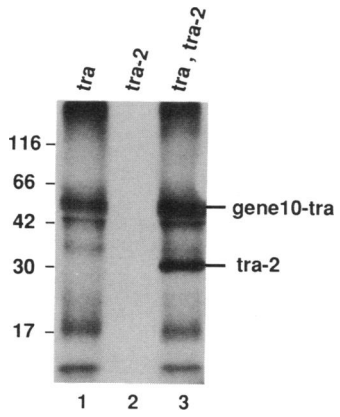

FIG. 5. Coimmunoprecipitation of tra and tra-2 proteins. The gene 10–tra and tra-2 proteins, individually or together, were crosslinked with the labeled C-type RNA and then treated with RNase A. The reaction mixtures were immunoprecipitated by antiserum to the gene 10 protein. Each immunoprecipitate was analyzed by SDS/PAGE. Lanes: 1, gene 10–tra; 2, tra-2; 3, gene 10–tra and tra-2. Positions corresponding to each protein are indicated at right. Molecular size markers (kDa) are shown at left.

In the present study, we have classified the 13-nt sequences into four groups. The A-, B-, and C-type motifs functioned efficiently as cis elements to promote the female-specific splicing of *dsx* pre-mRNA in cells. The D-type is different from the C-type by one nucleotide: the sixth nucleotide, A, in the C-type is replaced by G in the D-type (Table 1). However, the activation of the female-specific splicing was very weak when the D-type sequence was inserted into the R0 mutant as compared with other type sequences. The mutant 13-nt sequence, M1, is different from the A-type motif by only the first two nucleotides, but it was unable to activate the female-specific splicing. These results indicate that the primary sequences of the 13-nt sequences are extremely important for their function. The results led us to conclude that the 13-nt sequences act as cis regulatory elements on *dsx* alternative processing.

This conclusion is consistent with the results of UV-crosslinking experiments. Both the gene 10–tra fusion protein and the tra-2 protein bound efficiently to the A-, B-, and C-type 13-nt sequences, and less efficiently to the D-type. Thus, the 13-nt sequences except for the D-type seem to be preferred binding sites for the tra and tra-2 proteins, although we could not exclude the possibility that other binding sites exist in *dsx* pre-mRNA.

We have shown here that both tra and tra-2 proteins could bind the same 13-nt sequences *in vitro*. Since the female-specific *dsx* splicing requires the presence of both tra and tra-2 proteins, there may be some cooperative interaction of these proteins with *dsx* pre-mRNA *in vivo*. Presumably, the two proteins might bind to the 13-nt sequences cooperatively to allow activation of the female-specific *dsx* splicing. In this respect, the results obtained from the immunoprecipitation experiment strongly suggest protein–protein association between tra and tra-2 proteins. This association could be responsible for efficient binding of these proteins to the 13-nt sequences and for activation of the *dsx* female-specific splicing. The binding of both of these proteins to the *dsx* pre-mRNA may be a prerequisite for efficient association of other basic splicing machinery. This idea includes the possibility that the tra and tra-2 proteins are components of a subset of splicing machinery including small nuclear ribonucleoproteins.

As to which regions of the tra and tra-2 proteins are responsible for their binding to the 13-nt sequences, we have no direct evidence so far. However, as mentioned above, the tra-2 protein contains both an RBD and an R/S domain (9, 10), while the tra protein has only an R/S domain (8). A similar R/S domain exists in the product of the suppressor of white apricot [*su(w^a)*] gene of *Drosophila* (20). The *su(w^a)*-encoded protein was shown to have an autoregulatory function, thereby preventing removal of the first two introns from the *su(w^a)* pre-mRNA (20). Moreover, a human splicing factor, ASF/SF2 (21, 22), and a 70-kDa protein of U1 small nuclear ribonucleoprotein (15, 23, 24) contain both an R/S domain and an RBD. From these facts, it is likely that the RBD or R/S domain plays an important role in RNA binding by the tra and tra-2 proteins. Another possible role of the R/S domain may be in interaction with other proteins. It is possible that the tra and tra-2 proteins may interact with each other through the R/S domains. In addition, it is also possible that the R/S domain is a targeting signal that directs subnuclear localization to allow coconcentration of splicing components, as was recently suggested (25).

We can think of two mechanisms for splice site selection: positive and negative controls of splicing. As an example of negative control, the *Drosophila* Sex-lethal (*Sxl*) gene product binds specifically to a non-sex-specific default acceptor site of *tra* pre-mRNA to repress the use of this acceptor site in female flies (18). In addition, negative control of alternative splicing has been proposed in several cases (26). On the other

hand, there is no example of positive regulation of alternative splicing (or splice site selection) except for the case of *dsx* pre-mRNA. The female-specific acceptor site of *dsx* has a suboptimal polypyrimidine stretch that results in poor recognition of the acceptor site during the splicing reaction in males (11). The tra and tra-2 proteins may facilitate the recognition of the suboptimal acceptor as an efficient splicing signal. In addition to female-specific splicing, female-specific polyadenylylation has also been shown to be activated in the presence of the tra and tra-2 proteins (12). Deletion of the female-specific polyadenylylation signal of *dsx* did not affect the regulation of female-specific splicing (11). It has also been suggested that the female-specific polyadenylylation is regulated only in the presence of splicing (13). However, it is possible that the tra and tra-2 proteins regulate both splicing and polyadenylylation independently.

Note. Recently, Tian and Maniatis (27) have shown that the female-specific splicing of *dsx* pre-mRNA is activated *in vitro* by the tra and tra-2 proteins produced by a baculovirus system. According to their results, the tra-2 protein specifically binds to the RNA containing the 13-nt sequences that we have identified as cis-acting elements, but the tra protein shows specific RNA-binding activity only in the presence of HeLa nuclear extract.

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