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Protein-RNA and Protein-Protein Interactions of the *Drosophila* Sex-Lethal Mediated by Its RNA-Binding Domains[1]

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The *Drosophila* Sex-lethal (Sxl) contains two RNA-binding domains (RBDs) which belong to the RNA recognition motif (RRM) group. Sxl binds to a specific uridine-rich sequence which is believed to be the major *cis*-acting element for the splicing regulation of the *transformer* (*tra*) mRNA precursor. Here we show evidence supporting the previous suggestion that Sxl recognizes the sequence context downstream of the uridine-rich sequence. In addition, by means of UV-crosslinking assays with Sxl deletion constructs, we have demonstrated that Sxl RNA binding requires both of its RBDs for specificity and strength. Moreover, by the yeast two-hybrid analysis, we found that homodimeric interaction occurs between two Sxl molecules. Interestingly, the amino- and carboxy-terminal regions outside of the Sxl RBDs are dispensable for such dimerization, indicating that the protein-protein interaction is also mediated by RBDs. Coprecipitation experiments *in vitro* showed that the protein-protein interaction seems to be RNA-dependent but greatly enhanced by addition of the specific RNA containing the Sxl binding site, suggesting that the conformational change which is induced on binding to RNA may facilitate the interaction between Sxl molecules.

Key words: protein-protein interaction, RNA-binding domain (RBD), RNA recognition motif (RRM), Sex-lethal (Sxl), two-hybrid analysis.

In eukaryotes, post-transcriptional regulation of gene expression is achieved by numerous RNA-binding proteins, which can be classified into several groups (*1, 2*). Of these, the RNA-binding proteins with RNA recognition motifs (RRM) (*3-6*) are well-characterized and have been shown to be involved in many aspects of RNA processing events such as polyadenylation and pre-mRNA splicing (*2*). The RNA recognition motif consists of approximately 90 amino acids, is structurally characterized by the presence of two α helices and four β strands, and functions as an RNA-binding domain (RBD) (*6-8*). Two short sequences rich in hydrophobic residues, RNP1 and RNP2, which are present on the third and first β strands of the RRM, respectively, display the highest conservation in many proteins and are thought to be important core sequences for the RNA binding ability.

Sex-lethal (Sxl) is an RRM-type RNA-binding protein which has been shown to play a key role in the sex-determination and dosage compensation of *Drosophila melanogaster* (*9-12*). It is a protein of 354 amino acids containing two RBDs with asparagine-glycine-rich and hydrophobic residue-rich regions at the amino- and carboxy-termini, respectively (*13*). Functional Sxl protein is produced only in females since specific splicing of its mRNA precursor (pre-mRNA) requires initiation by an embryonic Sxl isoform which is transiently synthesized only in the very early stage female embryos (*14*). Once the functional adult-type Sxl is produced, it controls its own sex-specific splicing *via* a positive feedback and induces the *transformer* (*tra*) female-specific splicing, which eventually leads to feminization (*15*).

Molecular analyses have revealed that Sxl binding to a specific *cis*-acting element within the *tra* pre-mRNA is critical for the female-specific splicing (*16-20*). Sxl can also binds to multiple sites of the *Sxl* pre-mRNA *in vitro* and thereby seems to regulate its own pre-mRNA splicing (*21, 22*). These Sxl binding sites contain consecutive uridine residues and the cooperative interaction of Sxl molecules binding to such sequences has been suggested to be important for the *Sxl* pre-mRNA splicing autoregulation (*21, 23, 24*). An *in vitro* ligand RNA selection method was utilized to examine Sxl RNA binding specificity (*25*). UV-crosslinking analysis of the selected RNAs has suggested that Sxl preferentially binds to polyuridine stretches which are surrounded by purine residues and that this binding is facilitated by an AG dinucleotide located downstream of the stretches. Although the RBDs have been shown to play an important role for specific RNA binding, the contribution of other domains of the Sxl protein has not been addressed clearly. In particular, for the Sxl pre-mRNA splicing regulation, we speculated that one or more subregions of the Sxl protein might be involved in the specific protein-RNA and protein-protein interactions.

In this study, we examined the importance of the downstream AG dinucleotide for Sxl binding, which was

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suggested previously (*25*). In addition, we made several Sxl deletion mutants to examine their RNA binding ability by UV-crosslinking experiments and clarified the region(s) responsible for the specific RNA binding. Moreover, using the yeast two-hybrid system and the coprecipitation assays, we examined whether Sxl interacts with itself. These lines of analyses have revealed surprisingly that both the protein-RNA and protein-protein interactions are mediated by the two Sxl RBDs.

MATERIALS AND METHODS

Enzymes and Other Chemicals—Restriction enzymes and Klenow fragment were obtained from TOYOBO. DNA ligation and LA PCR kits were obtained from TAKARA Shuzo. Sequenase version 2.0 sequencing kit was obtained from United States Biochemicals. Radiolabeled nucleotides were purchased from Amersham.

Preparation of Fusion Proteins—For GST-RBDW, GST-RBD1, and GST-RBD2 fusion proteins, the *Sxl* cDNA fragments corresponding to amino acid residues 122-297, 122-208, and 202-297 were PCR-amplified, then cloned into the *Bam*HI site of the bacterial expression vector pGEX-3x (Pharmacia). The resultant plasmids were transformed into *Escherichia coli* XL1-blue. Fusion proteins were induced with 1 mM IPTG for 2 h and affinity-purified by glutathione-Sepharose according to the manufacturer's recommendations. GST-Sxl and T7-Sxl fusion proteins were prepared and purified as described previously (*25*).

In Vitro RNA Binding Analysis—The RNAs for *in vitro* binding analysis were synthesized by *in vitro* transcription with SP6 RNA polymerase in the presence of α-^{32}P-GTP and purified as previously described (*21, 25*). The TraE and TraEm sequences were introduced into *Eco*RI and *Sal*I sites in pSP73 using the following synthetic primers:

TraE-sense: 5′-AATTCTTTTTTGTTGTTTTTTTTTCTAGTG-3′
TraE-antisense: 5′-TCGACACTAGAAAAAAAAACAACAAAAAG-3′
TraEm-sense: 5′-AATTCTTTTTTGTTGTTTTTTTTTCTCGGG-3′
TraEm-antisense: 5′-TCGACCCGAGAAAAAAAAACAACAAAAAG-3′

RNA binding, UV-crosslinking, and SDS-PAGE were performed as described previously (*25*). The efficiency of label transfer was calculated by densitometry using a Fuji BAS 2000 Image Analyzer.

Two-Hybrid Analysis—Protein-protein interaction was analyzed by the method of Chevray and Nathans (*26*). Various cDNA fragments of Sxl were PCR-amplified using appropriate synthetic primers, introduced into the two-hybrid vectors, pPC86 and pPC97, and transformed into the yeast PCY2. β-Galactosidase activity was measured by the method of Guarente (*27*).

GST Pull-Down Experiment—T7-Sxl (1 μg) and GST-Sxl (1.5 μg) were incubated in 20 μl of D'K200T buffer (20 mM HEPES-NaOH pH 7.9, 5% glycerol, 200 mM KCl, 0.1% Triton X-100, 0.1 mM PMSF, 1 mM DTT) at 20°C for 60 min in the presence or absence of TraE RNA (2.5 μg) or MCS RNA (2.5 μg, a negative control RNA containing the multi-cloning site sequence of pSP73). For RNase treatment, RNaseA (5 μg/ml) was added to the incubation mixture. After incubation, 10 μl of glutathione-Sepharose (50% slurry) and 70 μl of D'K200T buffer were added to the reaction mixture, which was further incubated for 30 min. The glutathione-Sepharose beads were precipitated after washing five times with buffer D'K200T, then subjected to Western blot analysis using the T7 monoclonal antibody (Novagen). Relative binding of T7-Sxl to GST-Sxl was measured by densitometric analysis of the films.

RESULTS

Effect of the Downstream Sequence on Sxl RNA Binding—The results of the *in vitro* ligand RNA selection analysis suggested that Sxl binding to the polyuridine sequence may be positively influenced by the context of the downstream sequence which contains an AG dinucleotide (*25*). To test this possibility, the downstream AGU sequence of the *tra cis*-acting element (TraE), which was frequently observed in the *in vitro* selected RNAs, was altered to CGG by *in vitro* mutagenesis. The corresponding RNA was synthesized *in vitro* along with two wild-type RNAs, AF130 (*17*) and TraE (Fig. 1A), and these were tested for their affinity to Sxl by UV-crosslinking experiments. Sxl binding to the RNA containing the mutation (TraEm) was reduced by approximately twofold or less, as compared with the wild-type, AF130 and TraE RNAs (Fig. 1B). This supports our previous *in vitro* RNA selection analysis observations, indicating that Sxl is not a simple polyuridine-binding protein but that its binding is also influenced by the flanking sequences.

Localization of the Region for the Specific RNA Binding—To map the region responsible for Sxl RNA binding, three GST (glutathione S-transferase)-fusion proteins, GST-RBDW containing two RBDs (amino acid residues 122-297), GST-RBD1 containing the first RBD (122-208), and GST-RBD2 containing the second RBD (202-297), were overproduced in *E. coli* and affinity-purified by use of glutathione-Sepharose columns. The RNA binding abilities of these proteins were analyzed by UV-crosslinking, together with GST-Sxl (*25*) and T7-Sxl (*17*), which contain the full-length Sxl with GST and T7 tags, respectively (Fig. 2A). GST-Sxl, T7-Sxl, and GST-RBDW specifically bound to the RNA containing the *tra cis*-acting element, TraE (Fig. 2B, compare lanes 1, 2, and 4 with lanes 7, 8, and 10), whereas both GST-RBD1 and GST-RBD2 showed very weak binding to the RNA (lanes 5 and 6). These results indicate that both of the Sxl RBDs are required for efficient and specific RNA binding and that the regions outside of the RBDs are dispensable, although non-specific RNA binding appeared slightly higher with the GST-RBDW.

Homo-Dimeric Interaction of Sxl—We attempted to analyze the protein-protein interaction of Sxl using the yeast two-hybrid system (*26, 28*). A series of two-hybrid plasmids encoding Sxl deletion mutants fused downstream of the GAL4 DNA binding domain (DB) were constructed and individual plasmids were cotransformed into the yeast PCY2 with another two-hybrid plasmid encoding the full-length Sxl fused downstream of the GAL4 *trans*-activation domain (TA) (Fig. 3). The transformants were assayed for β-galactosidase activity to determine the presence or absence of protein-protein interaction. Significant levels of β-galactosidase activity were seen in the cotransformants

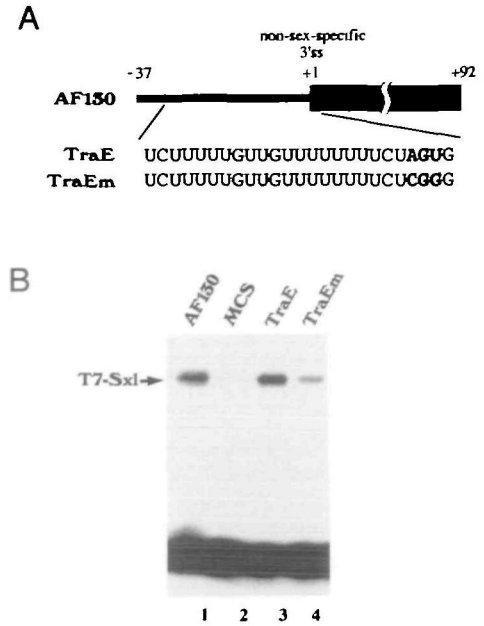

Fig. 1. **Alteration of the sequence downstream of the polyuridine stretch reduces Sxl binding efficiency.** (A) RNA sequences used for *in vitro* Sxl binding analysis. AF130 RNA (*17*) contains the *tra* non-sex-specific 3′ splice site. TraE RNA is a truncated version of AF130. The AGU sequence of TraE downstream of the polyuridine stretch is altered to CGG in TraEm RNA. (B) *In vitro* Sxl binding to the wild-type and mutant *cis*-acting element RNAs. T7-Sxl fusion protein was incubated with various RNAs, UV-irradiated followed by RNase treatment, electrophoresed on an 8% SDS-polyacrylamide gel and then autoradiographed. Lanes 1, AF130; 2, MCS (a negative control RNA containing the multi-cloning site sequence of pSP73); 3, TraE; 4, TraEm.

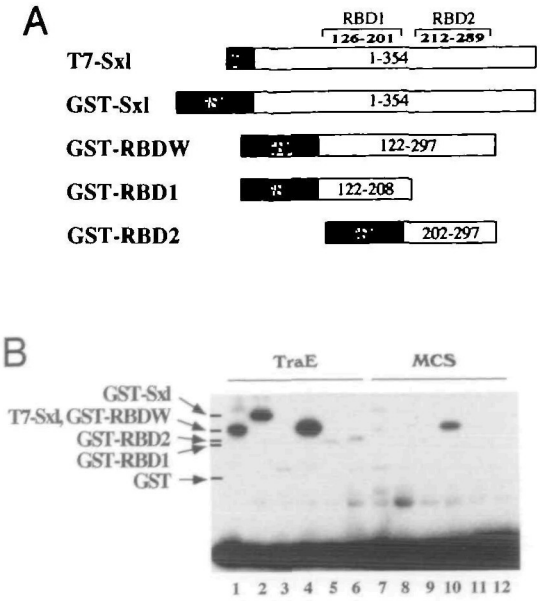

Fig. 2. ***In vitro* RNA binding analysis of Sxl deletion mutants.** (A) Schematic representation of the full-length Sxl fusion proteins and deletion mutants. Shaded boxes indicate the T7 and GST portion, and the location of the Sxl RBD1 and RBD2 are labeled. The numbers in open boxes indicate the amino acid residues of Sxl, starting with the initiation methionine. (B) UV-crosslinking experiment with Sxl fusion proteins. Each fusion protein was incubated with either *tra* *cis*-acting element RNA (TraE, lanes 1-6) or negative control RNA containing the multi-cloning site sequence (MCS, lanes 7-12), UV-irradiated followed by RNase treatment, electrophoresed on a 12% SDS-polyacrylamide gel and then autoradiographed. The position of each fusion protein is indicated on the right side of the figure. T7-Sxl, lanes 1 and 7; GST-Sxl, lanes 2 and 8; GST, lanes 3 and 9; GST-RBDW, lanes 4 and 10; GST-RBD1, lanes 5 and 11; GST-RBD2, lanes 6 and 12.

of four plasmids, DB(1-354), DB(99-354), DB(99-297), and DB(122-297) (Table I, no. 1, 3, 4, and 7), strongly suggesting that, as speculated, Sxl does possess homo-dimerization ability (*21*) and that this dimerization occurs only in the presence of both RBDs. Moreover, it appears that the N-terminal and C-terminal regions of the Sxl protein are not necessary for this interaction. To confirm these observations, we tested the transformants with three pairs of plasmids, TA(99-297) and DB(1-104), TA(99-297) and DB(99-297), and TA(99-297) and DB(291-354). As expected, only the transformant of the pair TA(99-297) and DB(99-297), which contained both of the Sxl RBDs, showed higher β-galactosidase activity (see Table I, no. 12-14).

Physical Interaction Dependent on the Presence of RNA—To confirm the data from the two-hybrid analysis, we attempted to detect the physical interaction of two Sxl molecules by the pull-down experiment using T7-Sxl and GST-Sxl (Fig. 4). After GST-Sxl had been incubated with T7-Sxl and precipitated by the affinity of GST portion to glutathione-Sepharose beads, T7-Sxl associated with GST-Sxl was measured by Western blot analysis using the T7 monoclonal antibody. A relatively weak interaction between T7-Sxl and GST-Sxl was observed when the incubation was done without any RNA added and when a non-specific MCS RNA was added (lanes 1 and 3). By contrast, addition of TraE RNA significantly enhanced the interaction by threefold or more (lane 4). When the incubation was done in the presence of RNase, however, even the weak interaction could not be detected (lane 2). Essentially the same results were obtained when GST-RBDW and T7-Sxl were used in the pull-down experiment (data not shown). In addition, since no positive band could be detected when GST and T7-Sxl were used (data not shown),

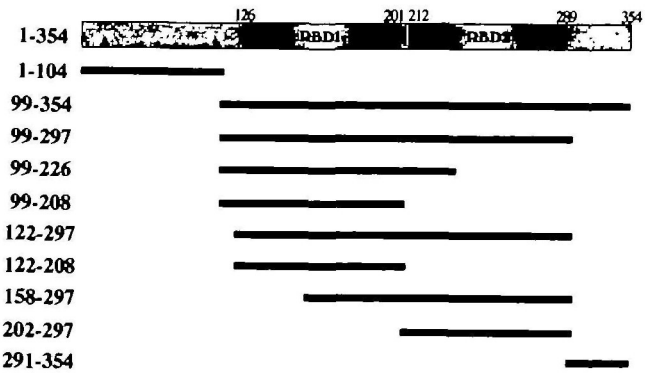

Fig. 3. **Schematic representation of the Sxl deletion mutants used for the yeast two-hybrid analysis.** The position of the Sxl RBDs are indicated. Deletion mutants are shown by thick bars. The number at the left indicates the amino acid residues of Sxl, starting with the initiation methionine.

TABLE I. **Protein-protein interaction of Sxl deletion mutants in the yeast two-hybrid system.**

No. of pairs	Transformants[a]		β-Galactosidase activity[b]
	Trans-activating (TA)	DNA-binding (DB)	
1	1-354	1-354	35.0
2	1-354	1-104	<1.0
3	1-354	99-354	10.2
4	1-354	99-297	13.5
5	1-354	99-226	1.2
6	1-354	99-208	<1.0
7	1-354	122-297	11.1
8	1-354	122-208	<1.0
9	1-354	158-297	<1.0
10	1-354	202-297	<1.0
11	1-354	291-354	1.0
12	99-297	1-104	<1.0
13	99-297	99-297	29.7
14	99-297	291-354	1.6
15	vector	vector	1.0

Yeast strain PCY2 was transformed with various combinations of the two-hybrid plasmids expressing Sxl deletion mutants. The β-galactosidase activity of each transformant was measured as described under "MATERIALS AND METHODS." [a]Each two-hybrid plasmid for transformation contains the Sxl coding region indicated as amino acid residue numbers. [b]β-Galactosidase activity is expressed relative to the background where two vector plasmids, pPC86 and pPC97, were cotransformed. Measurement of β-galactosidase activity was performed with three independent colonies for each combination.

two Sxl RBDs are responsible for the protein-protein interaction as well as the specific RNA binding. It is likely that the weak interaction observed in the reaction without exogenous RNA is mediated by the very low amount of non-specific RNAs contaminating the fusion proteins, and that the protein-protein interaction of Sxl is essentially RNA-dependent. Nevertheless, enhancement of the interaction by TraE RNA strongly suggests that the interaction is facilitated by the specific RNA binding at least *in vitro*.

DISCUSSION

To date, numerous RRM-type RNA-binding proteins containing multiple RBDs have been identified. Some have been shown to bind specific RNA sequences *via* a single RBD. An example is the human snRNP protein U1A, which requires only its first RRM for binding to U1 snRNA (*29, 30*). In contrast, we have shown here that both of the RBDs are indispensable for efficient and specific RNA binding of Sxl to the *tra cis*-acting element. Our conclusion is consistent with the results of a recent independent study on Sxl RNA binding by Kanaar *et al.* (*31*). By means of gel-shift assays using the *tra cis*-acting element, they also showed that both RBDs are required for the site-specific RNA binding of Sxl. In the case of the human hnRNPA1, each of its two RBDs, if taken separately, displays different RNA binding specificities. But the intact protein containing both of the RBDs binds to a unique sequence, demonstrating that the RNA binding specificity is not simply the sum of the binding specificities of each of the individual RBDs (*32*). Therefore, in some of the RRM-type RNA-binding proteins, it is suggested that the cooperative action of the multiple intramolecular RBDs seems to be important to determine the RNA binding specificity. It is likely that a similar cooperativity occurs between the two RBDs of Sxl

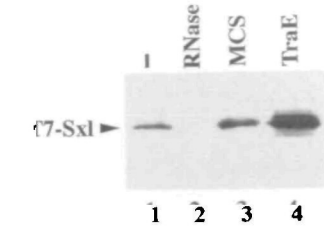

Fig. 4. **RNA-dependent protein-protein interaction of Sxl molecules *in vitro*.** T7-Sxl and GST-Sxl were incubated in the absence (lane 1) or presence of either RNase (lane 2), MCS RNA (lane 3), or TraE RNA (lane 4). After binding of GST-Sxl with glutathione-Sepharose beads followed by extensive washing, T7-Sxl bound to the beads was subjected to Western blot analysis using the T7 monoclonal antibody.

in the recognition of its target, including the sequence context downstream of the polyuridine stretch, and to strengthen the binding to the *tra cis*-acting element.

We have shown here that two Sxl molecules interact with each other *in vitro* as well as in the yeast cells, and that, unexpectedly, the interaction domain also lies within its RBDs. Other examples of such versatility in the function of the RBDs are the interaction of a fragment containing an RBD of the human U2 snRNP protein U2B″ with U2A′ (*33*) and the ability of the human SAP49, a spliceosome-associated protein with two RBDs, to interact with SAP145 even in the absence of the C-terminal proline-glycine-rich domain downstream of its RBDs (*34*). Crystallographic analysis of U1A has also suggested that interaction may occur between the RBDs, and that such interaction does not compromise the RNA-binding surface of the domain (*7*). Therefore, it is possible that protein-protein interaction between two Sxl molecules may occur in their RBDs, yet leaving their RNA binding ability intact.

Since the Sxl interaction *in vitro* seems to be RNA-dependent, the Sxl interaction observed in the yeast cells might also be dependent on unknown yeast RNAs. Alternatively, a very weak interaction may occur between Sxl molecules in cells that is independent of RNA, and which could be detected because of the high sensitivity of two-hybrid analysis. In either case, it is important that the *in vitro* interaction was found to be greatly enhanced only in the presence of the specific *tra* RNA. Stable binding to the true target RNA may change the Sxl conformation to facilitate and/or stabilize the homo-dimerization mediated by its RBDs, whereas an Sxl molecule which transiently binds to non-specific RNAs may not maintain such a proper conformation for a long time. It remains to be elucidated whether this RNA-dependent interaction is involved in the splicing regulation of *Sxl* pre-mRNA, which contains multiple Sxl binding sites.

The cooperativity in RNA binding has been implicated in the autoregulation of the Sxl pre-mRNA splicing. Wang and Bell have reported that the Sxl N-terminal region is essential for cooperative binding to the RNA containing two binding sites and is required for alternative splicing regulation of the *Sxl* pre-mRNA (*22*). Our results have revealed, however, that the protein-protein interaction of two Sxl molecules appears to be mediated through the two RBDs, not by the N-terminal region, both in the yeast cells and *in vitro*. How can one reconcile what seem to be two contradictory observations? An important factor which should not be

ignored is the difference in the assay systems used. The previous study used gel-shift assays in the presence of RNA ligands to analyze Sxl binding cooperativity. The two-hybrid system and the coprecipitation assays were principally utilized in this present work to analyze the interaction of two Sxl molecules. It is difficult, therefore, to directly compare the two observations, which may reflect two different phases of Sxl molecular interaction.

A possible explanation is that protein-protein interaction of Sxl molecules may be initiated through binding at independent sites, and after mutual binding to neighboring *cis*-acting sequences, further interaction between the N-terminal regions may stabilize their binding. In contrast to the adult-type Sxl, however, the embryonic Sxl isoform does not encode a large portion of the N-terminal asparagine-glycine-rich region (*14*). Therefore, at least during the stages where establishment of *Sxl* expression occurs, RBD-mediated interaction of Sxl after RNA-binding seems more important for the splicing regulation rather than interaction mediated by the N-terminal region. It will be of great interest to examine whether Sxl interacts with the products of other genes such as *fl(2)d* and *snf*, which have been shown to be involved in *Sxl* gene expression (*35-39*), and if so, which region of Sxl is responsible for the interaction.

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