4082–4086 Nucleic Acids Research, 1994, Vol. 22, No. 20

Characterization of RNA binding specificity of the *Drosophila* sex-lethal protein by *in vitro* ligand selection

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Received July 25, 1994; Revised and Accepted August 30, 1994

ABSTRACT

The *Drosophila* sex-lethal (Sxl) protein, a regulator of somatic sexual differentiation, is an RNA binding protein with two potential RNA recognition motifs (RRMs). It is thought to exert its function on splicing by binding to specific RNA sequences within *Sxl* and *transformer* (*tra*) pre-mRNAs. To examine the Sxl RNA binding specificity in detail, we performed *in vitro* selection and amplification of ligand RNAs from a random sequence pool on the basis of affinity with Sxl protein. After three cycles of selection and amplification, we cloned and sequenced 17 cDNAs corresponding to the RNAs selected *in vitro*. Sequencing showed that most of the RNAs selected contain polyuridine stretches surrounded by purine residues. *In vitro* binding analysis revealed that the sequences of the *in vitro* selected RNAs with relatively high affinity for Sxl show similarity to that of the *Sxl*- and *tra*-regulated acceptor regions, including the invariant AG sequence for splicing. These results suggest that Sxl recognizes and preferentially binds to a polyuridine stretch with a downstream AG sequence.

INTRODUCTION

Somatic sexual differentiation of *Drosophila melanogaster* is accomplished by a series of actions of sex determination gene products (see references 1–4 for reviews). One of these, sex-lethal (Sxl), plays a key role in this process and is also involved in both germline sex determination and dosage compensation. Functional Sxl protein is produced only in female flies by the female-specific splicing of its mRNA precursor (pre-mRNA) and it controls the sex-specific pre-mRNA splicing of *transformer* (*tra*) which is also a sex determination gene just downstream of *Sxl* in the genetic hierarchy (5–7). Moreover, Sxl controls its own female-specific splicing in a positive feedback manner (8–11). During splicing regulation, Sxl protein causes the skipping of an exon that contains translational stop codons, leading to the production of the female-specific mRNA with a long open reading frame encoding the functional Sxl or tra protein.

Molecular analyses of the splicing regulation have revealed that Sxl binds to the specific *cis*-acting element with a polyuridine stretch in *tra* pre-mRNA and that this binding inhibits the use of the non-sex-specific splice acceptor site, leading to female-specific splicing (6, 7, 12). Indeed, Sxl has two potential RNA recognition motifs (RRMs) which are features of various RNA binding proteins (5, 13–17). Sxl RRMs are important for RNA binding *in vitro* and for the splicing regulation of *tra* pre-mRNA *in vivo* (H.Sakamoto, unpublished data; K.Inoue, and Y.Shimura, personal communication). It has been suggested that similar *cis*-acting elements with polyuridine stretches are also involved in the autoregulation of *Sxl* pre-mRNA splicing (9–11). However, it has been shown that multiple *cis*-acting elements within introns that surround the *Sxl* male-specific exon are critical for the splicing regulation rather than the polyuridine stretch nearest the male-specific splice acceptor site, which has an extensive similarity with the *tra cis*-element with respect to sequence and location (9, 11). This observation suggests that the molecular mechanism underlying the splicing regulation of *Sxl* pre-mRNA differs from that of *tra* pre-mRNA, although the same Sxl protein is involved in both. In addition, it has been speculated that Sxl regulates expression of the gene(s) that is involved in the dosage compensation in a different way, such as at the level of translation rather than splicing (18). Thus, Sxl may control the RNA metabolism of its target genes in various ways, possibly through direct interaction with the *cis*-acting elements. Although previous studies have suggested that Sxl binds to the polyuridine sequence, it remains to be elucidated whether other sequence features are required for the specific and efficient binding of Sxl to the *cis*-acting elements.

To examine the RNA binding specificity of Sxl protein in detail, we applied *in vitro* selection and amplification of ligand RNAs from a random sequence pool (19, 20). Sequence and *in vitro* binding analyses of the selected RNAs showed that Sxl preferentially binds to a polyuridine stretch surrounded by purine residues and that the binding may be facilitated by an AG sequence downstream of the polyuridine stretch.

MATERIALS AND METHODS

Enzymes and biochemicals

Restriction enzymes, Klenow fragment, SP6 RNA polymerase, T4 DNA ligase and RNase inhibitor were obtained from Takara Shuzo, Japan. RNA PCR kit was obtained from Perkin-Elmer

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Cetus. Sequenase version 2.0 sequencing kit was obtained from United States Biochemicals. T7 RNA polymerase was from Toyobo, Japan. Glutathione–Sepharose and the GST fusion vector (pGEX-2T) were from Pharmacia. RNase-free DNase (RQ1) was from Promega. The oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer. [α-^{32}P]GTP and [α-^{32}P]dCTP were purchased from Amersham.

Preparation of fusion protein

A *Bst*BI–*Eco*RI fragment of the female-specific Sxl F1 cDNA (5) of about 1.2 kb was blunt-ended with Klenow fragment and cloned into the blunt-ended *Bam*HI site of the GST fusion vector pGEX-2T. The resulting plasmid encoded a fusion protein (GST–Sxl) in which the full-length Sxl protein with 9 additional amino acid residues derived from the 5′ non-coding region was linked to glutathione S-transferase. The plasmid was transformed into *Escherichia coli* XL1-blue and GST–Sxl was purified from the sonicated extract by using glutathione–Sepharose according to the manufacturer's recommendations. T7–Sxl fusion protein was prepared and purified as described (9).

Preparation of a degenerate RNA pool

The oligonucleotides were as follows (20). Linear N25: 5′-TGGGCACTATTTATATCAAC(N)$_{25}$AATGTCGTTGG-TGGCCC-3′, T7Univ: 5′-CGCGGATCCTAATACGACTCAC-TATAGGGGCCACCAACGACATT-3′, RevUniv: 5′-CCCG-ACACCCGCGGATCCATGGGCACTATTTATATCAAC-3′. The DNA template for a random RNA pool was prepared essentially as described by Tsai *et al.* (20), except for the following modification. PCR was performed using 5 ng of Linear N25 as template and 1 μg each of T7Univ and RevUniv as primers (94°C, 1 min; 54°C, 1 min; 72°C, 1 min; 9 cycles; ASTEC thermal cycler PC-800). The template (about 500 ng) was transcribed with T7 RNA polymerase. The RNA transcript was treated with RNase-free DNase and extracted by phenol/chloroform followed by ethanol precipitation.

Selection and amplification of ligand RNAs

GST–Sxl (about 2 μg) was bound to 5 μl of glutathione–Sepharose beads (50% slurry), then suspended in 100 μl of RNA binding buffer (20 mM HEPES–NaOH, pH 7.9, 200 mM KCl, 5% glycerol, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml aprotinin, 0.1% Triton X-100) supplemented with 1 μg of yeast RNA and 55 units of RNase inhibitor. RNA with a random sequence (about 500 ng) was added to the suspension, gently mixed at 4°C for 1 h and washed 5 times with 400 μl of RNA binding buffer by continuous centrifugation and resuspension. After a final wash and centrifugation, GST–Sxl was eluted from the Sepharose beads by adding 50 μl of GST elution buffer (10 mM glutathione, 50 mM Tris–HCl, pH 8.0) and incubating at room temperature for 15 min. After centrifugation, the supernatant was extracted 3 times with phenol/chloroform and RNA in the aqueous layer was precipitated with ethanol. One-fifth of the recovered RNA was amplified into double-stranded cDNA by reverse transcription combined with PCR (94°C, 1 min; 54°C, 1 min; 72°C, 1 min; 9 cycles) using 1 μg each of T7Univ and RevUniv primers according to the manufacturer's recommendations. RNA for the next selection was synthesized by transcription with T7 RNA polymerase using the recovered cDNA as a template. The selection and amplification procedure was repeated twice more.

Cloning and sequencing

After the final amplification, the cDNA was digested at *Bam*HI sites in the two primers, and cloned into pUC118. Plasmid DNA was prepared as described (21). The DNA was sequenced using Sequenase version 2.0 kit (United States Biochemicals) according to the manufacturer's recommendations.

In vitro binding analysis

For *in vitro* selected clones, each plasmid DNA, whch had been linearized downstream of the RevUniv primer sequence with an appropriate restriction enzyme, was transcribed with T7 RNA polymerase in the presence of [α-^{32}P]GTP as described (9). AF130 RNA and an RNA derived from the multi-cloning site sequence of the plasmid pSP73 (Promega) were synthesized with SP6 RNA and T7 RNA polymerases, respectively, as described (7). Labeled RNA was purified by denaturing gel electrophoresis. The binding reaction mixture [containing about 0.3 μg of T7–Sxl, labeled RNA (2×10^4 c.p.m.), 1 μg of yeast RNA and 55 units of RNase inhibitor in the RNA binding buffer] was incubated at 20°C for 20 min followed by UV light irradiation and RNase digestion and then analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) as described (9). The efficiency of label transfer to T7–Sxl with each selected RNA relative to that with AF130 RNA was calculated by densitometry using a Fuji BAS 2000 Image Analyzer.

RESULTS

Affinity selection of ligand RNAs of Sxl protein

We established a system by which RNAs having affinity for Sxl protein are selected from a random RNA pool using immobilized Sxl fused with glutathione S-transferase (GST–Sxl). Generation of the random RNA pool and amplification of the selected RNAs by reverse transcription combined with polymerase chain reaction (RT–PCR) are essentially the same as described by Tsai *et al.* (20). In brief, each RNA in the random pool was designed to have a 25 consecutive nucleotide random sequence between the primer binding sites for amplification by RT–PCR. At selection, we used the affinity of the GST portion of the fusion protein for glutathione–Sepharose to recover the RNAs that were bound by GST–Sxl (Fig. 1).

To determine whether the bacterially expressed GST–Sxl retains RNA binding ability, we first performed UV crosslinking analysis using a ^{32}P-labeled RNA (AF130) containing the *tra cis*-acting element as a probe (7). As shown in Fig. 2, GST–Sxl bound to the RNA with almost the same efficiency as another Sxl fusion protein (T7–Sxl) which has RNA binding specificity for both the *tra* and *Sxl cis* elements (9). The binding of GST–Sxl to AF130 RNA was specifically inhibited by synthetic poly(U) RNA, as it is with T7–Sxl (data not shown). The GST–Sxl did not bind significantly to an unrelated RNA with the multi-cloning site sequence (data not shown). These results show that the GST–Sxl used here retained specific RNA binding ability.

Sequences of *in vitro* selected RNAs

After three cycles of selection and amplification as shown in Fig. 1, we cloned and sequenced 17 cDNAs corresponding to the RNAs selected. Most of these clones contained the consecutive poly(T) stretch in the randomized region, i.e. polyuridine in the RNA sequence (Table 1). Appearance of this stretch in the selected RNAs is consistent with previous reports that Sxl protein

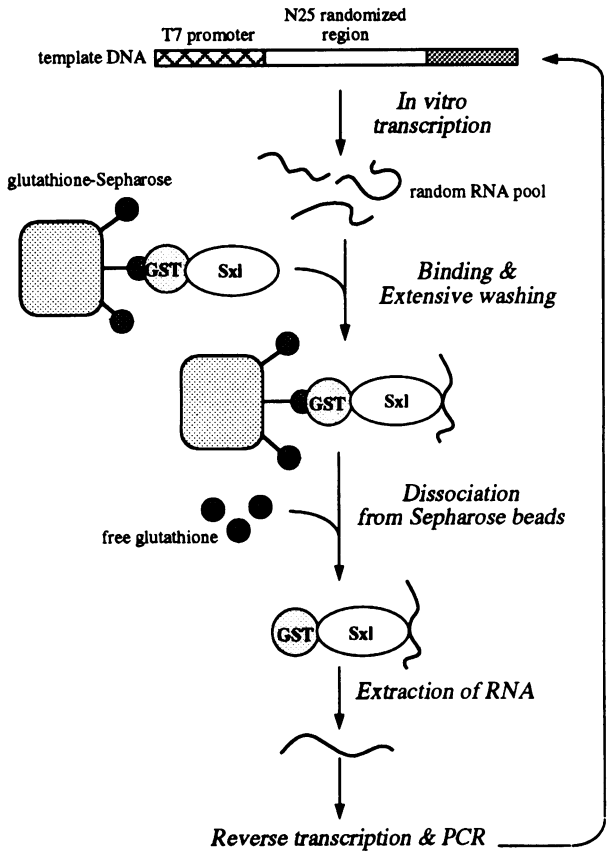

Figure 1. Schematic presentation of the selection and amplification of the ligand RNAs for Sxl using the GST fusion protein.

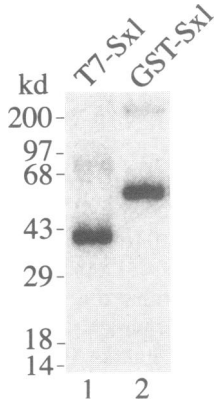

Figure 2. *In vitro* binding of T7−Sxl and GST−Sxl fusion proteins to the *tra cis*-acting element. Fusion proteins were incubated with AF130 RNA containing the *tra* element, irradiated with UV light followed by RNase digestion and electrophoresis on a 15% SDS−polyacrylamide gel. Lanes: 1, T7−Sxl; 2, GST−Sxl. The positions of molecular weight size markers (kD) are shown on the right.

binds to the *cis*-acting elements in *tra* and *Sxl* pre-mRNAs containing a polyuridine stretch (7, 9). This region in RNA sequences is frequently surrounded by purine residues, where $A(U)_nG$ is seen as a consensus. In more than half of the RNAs

Table 1. *In vitro* selected RNA sequences and their affinity for Sxl

Clone no.	Sequence	Binding efficiency (%)
1	AGAAUUUUUUUUAGUAUUUGCGCAA	105
2	auuUUUUUUUUUGAGUUUAGAGGAUAUCUUA	65
3	auuUUUUUUGCUAGUUUGUUUGGCGAUA	58
4	UAGAAUUUUUGUAGCUCGAGAACGC	4
5	CCGAGGAGAGGAUCUAAUUGUGUGU	30
6	UAUUUAAAUAGUUUAGCCGGGGUGC	16
7	auuUGGCUUAUGACAUGAUUUCUUUUg	55
8	auuUUUUUCUAAUAGAAGGGAUUAGCGG	36
9	AAUUUUUUUUUUUUUUGGAGAAAAUUCGAUAUG	79
10	auuUUUUUGAAUAAAUAAUAUAGCG	41
11	GAUCAAAUUUUUUUGUUACUUGACAA	40
12	auuUUAGAAAAUAAUUUUUUGCAUGAAC	50
13	auuUUUUGAGACAGCGGUU	10
14	GGAGCAAUGCAAGGGGGGAGUGGG	8
15	auuUUCUUUUGAg	55
16	auuUUUUGAAUAAUUUGUGGAGAAGCGG	28
17	auuUUUUUUAUGAGUUAAGGAUAAAUAUU	78

The polyuridine stretches surrounded by the purine residues are underlined. The binding efficiency of each RNA relative to that of AF130 RNA was calculated by densitometric analysis of the gels shown in Fig. 3.

containing polyuridine, the first two uridine residues were derived from the upstream primer sequence, possibly because of the high probability of generating long polyuridine stretches in the random RNA pool. Furthermore, in some clones the randomized region became shortened or extended, which was possibly caused by an unknown error during PCR amplification.

Binding of Sxl protein to *in vitro* selected RNAs

To determine whether the RNAs selected in our system are really bound by the Sxl protein, we synthesized them by *in vitro* transcription in the presence of [α-^{32}P]GTP and assayed the binding efficiency of each by UV crosslinking label transfer (Fig. 3). In this assay, we used a partially purified T7−Sxl fusion protein to precisely estimate the affinity of each RNA for Sxl protein, because our system might select RNAs with affinity for the GST portion of the GST−Sxl fusion protein. Equality in the amount of T7−Sxl in each binding reaction was confirmed by Coomassie gel staining after SDS−PAGE (data not shown). All of the selected RNAs were bound by T7−Sxl, although their binding efficiency varied significantly. The relative binding efficiencies of each RNA compared with that of AF130 RNA, which contains the native Sxl binding site of *tra* pre-mRNA (7), are shown in Table 1. Although RNAs with the relatively long polyuridine stretch tended to bind efficiently, several RNAs with the same length of polyuridine stretch widely differed in binding efficiency (Table 1, compare clones 8, 10 and 17).

Close inspection of the sequences of five RNAs selected *in vitro*, which had higher affinity for Sxl than the others (clones

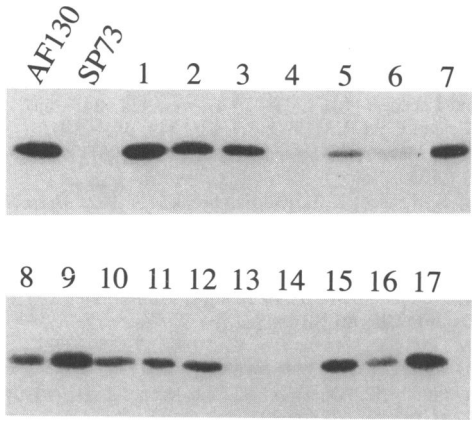

Figure 3. *In vitro* binding of T7−Sxl protein to the selected RNAs. The RNA species used is shown at the top of each lane (see also Table 1). AF130, RNA containing the *tra cis*-acting element; SP73, unrelated control RNA derived from the multi-cloning site sequence of the plasmid SP73; 1−17, RNAs derived from cDNA clones 1−17. The binding reaction and other procedures in this experiment are the same as described in the legend to Fig. 2.

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Sxl   GUAGACAUAUUUUUUUUCACAGCCCAGAAAGAAGCAGCC
tra   CUUUUUGUUGUUUUUUUUCU-AGUGUCACAUUGUGUGAAA
1        AGAAUUUUUUUU---AGUAUUUGCGCAA
9     AAUUUUUUUUUUUUUGG-AGAAAAUUCGAUAUG
17       auuUUUUUAUGAGUUAAGGAUAAAUAUU
2     auuUUUUUUUUUG--AGUUUAGAGGAUAUCUUA
3        auuUUUUUUGCUAGUUUGUUUGGCGAUA

consensus AUnNnAGU
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Figure 4. A comparison of the selected RNA sequences having higher affinity for Sxl with the sequences of the regulated acceptor region of *Sxl* and *tra* pre-mRNAs. The regions with sequence similarity are boxed. Lower case letters represent the residues derived from the upstream primer sequence used for amplification. The consensus sequence for the Sxl high-affinity binding site is shown below.

1, 9, 17, 2 and 3 in order based on binding efficiency), revealed that they have a degree of similarity to the *Sxl*- and *tra*-regulated acceptor regions (Fig. 4). All five RNAs contain the AG dinucleotide downstream of the polyuridine stretch, as do the *Sxl* and *tra* acceptor regions, having the consensus sequence AU_nN_nAGU. This observation suggested that the downstream AG dinucleotide is also important for the efficient binding of Sxl, as well as the polyuridine stretch. It may be possible that RNAs without a prominent polyuridine stretch (clones 5, 6 and 14) were selected by the weak affinity of the AG dinucleotide for Sxl, because they are characterized by having two or three copies of the AG dinucleotide, although we could not exclude the possibility that such RNAs without a polyuridine resulted from non-specific Sxl binding.

DISCUSSION

By means of *in vitro* selection and amplification, we extracted the sequences from a random RNA pool to which Sxl binds. Most of the sequences contain a polyuridine stretch surrounded by purine residues as a consensus. UV crosslinking demonstrated that all of the selected RNAs are bound by the Sxl protein, although their affinity varied widely, depending upon their sequence context. This variety may be due to the limited cycles of selection and amplification performed here. Therefore, the selected sequences should converge into the most similar one if more cycles are performed. Nevertheless, our findings on the features of the selected sequences and their affinity for Sxl uncovered several previously unknown properties of Sxl binding.

Firstly, by comparing the selected RNAs with the higher affinity for Sxl with the *Sxl*- and *tra*-regulated acceptor site regions, we identified a consensus, AU_nN_nAGU, for efficient Sxl binding, suggesting that Sxl can recognize and bind to the AG dinucleotide downstream of the polyuridine stretch. In general, the AG dinucleotide, which is invariant among the acceptor sites of essentially all pre-mRNAs, acts as an essential *cis*-acting element for splicing (22, 23). Thus, it is possible that Sxl binding to the acceptor site region of *tra* pre-mRNA masks not only the polyuridine but also the AG dinucleotide and blocks the default splicing in male flies. This notion is similar to the blockage model (6, 7), but it seems to more favorably explain the mechanism of splicing inhibition and the strict discrimination of the target RNA from other unrelated species by the Sxl protein, because of the longer sequence determinant required for efficient Sxl binding.

Secondly, Sxl bound polyuridine containing less than 8 consecutive U residues, such as U_7, U_6, U_3CU_4 or U_4CU_4, although the binding efficiency was relatively medium or low compared with that of polyuridine containing more consecutive U residues. This observation is suggestive for considering the mechanism underlying the regulation of *Sxl* pre-mRNA splicing. The multiple *cis*-acting elements primarily responsible for Sxl autoregulation are located within the introns surrounding the male-specific exon of *Sxl* pre-mRNA (9, 11). The *cis*-acting elements contain polyuridine with various lengths of consecutive U residues. Even if the affinity of individual polyuridines in these *cis*-acting elements for Sxl is relatively low, multiplication of the weak sites may enhance Sxl binding to them. Of course, a prerequisite is needed for this idea that multiple Sxl molecules on the same pre-mRNA interact . We reported that Sxl seems to form a homodimer *in vitro* (9). In addition, our preliminary data from the yeast two-hybrid analysis suggested that the Sxl protein forms a homodimer and a heterodimer with U2AF, which is an essential splicing factor (H.Sakamoto, unpublished data). Thus, interactions between multiple Sxl molecules binding to the distant sites in introns and between Sxl and other splicing factor(s) may induce a conformational change of *Sxl* pre-mRNA and lead to female-specific exon skipping, as occurs in the artificial exon skipping system (24). In addition, multiple binding of Sxl to weak sites may control other aspects of gene expression, including the translational regulation that has been speculated with the *Drosophila msl-1* gene, because many polyuridine stretches are dispersed within the 3′ untranslated region of its mRNAs (18).

Thirdly, Sxl RNA binding specificity may be a composite of two separable entities: one for the polyuridine stretch and the other for the AG dinucleotide, because we obtained RNAs without polyuridine and those without the downstream AG dinucleotide in our ligand selection system. It is likely that these two kinds of binding are attributed to two distinct Sxl RRMs, as with U1A. This protein has two distinct RRMs, one of which is responsible for binding to U1 snRNA and the other for the specific sequence located in the 3′ untranslated region (20, 25,

26). However, the overall RNA binding specificity of hnRNP A1 is not a simple sum of the specificity of its two distinct RRMs (27). The high-affinity binding sequence of hnRNP A1 resembles the vertebrate splice acceptor consensus and contains the AG dinucleotide, like Sxl (27). Also, as speculated, there is a functional similarity between hnRNP A1 and Sxl, in that both proteins cause selection of the distal splice site in splicing (9). In addition, another splicing regulator, SF2/ASF, can antagonize hnRNP A1 and bind to the purine-rich sequence termed ERS or ESE, which also contains AG dinucleotide (28–30). Recognition of the AG dinucleotide may be a general feature for a group of splicing regulators that contain RRMs.

In conclusion, we established an *in vitro* selection and amplification system using the affinity of GST fusion protein for glutathione–Sepharose. Our system is analogous to those reported previously (20, 27), but more versatile in that it does not require a specific antibody against a given protein of interest at selection. We suggest that the AG dinucleotide is important for the efficient and specific binding of Sxl, as well as the polyuridine stretch. A more detailed examination is required to determine the importance of the AG dinucleotide for Sxl binding.

ACKNOWLEDGEMENTS

We thank Dr Hideaki Shiraishi for valuable experimental advice. This work was supported in part by research grants from the Ministry of Education, Science and Culture of Japan, Kato Memorial Bioscience Foundation, and Hyogo Science and Technology Association.

NOTE ADDED

Recently, Samuels *et al.* (31) have also shown that the Sxl protein binds preferentially to the polyuridine sequence *in vitro* and that the binding is enhanced by the presence of an adenosine residue at the 5′ end of the polyuridine. However, in contrast to our results, theirs suggested that Sxl does not recognize the AG dinucleotide sequence downstream of the polyuridine.

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