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RNA Binding by Sxl Proteins In Vitro and In Vivo

MARK E. SAMUELS,[1]* DANIEL BOPP,[1]† RICHARD A. COLVIN,[2] ROBERT F. ROSCIGNO,[2] MARIANO A. GARCIA-BLANCO,[2] AND PAUL SCHEDL[1]

Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544,[1] and Section of Cell Growth, Department of Microbiology, Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710[2]

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Sxl has been proposed to regulate splicing of specific target genes by directly interacting with their pre-mRNAs. We have therefore examined the RNA-binding properties of Sxl protein in vitro and in vivo. Gel shift and UV cross-linking assays with a purified recombinant MBP-Sxl fusion protein demonstrated preferential binding to RNAs containing poly(U) tracts, and the protein footprinted over the poly(U) region. The protein did not appear to recognize either branch point or AG dinucleotide sequences, but an adenosine residue at the 5′ end of the poly(U) tract enhanced binding severalfold. MBP-Sxl formed two shifted complexes on a *tra* regulated acceptor site RNA; the doubly shifted form may have been stabilized by protein-protein interactions. Consistent with its proposed role in pre-mRNA processing, in nuclear extracts Sxl was found in large ribonucleoprotein (RNP) complexes which sedimented significantly faster than bulk heterogeneous nuclear RNP and small nuclear RNPs. Anti-Sxl staining of polytene chromosomes showed Sxl protein at a number of chromosomal locations, among which was the *Sxl* locus itself. Sxl protein could also be targeted to a new chromosomal site carrying a transgene containing splicing regulatory sequences from the *Sxl* gene, following transcriptional induction. After prolonged heat shock, all Sxl protein was restricted to the heat-induced puff at the hs93D locus. In contrast, a presumptive small nuclear RNP protein was observed at several heat shock puffs following shock.

The *Sex-lethal* (*Sxl*) gene in *Drosophila melanogaster* governs sexual development by controlling pre-mRNA splicing of specific genes (9, 12, 16, 41) (for reviews, see references 2, 3, 6, 27, 39, and 63). The precise alternative splicing events for regulation of the downstream target gene *transformer* (*tra*) and for the autoregulation of the *Sxl* gene itself are known. The regulated *tra* splice utilizes a common donor site and one of two acceptor sites. The proximal acceptor site is used by default in males and does not require *Sxl* activity (9, 12, 40, 44). *Sxl* functions in females to prevent the use of this site, leading to alternative splicing to the distal acceptor (62). In contrast to *tra* regulation, *Sxl* regulation involves the inclusion of a complete exon in males and its exclusion in females (4, 5, 10, 34, 56). Both the *tra* and *Sxl* alternative splices have been reconstructed in *Drosophila* tissue culture cells in cotransfection assays with an expressed *Sxl* cDNA (32, 53).

Despite the detailed differences between *Sxl* and *tra* splicing regulation, attention has focused on the homologous acceptor sites, which in both genes contain a conserved U_8 as part of the polypyrimidine tract. Mutation of this poly(U) tract in the *tra* gene was shown to abrogate *Sxl* regulation in vivo (32, 53). This acceptor site is also highly conserved in the *tra* gene from *Drosophila virilis* (45). These results suggested that Sxl gene products might bind to the regulated *tra* acceptor site, thereby preventing its use by the general splicing machinery. More specifically, Sxl binding might interfere with the action of general splicing factor U2AF, since U2AF has been hypothesized to interact with acceptor site polypyrimidine tracts as a prerequisite to small nuclear ribonucleoprotein (snRNP) U2 binding (69). Sxl protein was shown by UV cross-linking to bind to *tra* RNA containing this acceptor site (32). Subsequently, Valcárcel et al. showed elegantly that Sxl protein could compete with U2AF for binding to a poly(U)-rich pyrimidine tract and could also inhibit splicing in vitro (66). The poly(U) tract at the *Sxl* male exon acceptor site has similarly been shown to be capable of mediating splicing regulation in vivo. Thus, when the male exon was placed in a heterologous context, regulation in females was observed and like *tra* regulation was dependent on the poly(U) tract at the acceptor site (29).

These results notwithstanding, *Sxl* autoregulation appears to be more complicated than *tra* regulation. In experiments employing larger regions of genomic *Sxl* sequence, including the true *Sxl* flanking exon splice junctions, the male exon acceptor site poly(U) tract appeared largely dispensable for splicing regulation. Instead, surrounding intron sequences seemed to be critical for proper male exon skipping in females (30, 53). Multiple poly(U) tracts exist in these introns and may serve as Sxl binding sites. Thus, the same primary RNA sequence may be involved in both *Sxl* and *tra* regulation, but direct steric competition between Sxl and U2AF at the acceptor site may not be a universal aspect of Sxl's regulatory activity. The presence of additional potential Sxl protein-binding sites around the regulated *Sxl* splice may explain why *tra* regulation is normally incomplete, whereas *Sxl* autoregulation is extremely tight, such that no male RNA is detected in wild-type female flies (5, 8).

To better understand splicing regulation by Sxl, we have studied the biochemical properties of Sxl protein in greater detail. Using a purified recombinant Sxl fusion protein, we have examined the dependence of RNA binding on the length of the poly(U) tract and on the presence of additional splice acceptor site sequences. We have also begun to address the issue of protein cooperativity and its possible role in regulating splicing. We show that Sxl protein can be targeted to specific polytene chromosome sites containing regulatory splicing signals, and we identify Sxl-containing RNP complexes in embry-

* Corresponding author. Phone: 609-258-6736. Fax: 608-258-5323.
† Present address: Zoologisches Institut der Universität Zürich, CH-8057 Zürich, Switzerland.

onic nuclear extracts which appear to be qualitatively different from general heterogeneous nuclear RNPs (hnRNPs).

MATERIALS AND METHODS

Purification of MBP-Sxl fusion protein. The Sxl open reading frame from cDNA MS3 (58) was amplified by PCR and cloned into the *Stu*I site of the *malE* gene (encoding MBP, maltose-binding protein) (25) in pMAL-c (New England Biolabs), juxtapositing the presumptive Sxl initiating methionine immediately adjacent to the factor Xa cleavage site (43).

JM101 cells harboring the malE-Sxl plasmid (pDH13) were grown at 37°C. Exponential-phase cultures were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h, harvested, washed, and frozen at −70°C as cell pellets.

A standard preparation began with 2-liter cultures of JM101(pDH13). Cells were thawed in 50 ml of 50 mM Tris-HCl (pH 7.9)–0.5 mM EDTA–0.5 mM dithiothreitol (DTT)–1 mM phenylmethylsulfonyl fluoride–1 mM benzamidine–1 μg of leupeptin per ml–1 μg of antipain per ml–0.1% Nonidet P-40 (NP-40)–0.2 mg of lysozyme per ml. After 15 min on ice, cells were lysed by two rounds of freeze-thaw. Chromatin was sheared by sonication, and the cell lysate was clarified by centrifugation at 14,000 × *g* for 20 min. At this stage, the bulk of fusion protein was soluble.

The clarified lysate was applied to an 80-ml DEAE-Sephacel (Pharmacia) column equilibrated in buffer C+50 (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-NaOH [pH 7.9], 15% glycerol, 0.1 mM EDTA, 0.5 mM DTT, 0.1% NP-40, 50 mM NaCl). The column was washed with C+50, and the fusion protein was eluted with a salt gradient of 50 to 600 mM NaCl in buffer C. Fractions containing the fusion protein were pooled and applied to a 6-ml poly(U)-agarose (Pharmacia) column equilibrated in buffer C+500 (same as buffer C+50 but with 500 mM NaCl). The column was successively washed with 10 volumes each of buffer C+500 and buffer C+1 M NaCl containing 1 M urea, and the fusion protein was eluted with buffer C+1 M NaCl containing 2 M urea. Proteolytic fragments coeluting from the poly(U)-agarose column were removed by chromatography on Sephacryl S-200 (150-ml column; Pharmacia) equilibrated in buffer C+50. Finally, gel-filtered protein from several preparations was pooled and concentrated by rechromatography on poly(U)-agarose. The MBP-Sxl fusion was finally dialyzed against buffer C+40 and frozen in aliquots at −70°C, a temperature at which it remained stable for at least a year.

The concentration of the MBP-Sxl fusion preparation was determined by measuring A_{280} after dilution into 6 M guanidine-HCl–20 mM HEPES-NaOH, pH 7.9, by using an extinction coefficient (ε) of 85,440 calculated from the amino acid sequence (22). The measured concentration of the fusion protein by this method was 1.1 mg/ml, for a final yield of 10 mg of purified protein from 4 liters of bacterial cultures. An aliquot was also analyzed by acid hydrolysis and amino acid derivatization, giving a value of 0.6 to 0.9 mg/ml.

The concentration of active fusion protein was independently determined by saturation RNA binding by a gel shift assay (see below). This method gave a concentration of active protein of 0.8 mg/ml, in good agreement with the physical values.

Unfused native Sxl protein was expressed by cloning the MS3 open reading frame into the pT7-7 vector (64) and growing it in strain BL21(DE3). pT7-7 was a generous gift from S. Tabor. Cells were induced with IPTG and lysed as described above for the MBP-Sxl fusion. Sxl protein was precipitated by gradual addition of solid NaCl to 0.5 M and redissolved in buffer B containing 10 mM NaCl (buffer B was similar to buffer C but lacked NP-40). Sxl was purified by chromatography on DEAE-Sephacel and poly(U)-agarose as described above, except that buffer B was substituted for buffer C. During chromatography and the final dialysis, a minimum of 0.5 M urea was included to maintain solubility of the unfused protein. The concentration of the Sxl preparation was determined by measuring A_{280} after dilution into guanidine as described above, using an ε of 20,720.

Cleavage of the MBP-Sxl fusion protein. An aliquot of the fusion protein (8 μg) was adjusted to 0.15 M NaCl–2 mM $CaCl_2$ and incubated with 0.2 U of factor Xa protease (New England Biolabs) for 1 h at room temperature. The cleavage products were used immediately for RNA-binding reactions, or else the reaction was stopped with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer.

Band shift analysis. Templates for transcription of RNA probes were derived by mutagenesis of the PIP4 WT splicing construct as described by Roscigno et al. (51). In the present work, the PIP4 variants have been renamed according to the lengths of U tracts at the splice acceptor site. For concordance with the original plasmid names, compare the sequences given in Table 1 with those in Table 1 of the paper by Roscigno et al. (51). ΔBP in the present work corresponds to variant 62B in reference 51. TraWT and TraMut were also provided by R. F. Roscigno. ΔAG was constructed in the present work by mutagenesis of PIP4 WT.

Template DNAs were linearized with *Hin*dIII and transcribed with T7 RNA polymerase (U.S. Biochemicals, Promega, or Pharmacia) by using [α-^{32}P]UTP (ICN) at 10 to 20 Ci/mmol for standard reactions, 1 Ci/mmol for RNA saturation experiments, or in trace amounts for quantitation of synthesis of cold competitor RNAs.

The MBP-Sxl fusion protein was diluted to various concentrations with 10 mM HEPES-NaOH (pH 7.9)–10% glycerol–0.1 mM EDTA–0.5 mM DTT–0.1% NP-40. RNA-binding reactions were carried out in 10-μl volumes containing 1 μl of diluted fusion protein; RNA probe at 10^{-11} to 10^{-10} M; 10 mM HEPES-NaOH, pH 7.9; 10% glycerol; 0.1 mM EDTA; 0.5 mM DTT; and 0.12 M NaCl. Competitor RNAs were added prior to protein addition, as noted in the figure legends. Probe and competitor RNAs were preheated for 5 min at 65°C and quenched on ice to remove secondary structures; this step was essential for native gel electrophoresis. After 10-min binding incubations at room temperature, 5-μl aliquots were loaded onto 1.5-mm-thick, 0.5× Tris-borate-EDTA (TBE)–6% (80:1) polyacrylamide nondenaturing gels that had been equilibrated overnight in a cold room and preelectrophoresed for 20 to 30 min at 100 V. For some experiments, 4% (39:1) polyacrylamide gels were used. Typically, samples were applied while the gels were being electrophoresed at 200 V, with electrophoresis continuing for 60 to 90 min after loading. Gels were dried and autoradiographed or quantitated by phosphoimager scanning (Molecular Dynamics). Curve fitting of the quantified data was carried out by using SigmaPlot (Jandel Scientific). Note that for some RNAs tested, the probe concentration was in the range of the fitted dissociation constant (K_d); thus, the protein concentration giving 50% binding was not directly equivalent to the K_d.

To control for nonequilibrium effects during loading, a set of binding reaction mixtures were loaded onto gels without voltage prior to a 10-min incubation in the well and then standard electrophoresis. No difference in the amount of shifted RNA was seen when these reaction mixtures were compared with reaction mixtures loaded while the gel was

being electrophoresed (data not shown). In some experiments, there was clearly loss of shifted products during electrophoresis (observed as smears of radioactive material migrating between the bound and free RNA bands). Therefore, free RNA bands were regularly used for quantitation (14). As noted in Results, numerous experiments with the PIP4 SxlWT RNA gave fairly similar results over a range of RNA probe concentrations. However, the nonspecific aggregation of all RNAs, observed at protein concentrations above about 15 nM, set an upper limit on measurable K_ds with the simple band shift assay.

UV cross-linking. RNA binding reactions were performed as described above, with NaCl replacing KCl to avoid precipitation of SDS. Proteins were cross-linked to RNA for 5 min on ice in a Stratalinker (Stratagene). RNA was digested for 20 min at room temperature with 0.5 mg of RNase A per ml, 2× SDS-PAGE sample buffer was added, and samples were boiled and loaded on SDS–10% polyacrylamide gels. The synthetic polymers poly(U), poly(C · U), and poly(A · C · U) used in competition experiments were from Sigma.

CMCT footprint. Nonradioactive Sxl and Tra RNAs were transcribed in vitro with T7 RNA polymerase. RNA was incubated with MBP-Sxl fusion or unfused Sxl protein for 5 min at room temperature in 0.1 M HEPES-KOH (pH 7.9)–1 mM DTT–0.1% NP-40. Following incubation, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-methyl-*p*-toluenesulfonate (CMCT; Sigma) was added to a final concentration of 21 mg/ml in a 20-μl final volume. CMCT reacts with position N-1 of guanine and position N-3 of uracil. Reaction mixtures were incubated at 30°C for 12 min. Reaction mixtures were then extracted once with phenol-chloroform and once with chloroform, and then they were ethanol precipitated (15). Modifications were detected by primer extension with murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) as described elsewhere (42). The labelled cDNAs were separated in a 15% denaturing polyacrylamide sequencing gel.

Nuclear extracts and RNP analysis. Oregon R wild-type flies were grown at 25°C in population cages. Nuclei from 3- to 16-h embryos were prepared essentially as described by Soeller et al. (61). Nuclei were washed with TEN (10 mM Tris-HCl [pH 7.9], 1.5 mM EDTA, 100 mM NaCl) and resuspended in TMND (10 mM Tris-HCl [pH 7.9], 1.5 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mM $Na_2S_2O_5$, 1 μg of leupeptin per ml, 1 μg of antipain per ml). The nuclei were lysed by sonication, clarified, and sedimented through 10 to 45% sucrose gradients as described by Risau et al. (49). Nuclei from 2 g of embryos were sonicated in 2.5 ml of TMND, and 0.5 ml of clarified lysate was applied to each 9-ml gradient. Additional treatments of clarified extracts prior to sedimentation are as described in the figure legends. Gradients were centrifuged at 20,000 rpm in a Beckman SW41 rotor for 12 to 16 h. Fractions (1 ml) were collected, and protein was concentrated by precipitation with sodium deoxycholate and trichloroacetic acid and assayed by Western blot (immunoblot) of SDS–10% polyacrylamide gels with anti-Sxl monoclonal antibody m114 (10), a mixture of antibodies 8D2 (anti-hrp40) and 10D5 (anti-hrp48) (38), or anti-U2 B″ antibody 4G3 (26). 8D2 and 10D5 were a kind gift of Michael Matunis and Gideon Dreyfuss, and 4G3 was from the laboratory of Walther van Venrooij. Proteins on blots were visualized by reaction with a biotinylated anti-mouse secondary antibody (Vector Laboratories), streptavidin-horseradish peroxidase, and ECL reaction mix (Amersham).

Polytene chromosome staining. Polytene chromosomes were stained as described elsewhere (1), with either anti-Sxl monoclonal antibody m104 (10) or antibody 4G3. Antibody was visualized either with biotinylated anti-mouse antibody followed by streptavidin-horseradish peroxidase (Vector Laboratories) or by immunofluorescence, using rhodamine-conjugated anti-mouse antibody (Immunojackson Laboratories). Where noted, heat shock assays were performed by submerging larvae in sealed microcentrifuge tubes at 37°C.

Flies used for staining included wild-type Oregon R, or else females homozygous for *Sxl* deficiency 7B0 (55) (the distal 7B0 breakpoint has been mapped to the 3′ end of the *Sxl* transcription unit [56, 57]), carrying Dp(2)Sxl^+ for viability. Also tested were transgenic flies carrying either CH13A or hs83-*lacZ*. CH13A is a P element containing *Sxl* genomic DNA from the 5′ end of early exon 1 through the middle of exon 5, fused to *lacZ* and driven by the hs83 promoter, inserted at polytene region 11A (28). The hs83-*lacZ* construct has the *lacZ* gene driven by the hs83 promoter without *Sxl* sequences and is inserted at polytene site 56D (24).

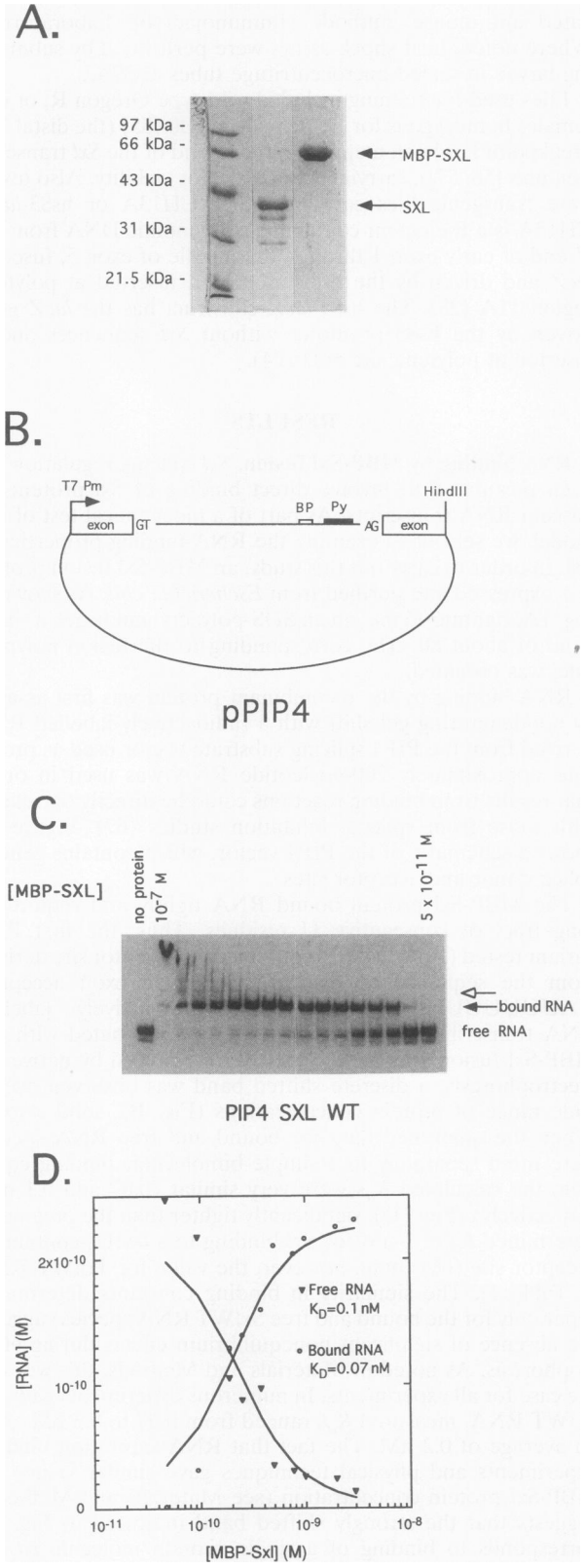
RESULTS

RNA binding by MBP-Sxl fusion. *Sxl* splicing regulation has been postulated to involve direct binding of Sxl proteins to nascent RNA transcripts. As part of a biochemical test of this model, we set out to examine the RNA-binding properties of Sxl. In order to carry out this study, an MBP-Sxl fusion protein was expressed and purified from *Escherichia coli*. As shown in Fig. 1A, rightmost lane, on an SDS-polyacrylamide gel, a single band of about 80 kDa, corresponding to the fusion polypeptide, was obtained.

RNA binding by the recombinant protein was first assayed by nondenaturing gel shift with a radioactively labelled RNA derived from the PIP4 splicing substrate vector used as probe. This approximately 200-nucleotide RNA was used in order that results from binding reactions could be directly correlated with those from splicing inhibition studies (67). Figure 1B shows a schematic of the PIP4 vector, which contains generic splice donor and acceptor sites.

The MBP-Sxl protein bound RNA tightly and required a long tract of consecutive U residues. Thus, the first PIP4 variant tested (PIP4 SxlWT) contained an acceptor site derived from the sequence of the *Sxl* gene male exon acceptor, UAUUUUUUUUCACAG. When radioactively labelled RNA transcribed from PIP4 SxlWT was incubated with the MBP-Sxl fusion and the products were resolved by native gel electrophoresis, a discrete shifted band was observed over a wide range of protein concentrations (Fig. 1C, solid arrow). When the quantified data for bound and free RNA species were fitted separately to a simple bimolecular binding equation, the calculated K_ds were very similar (0.07 and 0.1 nM, respectively) (Fig. 1D), significantly tighter than the previously determined K_d of 1 nM for Sxl binding to a *tra* U_8-containing acceptor site (66) (note, however, the value for TraWT RNA in Table 1). The similarity in binding constants determined separately for the bound and free SxlWT RNA species suggests the absence of significant nonequilibrium effects during electrophoresis. As noted in Materials and Methods, this was not the case for all experiments. In numerous experiments with the SxlWT RNA, measured K_ds ranged from 0.07 to 0.3 nM, with an average of 0.2 nM. The fact that RNA saturation binding experiments and physical techniques gave similar values for MBP-Sxl protein concentration (see Materials and Methods) suggests that the strongly shifted band indicated in Fig. 1C corresponds to binding of a single protein molecule to the RNA. However, this stoichiometry has not been rigorously proved.

When the U_8 tract was disrupted, specific MBP-Sxl binding



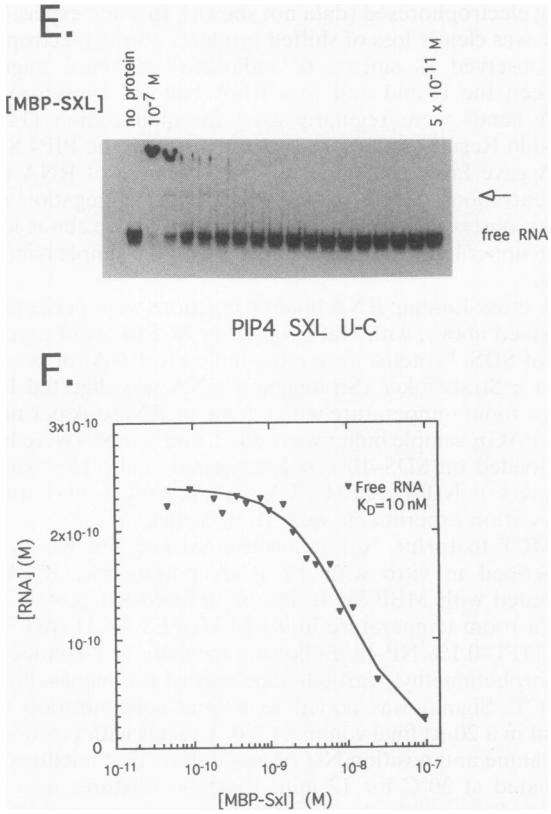


FIG. 1. Band shifts with MBP-Sxl fusion protein. (A) SDS-polyacrylamide gel of purified MBP-Sxl fusion protein and unfused native Sxl, prepared as described in Materials and Methods, stained with Coomassie blue. (B) Schematic of PIP4 plasmid vector used as a template for RNA probe synthesis. The distance from the T7 transcription initiation site to the *Hin*dIII runoff site is approximately 200 bp. Sequence variations at the splice acceptor site, including changes to the branch point, polypyrimidine tract, and AG dinucleotide, are as described in the text and in Table 1. Other than poly(U) tracts introduced at the acceptor site, there are no more than three consecutive uridine residues anywhere in PIP4 transcripts. (C) Titration of MBP-Sxl with PIP4 SxlWT RNA. Serial dilutions of MBP-Sxl protein (see panel D for actual concentrations) were incubated with 0.23 nM SxlWT RNA (see text for polypyrimidine tract sequence), and the products were resolved by native gel electrophoresis as described in Materials and Methods. (D) Quantitative analysis of SxlWT RNA gel shift data. Bound and free RNA bands from the gel shown in panel C were quantitated, and the data were fitted to a bimolecular binding equation. K_ds were determined independently for the bound and free RNAs. Data for the higher protein concentrations were omitted from the curve fit because of aggregation effects that artificially reduced the apparent amount of bound complex. (E) Titration of MBP-Sxl with PIP4 SxlMut RNA. Gel shift experiments were performed as described above, with serially diluted MBP-Sxl (concentrations given in panel F) and 0.24 nM SxlMut RNA (SXL U-C) (see text for sequence). (F) Quantitative analysis of SxlMut RNA gel shift data. The free RNA band from the gel shown in panel E was quantitated, and the data were fitted to a bimolecular binding equation.

was essentially eliminated. As shown in Fig. 1E, a gel shift titration series with RNA from PIP4 SxlMut (acceptor site sequence UAUUCUCUCUCACAG) showed absolutely no shifted product corresponding to the major SxlWT bound form. However, at high protein concentrations small amounts of a higher shifted form were observed (Fig. 1E, open arrow). On the basis of the mobility of this shifted form, we believe it

TABLE 1. K_ds of PIP4 RNAs

Variant	Acceptor Site Sequence	Apparent K_D[a]	Shifted band[b]
SxlWT	UACUUAU.....CCCUAUUUUUUUUCACAG	0.2	+++
SxlMut	CCCUAUUCUCUCUCACAG[c]	>10[d]	- -
PIP4 WT	CCCUUUUUUUUCCACAG	2-3	++
ΔBP	UGCUGGC.....CCCUUUUUUUUCCACAG	0.8	++
ΔAG	CCCUUUUUUUUCCACAC	4	++
U_7	CCCCUUUUUUUCCACAG	4	+
U_6	CCCUUUUUUCUCCACAG	4	+
U_5	CCCCUUUUUCUCCACAG	5-6	+/-
U_4	CCCCUUCUUUUCCACAG	5-6	-/+
U_3	CCCUUUCUUCUCCACAG	>15[d]	- -
U_0[e]	CCCCCCCCCCCCCACAG	n.d.	- -
U_7(5'A)	CCCAUUUUUUUCCACAG	0.8	++
U_6(3'A)	CCCUUUUUUAUCCACAG	n.d.	+
TraWT	CCCUCUUUUUGUUGUUUUUUUUCUAG	0.8[f]	+++
TraMut	CCCUCUUUUUGUUGUUCUCUCUCUAG	5-6	+/-

[a] Determined from quantitation of free RNA bands in native gel shift assays using MBP-Sxl serially diluted as shown in Fig. 2. Values are in units of 10^{-9} M.

[b] Estimated qualitatively with PIP4 WT RNA shifts run on each gel to control for gel variability. Compare Fig. 1C, 1E, and 2 for examples of +++, −−, and +, respectively.

[c] All variants except ΔBP are the same as SxlWT at the branch point.

[d] Minimum estimate, not considering effects of protein aggregation (see text).

[e] There is one additional U_3 tract elsewhere in the PIP4 transcript.

[f] Based on release of free RNA, assuming bimolecular binding with one high-affinity site (an approximation made for comparison with other PIP4 variants). The value is similar to that determined by Valcárcel et al. (66).

corresponds to binding of two or more fusion protein molecules to the RNA. Small amounts of this higher shifted form were also observed at high protein concentrations with the SxlWT RNA (Fig. 1C, open arrow), as well as with all other PIP4 RNAs tested (see below). At the highest protein concentrations tested (>10 nM range), the input RNA was observed to aggregate at the top of the gel with all probes tested.

The K_d for the SxlMut probe was calculated from the free RNA band to be 10 nM (Fig. 1F), compared with 0.2 nM for SxlWT. Since the SxlMut RNA titration included the range in which aggregation of RNA took place, the calculated K_d for SxlMut is underestimated. The specificity ratio for the fusion protein is thus a minimum of 50-fold.

Unfused recombinant Sxl protein was also made in bacteria (Fig. 1A). Like the MBP-Sxl fusion, unfused Sxl protein generated a strongly shifted complex with the SxlWT RNA but not with the SxlMut RNA (data not shown). However, the unfused protein proved difficult to work with routinely in gel shift experiments, in part because of its greater tendency to aggregate.

UV cross-linking of Sxl protein to RNA. In order to verify that the RNA-binding activity of the MBP-Sxl fusion protein did indeed reside in the Sxl moiety, free Sxl polypeptide was released from the fusion by proteolytic cleavage with Factor Xa. Cleavage produced two major bands as expected, a 42-kDa MBP fragment and a smaller 38-kDa Sxl fragment, together with some minor Sxl breakdown products (data not shown). The Factor Xa cleavage products were assayed for specific RNA binding by UV cross-linking (see Materials and Methods). The released Sxl polypeptide efficiently cross-linked to a PIP4 SxlWT RNA probe, whereas cross-linking to the released MBP fragment was not observed (data not shown). No cross-linking to a PIP4 SxlMut RNA probe was observed. Cross-linking of released Sxl to the SxlWT RNA probe was efficiently inhibited by competition with nonradioactive SxlWT RNA but not with cold SxlMut RNA. Synthetic poly(U) competed

extremely efficiently, whereas poly(A · C · U) had no effect on the cross-linking. Poly(C · U) inhibited SxlWT cross-linking weakly. Similar results were also obtained with the uncleaved MBP-Sxl and the unfused Sxl proteins (data not shown). Together, these results confirmed that RNA binding of the fusion protein was mediated via Sxl, and that there was a strong preference for poly(U).

RNA binding by Sxl requires a minimal poly(U) tract. The sequence requirements for RNA binding by the MBP-Sxl fusion were next examined in greater detail. Besides the affinity for a poly(U) tract, it seemed possible that Sxl might contain additional sequence recognition properties. In particular, the acceptor site branch point and AG dinucleotide were considered good candidates for recognition targets. Binding to either of these sequences in concert with a poly(U)-containing pyrimidine tract would greatly increase Sxl's specificity for splice junctions. In addition to testing for binding to these splice site elements, we also determined the minimal length of polyuridylate required for efficient Sxl binding.

To address these questions, gel shift titrations were performed by using MBP-Sxl with a number of PIP4 variant RNA probes. These mutants were available as part of a series generated from PIP4 WT to assay polypyrimidine tract requirements for pre-mRNA splicing (51). Because many of the variants tested failed to generate discrete high-affinity shifted products, for standardization in these experiments, the free RNA band was quantitated (see Materials and Methods). Failure to yield a shifted band was in no case artifactually due to protein aggregation, as protein dilutions were extended through the range at which aggregation was not observed (see Fig. 2). The measured K_ds for these variants, together with their sequences, are shown in Table 1. It was essential for analysis of the variants that PIP4 WT RNA itself was tightly bound by the fusion protein (K_d = 2 to 3 nM). This was not surprising, as PIP4 WT coincidentally contains a U_8 tract derived from an adenovirus splice site.

Decreasing the number of consecutive U residues at the polypyrimidine tract from 8 to 0 reduced affinity from 2 to 3 nM to the limit of 15 nM (Table 1; compare constructs PIP4 WT and U_7 to U_0). As noted in Materials and Methods, the K_ds determined from the free RNAs were subject to certain limitations. Quantitation of the free RNA species tended to underestimate the differences among weakly binding substrates, probably because of variability between gels and nonequilibrium effects during electrophoresis, as well as protein aggregation at higher concentrations. Therefore, Table 1 also indicates semiquantitatively the amount of singly shifted RNA observed with each substrate (column labelled Shifted band). Note that U_7 and U_6, whose free RNA measurements suggested K_ds only slightly different from those of PIP4 WT, actually gave much less of the shifted band. Moreover, as shown in Fig. 2, U_7 itself gave significantly more bound RNA than U_6.

Mutations at the branch point (ΔBP) and the acceptor site AG dinucleotide (ΔAG) had little effect on RNA binding relative to that of the PIP4 WT RNA (although the K_ds of these RNAs differed slightly from that of PIP4 WT, ΔBP and ΔAG showed amounts of shifted band which were almost identical to those of PIP4 WT, as noted in Table 1). These results appear to rule out additional splice acceptor site recognition activity by Sxl.

A completely unpredicted observation was the effect of an A residue immediately 5′ to the poly(U) tract, which increased affinity. This was first noted in comparing the binding of SxlWT and PIP4 WT: both of these RNAs contain a U_8 tract, yet SxlWT bound significantly more tightly to the MBP-Sxl protein

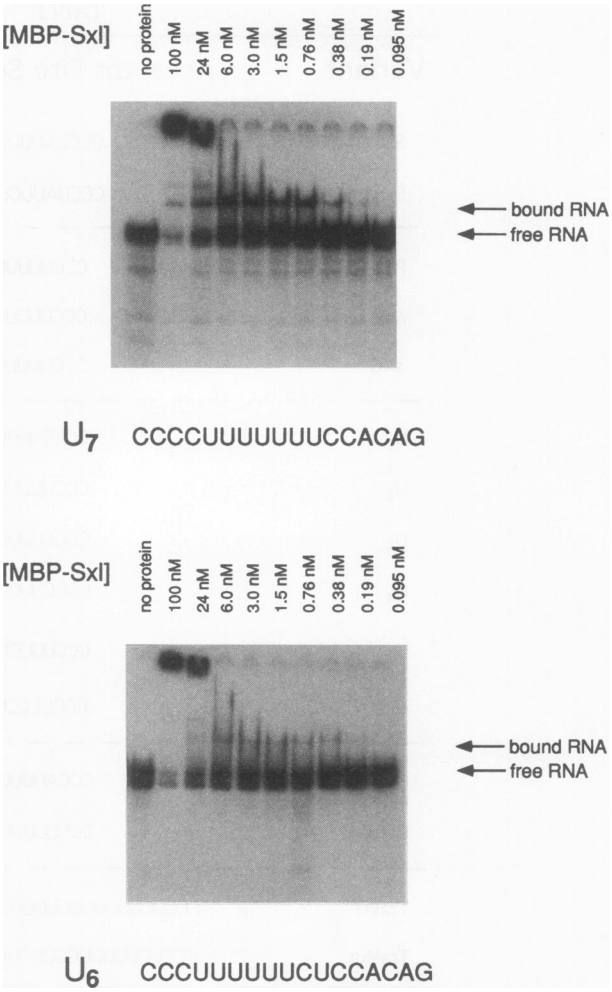


FIG. 2. Gel shifts with U_7 and U_6 RNAs. MBP-Sxl at the indicated concentrations was incubated with 0.14 nM U_7 or 0.2 nM U_6 RNA probe, and the products were resolved by native gel electrophoresis.

than did PIP4 WT (0.2 versus 2 to 3 nM). That this difference in binding involved the upstream A residue was supported by comparing the variants U_7(5′A) and U_7. Not only did the U_7(5′A) variant bind more tightly than U_7 (0.8 versus 4 nM) and yield more of the shifted band, but U_7(5′A) consistently bound better than PIP4 WT itself, even though the mutant contained a shorter stretch of only seven consecutive U residues. In contrast, U_6(3′A) bound similarly to U_6. This suggests that the position of the A residue relative to the poly(U) tract is important for enhancement of binding.

As mentioned above, doubly shifted RNA bands as well as larger protein-RNA aggregates were observed at high protein concentrations with all the probes tested. These apparently represent nonspecific complexes (but see Discussion).

Because the estimates of absolute affinity for the weakly binding RNAs were complicated by the effects noted above, competition experiments were also performed to provide a qualitative ordering of affinities. Gel shifts were done with PIP4 WT RNA as the probe and with various nonradioactive competitor RNAs. As seen in Fig. 3, the competitors titrated in a series consistent with their affinities as previously measured. Thus, SxlWT competed most efficiently, followed by U_7(5′A). ΔBP and ΔAG competed similarly to PIP4 WT, while U_7

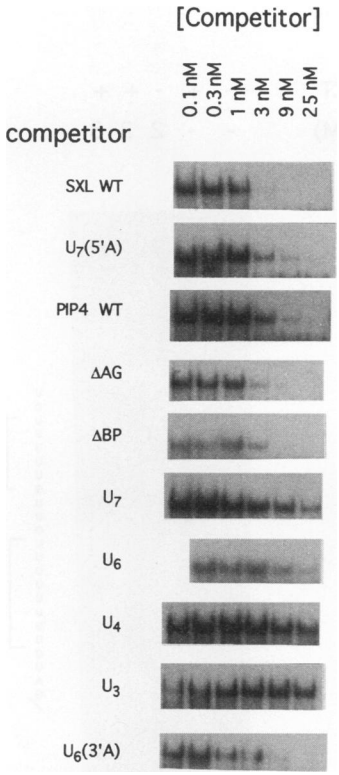


FIG. 3. Native gel shift competitions. Nonradioactive competitor RNAs were included at the indicated concentrations in binding reaction mixtures containing 1.5 nM MBP-Sxl protein and 0.2 nM radioactive PIP4 WT RNA probe. At this concentration of MBP-Sxl, aggregation effects were minimal (cf. Fig 2, 1.5 nM lanes). The products were resolved on nondenaturing gels. Only the bound PIP4 RNA band is shown; as bound RNA was inhibited by competition, a corresponding increase in free RNA was also observed (data not shown). See Table 1 for sequences of the PIP4 variants used as competitors. Differences in band intensities for the various competitors reflect variations in exposure time between experiments, or differences in the amount of total shifted probe at the given protein concentration because of variability between gels. These effects are not relevant to the relative efficiencies of competition of the PIP4 variants. Note that the U_6 sample in the first lane was lost.

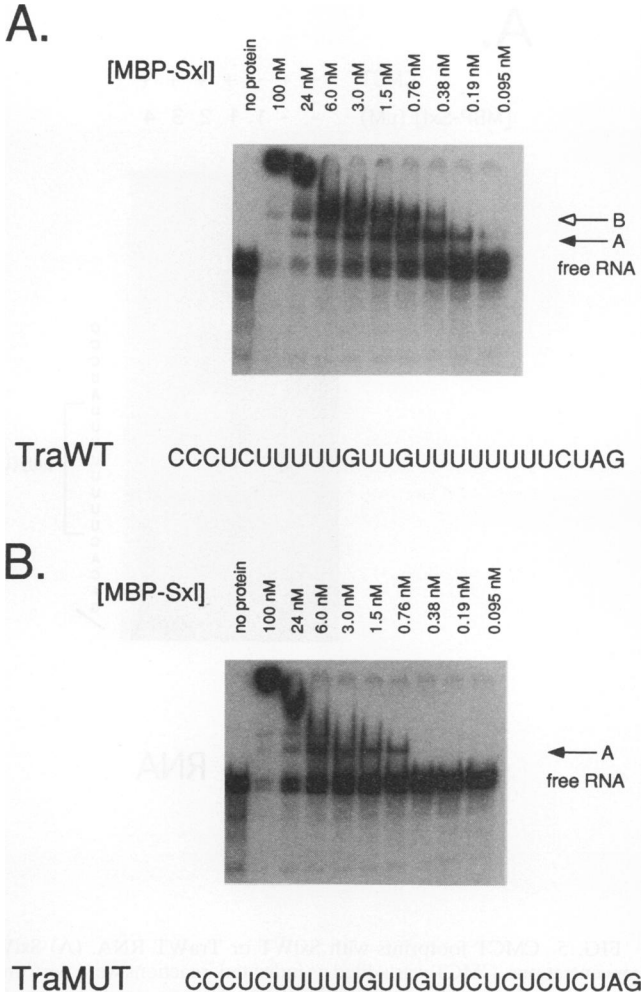


FIG. 4. Gel shifts with Tra RNAs. MBP-Sxl at the indicated concentrations was incubated with 0.17 nM TraWT or 0.14 nM TraMut RNA, and the products were resolved by native gel electrophoresis.

competed more poorly than PIP4 WT. As expected, U_6, U_4, and U_3 competed extremely inefficiently. U_6(3′A) appeared to compete more efficiently than expected from its gel shift. This discrepancy may have resulted from gel variability.

Sxl forms two complexes on a *tra* acceptor site. Although binding of Sxl protein to a *tra*-regulated acceptor site has previously been demonstrated (66), it was of interest to compare binding to the *Sxl* male exon acceptor site and the *tra* acceptor site in the same context. Therefore, a variant containing the regulated *tra* acceptor in PIP4 was constructed. Surprisingly, when gel shift titrations were carried out with RNA from PIP4 TraWT, two shifted products were observed (Fig. 4A). The higher-affinity form (Fig. 4A, solid arrow A) comigrated with the strongly shifted forms described above. The upper shifted form (Fig. 4A, unshaded arrow B), of lower affinity, comigrated with the minor doubly shifted forms described above as relatively nonspecific (Fig. 1E, SxlMut RNA open arrow). For the TraWT RNA, however, this upper shifted form constituted a major product at intermediate protein concentrations. We believe this upper form for TraWT RNA represents binding of additional protein molecule(s) to the U_5 tract just 5′ of the U_8 tract at the acceptor site. When the U_8 tract was completely eliminated by introduction of three C residues (TraMut RNA), a residual singly shifted form still was observed on gels (Fig. 4B, solid arrow), presumably resulting from binding to this U_5 tract.

Because of indications that Sxl protein-protein interactions might be occurring (see Discussion), we attempted to directly measure cooperativity with the TraWT RNA. The presumptive K_d of the U_5 site was determined from experiments with TraMut RNA (Table 1), followed by curve fitting to the two shifted complexes of TraWT. The experiment documented in Fig. 4A suggested that complex B was stabilized three- to fivefold, presumably by protein-protein interactions, in comparison with the homologous complex A in Fig. 4B (data not shown). However, it was noted that quite small changes in the K_d assumed for the weaker site substantially affected the cooperativity constant calculated for TraWT, between values of 1 (no cooperativity) and 5. Because of the difficulties noted above in measuring K_ds of the weaker binding substrates, the question of cooperativity was not clearly resolved with the Tra RNAs.

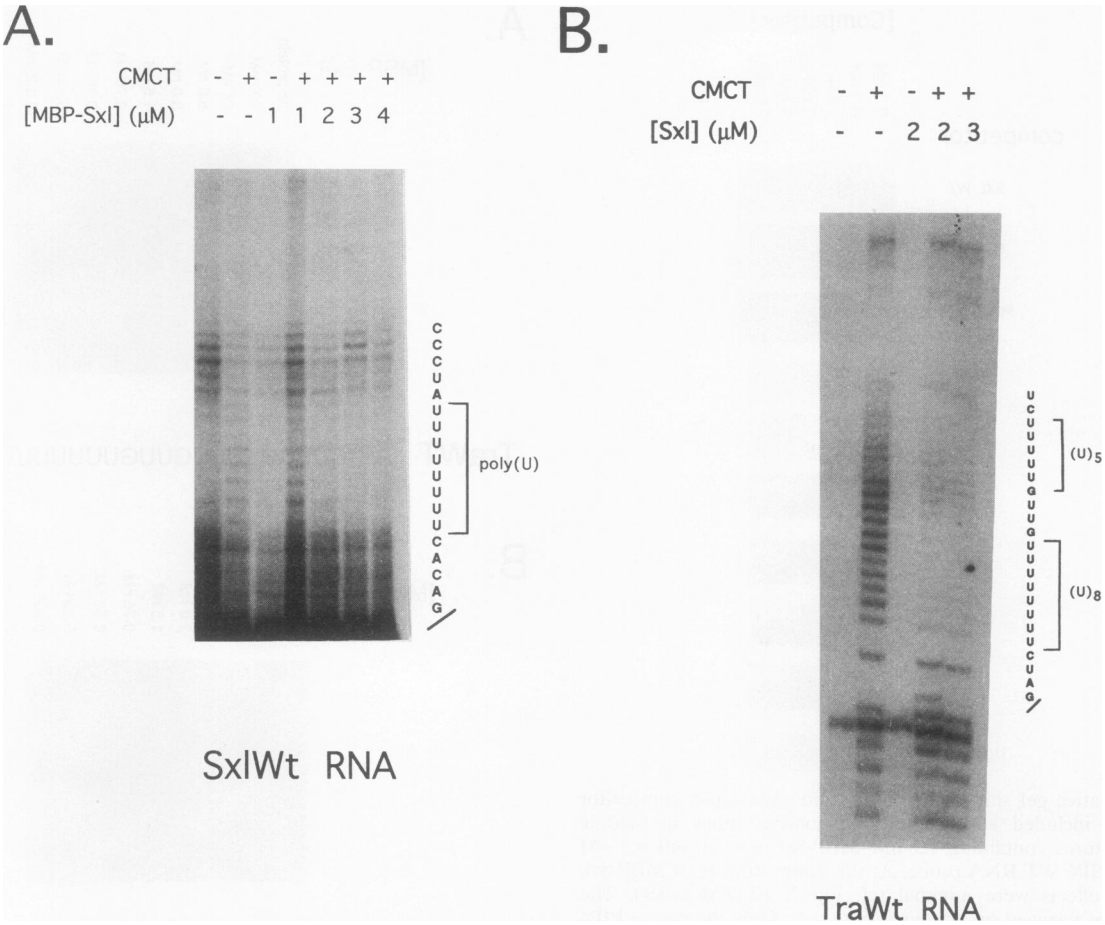


FIG. 5. CMCT footprints with SxlWT or TraWT RNA. (A) SxlWT RNA (12.5 nM) was incubated with MBP-Sxl protein at the indicated concentrations. CMCT was added as indicated, reactions were performed, and products were prepared and resolved on denaturing polyacrylamide gels as described in Materials and Methods. Alignment with the poly(U)-containing splice acceptor site sequence is shown. (B) TraWT RNA (50 nM) was incubated with unfused Sxl protein at the indicated concentrations. CMCT reactions were performed as described above. Alignment with the splice acceptor site sequence is shown.

It is unclear why doubly shifted complexes were not observed in previous work with a *tra* acceptor site RNA (66). Possibly this reflects differences in the RNA sequences outside the acceptor site, or differences in the protein preparations. Alternatively, the different gel concentration employed in the present study may have affected the stability of higher-order complexes during electrophoresis.

CMCT footprint of Sxl on RNA. In order to verify that binding was occurring over the poly(U) tracts, as suggested from the mutant RNA data, CMCT footprint experiments were performed following binding of either MBP-Sxl fusion or unfused Sxl protein to SxlWT or TraWT RNAs. As expected, MBP-Sxl protein showed a strong footprint over the U_8 tract of the SxlWT RNA (Fig. 5A). Similarly, unfused Sxl showed a footprint over the U_8 tract of the TraWT RNA (Fig. 5B). In addition, there appeared to be weaker protection of the U_5 tract of TraWT (Fig. 5B). This supports the hypothesis that complex B observed in gel shifts with TraWT RNA resulted from binding of the MBP-Sxl fusion to the U_5 region. However, protein and RNA concentrations were sufficiently different in the gel shift and footprint assays that direct correlation of shifted complexes with the footprint could not be made.

Sxl protein resides in large RNP complexes in vivo. As a splicing regulatory factor, Sxl is predicted to associate with nascent transcripts. We performed experiments to identify RNP complexes formed in vivo containing Sxl proteins, and to determine whether such complexes could be identified with other RNA packaging or splicing components. RNP complexes containing nascent transcript RNA have been extensively studied by sucrose gradient centrifugation of extracts from *Drosophila* cells (48, 49, 60). Embryonic nuclear extracts were therefore prepared under low-salt-concentration conditions and fractionated by sucrose gradient sedimentation, and Sxl proteins in the gradient fractions were detected by Western blotting.

Sxl proteins were found to sediment in very large aggregates, being detected across the bottom half of the gradients (Fig. 6A). These aggregates appeared to include RNA, since treatment of the extracts with RNase prior to sedimentation caused release of Sxl proteins to the uppermost fractions (Fig. 6B), where they were partially degraded. Addition of NaCl to 0.4 M similarly released Sxl to the top of the gradients (Fig. 6C), while DNase treatment had no effect (data not shown).

In such extracts, fragmented nascent RNA has been shown to fractionate in a broad band of about 40 to 80S, cosedimenting with general hnRNA packaging proteins (hrps) (48). In order to compare the sedimentation profile of Sxl with those of bulk hnRNA packaging proteins, aliquots of the gradient

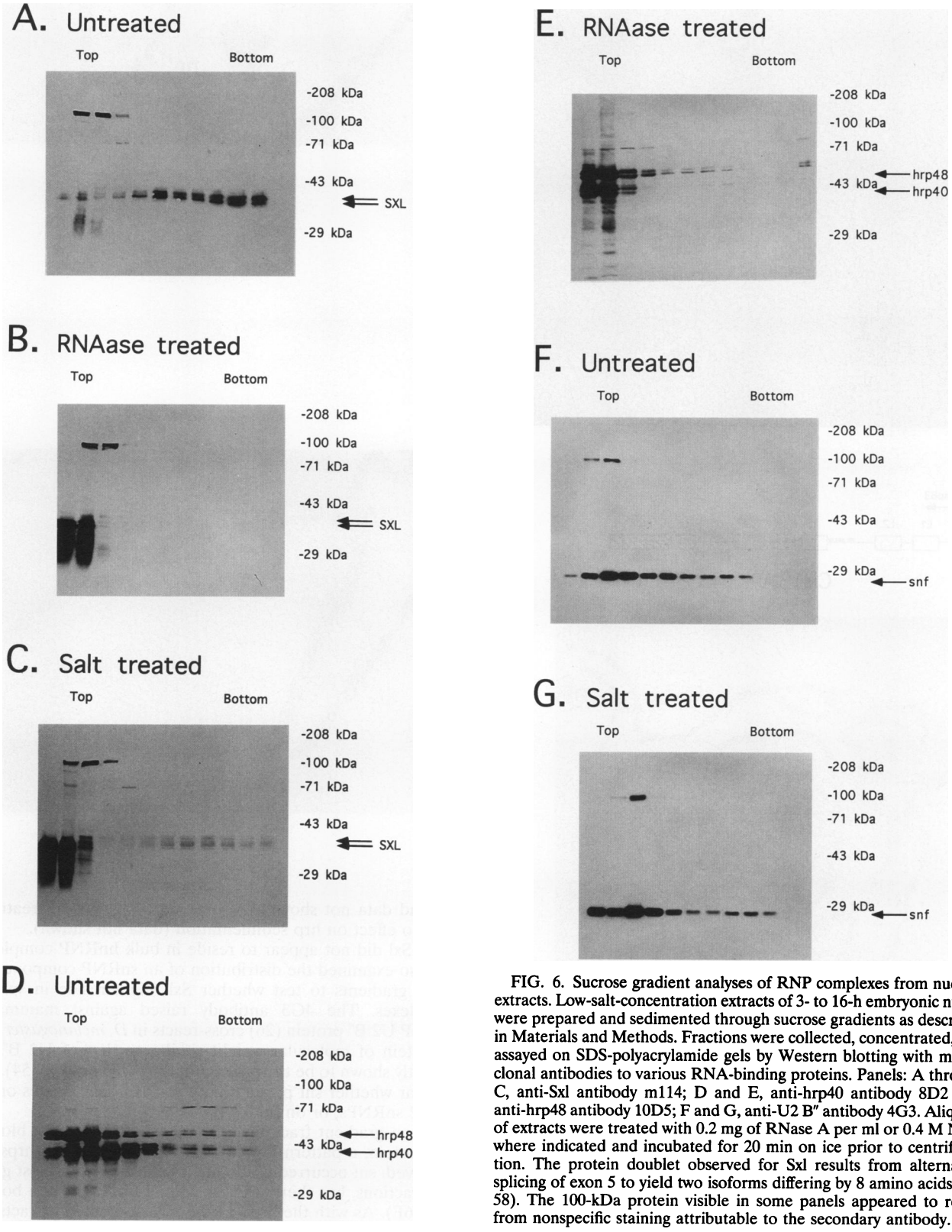
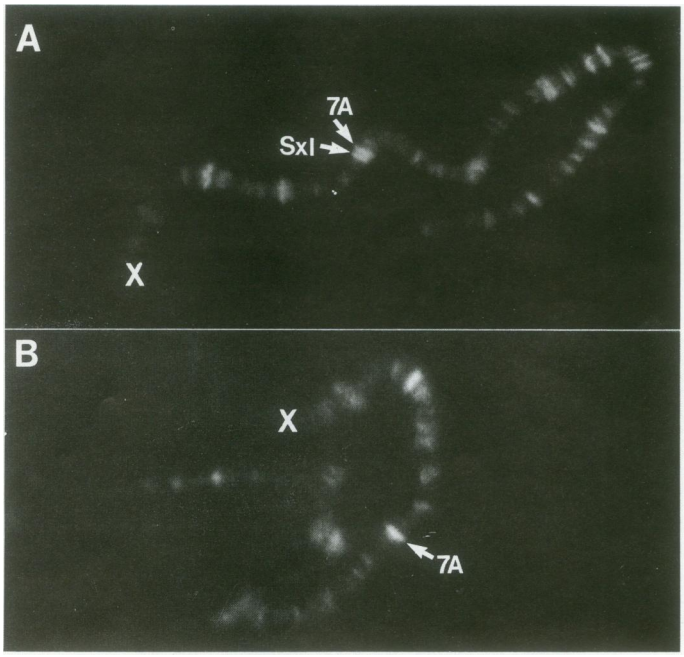
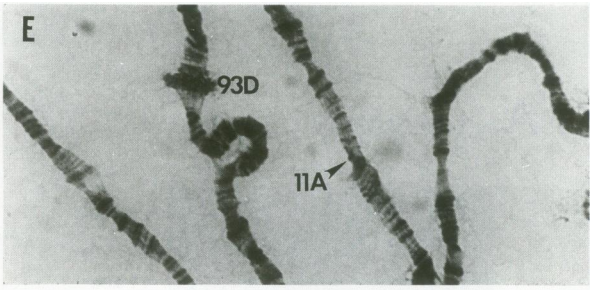
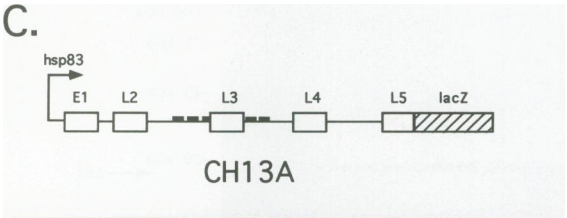
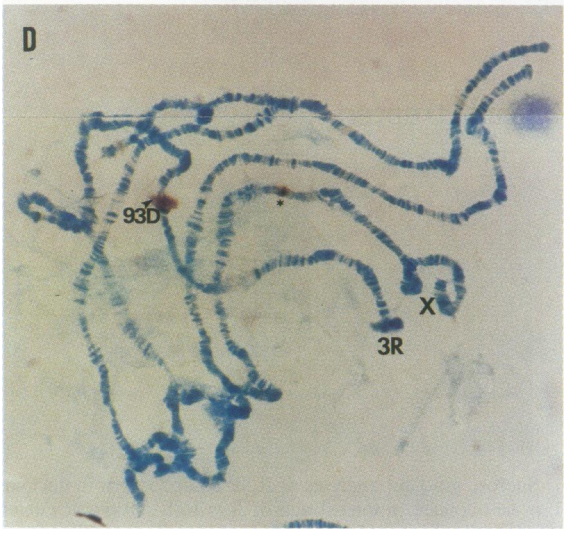


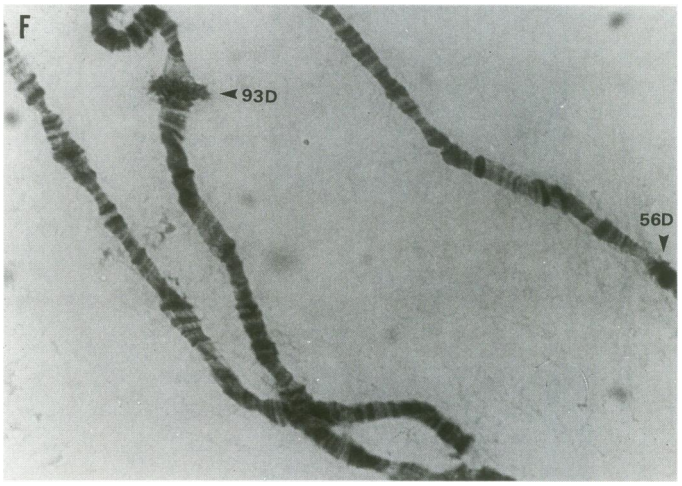
FIG. 6. Sucrose gradient analyses of RNP complexes from nuclear extracts. Low-salt-concentration extracts of 3- to 16-h embryonic nuclei were prepared and sedimented through sucrose gradients as described in Materials and Methods. Fractions were collected, concentrated, and assayed on SDS-polyacrylamide gels by Western blotting with monoclonal antibodies to various RNA-binding proteins. Panels: A through C, anti-Sxl antibody m114; D and E, anti-hrp40 antibody 8D2 plus anti-hrp48 antibody 10D5; F and G, anti-U2 B″ antibody 4G3. Aliquots of extracts were treated with 0.2 mg of RNase A per ml or 0.4 M NaCl where indicated and incubated for 20 min on ice prior to centrifugation. The protein doublet observed for Sxl results from alternative splicing of exon 5 to yield two isoforms differing by 8 amino acids (10, 58). The 100-kDa protein visible in some panels appeared to result from nonspecific staining attributable to the secondary antibody.











fractions previously assayed for Sxl were Western blotted for two hnRNPs, hrp40 and hrp48. hrp40 has recently been shown to be the product of the *squid* gene (33). As shown in Fig. 6D, these hnRNPs sedimented primarily in the uppermost half of the gradient, quite differently from Sxl. Some trailing of the hrps throughout the gradient was also observed. As with Sxl, treatment with RNase or high salt concentrations released both hrps to the uppermost fractions of the gradient, although unlike Sxl they were refractory to proteolytic degradation (Fig. 6E and data not shown). Also as with Sxl, DNase treatment had no effect on hrp sedimentation (data not shown).

As Sxl did not appear to reside in bulk hnRNP complexes, we also examined the distribution of an snRNP component in these gradients to test whether Sxl might reside in splicing complexes. The 4G3 antibody raised against mammalian snRNP U2 B″ protein (26) cross-reacts in *D. melanogaster* with a protein of molecular weight similar to that of U2 B″ (1), recently shown to be the product of the *snf* gene (19a, 54). It is unclear whether snf protein in *D. melanogaster* resides on U1 or U2 snRNPs, or on both.

When gradient fractions were assayed by Western blot for snf protein, a pattern broadly similar to that of the hrps was observed. snf occurred predominantly in the uppermost gradient fractions, but there was substantial trailing to the bottom (Fig. 6F). As with the hrps, in the RNase-treated extracts, snf was shifted more towards the top of the gradients. Unlike what was observed with the hrps, however, salt treatment only partially shifted snf (Fig. 6G). These data suggest that the

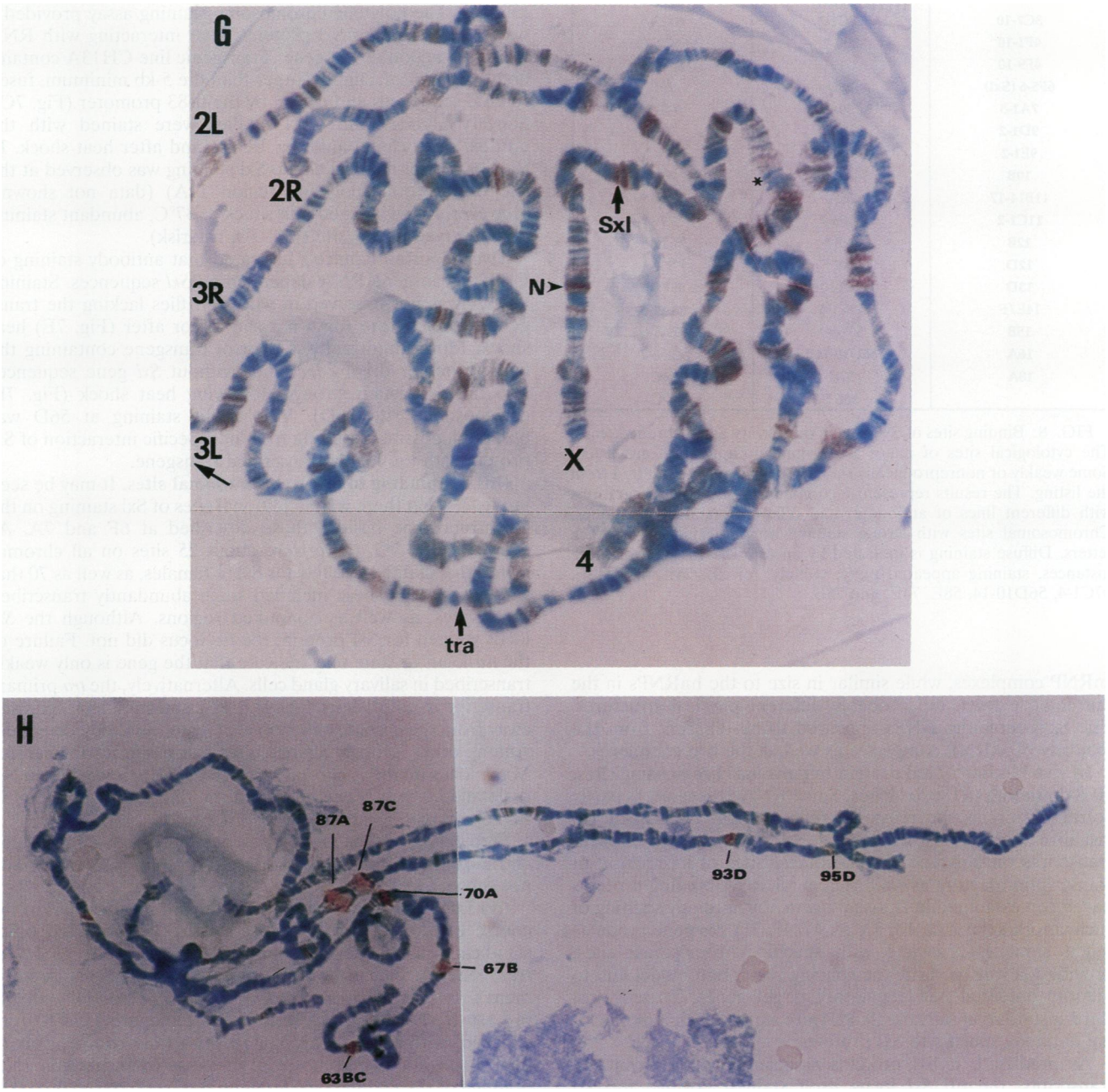


FIG. 7. Polytene chromosome staining with anti-Sxl antibody m104 (panels A through G) and anti-U2 B″ antibody 4G3 (panel H). (A) Wild-type Oregon R female with rhodamine-conjugated anti-mouse secondary antibody, visualized by immunofluorescence. (B) Sxl Df(7B0)/Df(7B0); Dp2(Sxl^+) female with rhodamine-conjugated anti-mouse secondary antibody. (C) Schematic of CH13A, a construct containing 7 kb of *Sxl* genomic sequence from early exon E1 to late exon L5, fused to *lacZ* and driven by the heat shock 83 promoter. Small black boxes indicate the approximate positions of poly(U) tracts in introns surrounding the *Sxl* male exon (exon L3). (D) Staining of transgenic line CH13A, containing a P element carrying CH13A inserted at polytene site 11A (marked with an asterisk), following 60 min of heat shock at 37°C. Visualization of Sxl protein by biotin-conjugated anti-mouse secondary antibody and streptavidin-horseradish peroxidase. (E) Wild-type female following 60 min of heat shock, stained for Sxl protein as described in the legend to panel D. Site 11A is indicated for comparison with panel D. (F) Staining of transgenic line hs83*lacZ*, containing a P element carrying the *lacZ* gene driven by the heat shock 83 promoter, following 60 min of heat shock. Only the regions containing the insertion site at 56D and the heat shock 93D locus are shown. Visualization of Sxl protein was done as described in the legend to panel D. (G) Wild-type female, no heat shock, showing all chromosome arms identified at termini. Sites of the *Sxl* and *tra* genes are indicated, as are the approximate site of the *Notch* gene and interval 11A (asterisk, insertion site of CH13A in the transgenic line). Visualization of Sxl protein was done as described in the legend to panel D. (H) Wild-type female following heat shock, stained for snf protein (secondary antibody and stain are as described in the legend to panel D). Major heat shock puffs are indicated.

X	2	3
2B *	22B3-5	62F1-2
3C7-10	23C1-2	63E3-5
4F1-10	25C *	**64C9-12**
4F9-10	27E-1	66B1-3
6F5-6 (Sxl)	**28D**	70F
7A1-5	**30A**	**74B ***
9D1-2	32D1-2	**74E ***
9E1-2	**33E**	**75B ***
10B	**34A5-6 ***	76D
11B14-17	35A	78A5-7
11C1-2	**36F6-7**	79B
12B	**42B ***	82C
12D	**43E ***	83F
13D	**47A10-16**	**85F1-5**
14E/F	**47C1-4**	**88A4-5**
15B	50E6-9	92A1-6
16A	**56D10-14 ***	93D6-7
18A	57D	94C
	58E *	97C

FIG. 8. Binding sites of Sxl protein on salivary gland chromosomes. The cytological sites of major Sxl protein accumulation are listed. Some weakly or nonreproducibly stained sites have been omitted from the listing. The results represent a compilation of many preparations with different lines of antibodies and different staining techniques. Chromosomal sites with strong staining are indicated by boldface letters. Diffuse staining is indicated by an asterisk. In some of these instances, staining appeared puffy; namely, for 2B, 25C, 42B, 43E, 47C1-4, 56D10-14, 58E, 74E, and 75B.

snRNP complexes, while similar in size to the hnRNPs in the untreated extract, are probably different physical structures. The Sxl-containing RNPs appeared to be different from the majority of snRNP complexes as well as the hrp complexes.

In vivo binding of Sxl protein to polytene chromosome sites. (i) Sxl binding to the *Sxl* locus. Since RNA processing is partly cotranscriptional, we reasoned that it might be possible to visualize a direct association of Sxl proteins with nascent transcripts on polytene chromosomes. Sites of interaction of transcription factors as well as general RNA-binding proteins have been examined in *D. melanogaster* by antibody staining of such chromosomes (1, 18, 37, 38, 47, 49, 71). Sxl gene products might similarly associate with specific chromosomal sites, providing a way to verify the splicing regulation model and to identify potential new regulatory target genes. To test this, third-instar larval salivary glands were stained with a monoclonal antibody specific to Sxl proteins.

As predicted, if Sxl proteins associated with nascent *Sxl* transcripts, the 6F *Sxl* locus itself stained positive for Sxl protein. Figure 7A shows a rhodamine stain of this region of a wild-type female X chromosome. Two bands stained for Sxl protein in this region, one at polytene interval 6F (Fig. 7A, labelled arrow marking the site of *Sxl*) and the other at interval 7A. In females homozygous for *Sxl* allele 7B0, a small deletion removing most of the *Sxl* transcription unit together with some upstream sequences, staining at 6F was lost, although the 7A staining was retained (Fig. 7B) (the females carried a duplication of Sxl^+ on the second chromosome for viability; this duplication was not clearly identified in any of the chromosome spreads for Sxl staining). Thus, for the *Sxl* regulatory target, protein-directed antibody staining of the chromosomal site depended on sequences near or within the target gene.

(ii) Sxl binding to RNA from a transgene containing splicing regulatory signals. Within *Sxl*, fully regulated splicing requires a region of about 5 kb, including the male-specific exon, surrounding intron sequences, and parts of the flanking exons (30, 53). The polytene chromosome-staining assay provided a way to test whether Sxl proteins were interacting with RNA from this region of the gene. Transgenic line CH13A contains about 7 kb of *Sxl*, slightly larger than the 5-kb minimum, fused to *lacZ* sequences and driven by the hs83 promoter (Fig. 7C). Salivary glands from CH13A flies were stained with the anti-Sxl monoclonal antibody before and after heat shock. In the absence of heat shock, no Sxl staining was observed at the site of insertion (polytene region 11A) (data not shown). However, following a 60-min shock at 37°C, abundant staining was observed at this site (Fig. 7D, asterisk).

Two important controls indicated that antibody staining of CH13A transgene RNA depended on *Sxl* sequences. Staining at 11A was not observed in wild-type flies lacking the transgene, either before (data not shown) or after (Fig. 7E) heat shock. More importantly, a control transgene containing the hs83 promoter driving *lacZ* but without *Sxl* gene sequences also failed to stain strongly following heat shock (Fig. 7F, chromosomal site 56D). Very slight staining at 56D was observed, possibly resulting from nonspecific interaction of Sxl protein with the highly transcribed transgene.

(iii) Sxl binding to other chromosomal sites. It may be seen in Fig. 7A that there were additional sites of Sxl staining on the X chromosome besides those described at 6F and 7A. As shown in Fig. 7G, there were about 25 sites on all chromosomes that stained strongly for Sxl in females, as well as 70 that stained weakly. These included some abundantly transcribed (puff) sites, as well as nonpuffed regions. Although the *Sxl* locus stained for Sxl protein, the *tra* locus did not. Failure of the *tra* locus to stain may indicate that the gene is only weakly transcribed in salivary gland cells. Alternatively, the *tra* primary transcript is quite short (about 2 kb) and may have diffused away from the chromosome too rapidly for antibody detection. Among other strongly stained sites was a site at or near the *Notch* locus on the X chromosome (Fig. 7G, arrow labelled N). A detailed listing of the major Sxl staining sites is provided in Fig. 8 and will be submitted to Flybase.

Predictably, Sxl staining was not observed on polytene chromosomes of males, who lack Sxl protein expression (data not shown).

(iv) Effects of heat shock on Sxl and snf localization. In the course of studying the transgenic lines, we observed other significant effects besides induced staining of the CH13A transgene. Specifically, prolonged (>60-min) heat shock treatment also resulted in the loss of Sxl from essentially all the previously stained chromosomal sites (compare Fig. 7G [unshocked] with Fig. 7D through F). In a time course assay of heat shock, not all chromosomal sites lost Sxl at the same rate. Staining of the endogenous *Sxl* gene persisted longer than that of most other sites, although ultimately *Sxl* staining too was eliminated. The 3C site likewise persisted through intermediate times of heat shock, as did several other sites (data not shown). This gradual loss of Sxl staining with the onset of heat shock further supports the interpretation that the staining was due to Sxl binding to nascent transcripts, since general transcription is turned off by heat shock.

Unexpectedly, in the CH13A transgenic line, one other major new site of Sxl staining, the hs93D locus (Fig. 7D, labelled arrow), arose following heat shock. Heat-shocked wild-type and hs83-*lacZ* females also showed the same hs93D staining (Fig. 7E and F), together with loss of staining from other sites.

The effect of heat shock on localization of the putative snRNP component snf was also examined by staining polytene

chromosomes of unshocked and shocked larvae with antibody 4G3. 4G3 staining of unshocked chromosomes has previously been reported (1). As seen in the earlier work, and as expected for a presumptive splicing factor, 4G3 stained almost uniformly across all chromosomes in wild-type males and females maintained at the standard temperature (data not shown). Upon heat shock, however, snf staining became restricted primarily to heat shock puffs, although not uniquely to 93D (Fig. 7H).

DISCUSSION

RNA binding by MBP-Sxl fusion protein in vitro. We have carried out the first systematic in vitro RNA-binding study of Sxl. A preference of Sxl for poly(U)-containing RNA has previously been reported (32, 53, 66). However, other RNA recognition motif (RRM) proteins show a similar preference (65), including, for example, the product of the *Drosophila* nervous system regulatory gene *elav* (50) and the general splicing factors U2AF (66) and PSF (46). Since these proteins presumably coexist with Sxl in the nuclei of some cells, they must each possess additional elements of specificity, either in terms of RNA binding, protein-protein interactions, or both. For this reason, we looked in more detail at the sequence specificity of Sxl. In good agreement with previous work, we found that the MBP-Sxl fusion protein bound strongly to RNAs containing long poly(U) tracts (Table 1) to generate a shifted protein-RNA complex. As the length of the U tract was decreased, binding was steadily weakened. These results are consistent with those obtained in in vivo studies of splicing of the *tra* gene, and of the *Sxl* gene when portions were placed in a heterologous context in vivo, as well as with those obtained in in vitro splicing experiments. In these cases, regulation of splicing by Sxl gene products depended on the presence of a long poly(U) tract directly at the splice acceptor site, which was thereby repressed (29, 32, 62, 66).

High-affinity binding of MBP-Sxl to RNA was not strongly affected by mutation of either the branch point or the AG dinucleotide. Initially, it seemed quite plausible that Sxl might recognize one of these sequences in addition to poly(U), given that Sxl action was presumably restricted to splice acceptor sites. Since Sxl has more recently been shown to be capable of acting via sequences relatively far from splice junctions (30, 53), it is not quite as surprising that branch point and AG recognition play no major role in Sxl binding.

We did note a significant and unexpected modulatory effect on binding by sequences neighboring the poly(U) stretch. Specifically, an A residue immediately 5′ of the poly(U) tract was shown to strengthen binding of the MBP-Sxl fusion protein severalfold. It is not clear whether the protein specifically recognizes this adenosine residue or whether the A residue has a more general effect on the RNA structure, possibly interrupting a pyrimidine-specific structure involving the adjacent run of 3 C residues in the PIP4 vector sequence.

Some of the PIP4 variant RNAs have also been tested for binding to the other polypyrimidine tract binding proteins PTB and hnRNP C, with the result that each of these proteins behaves differently with respect to introduction of cytidine or adenosine residues at specific positions within a poly(U) tract, although all the proteins show the strongest preference for uninterrupted uridines (51). In in vitro splicing assays, SxlWT RNA functioned more poorly than did PIP4 WT, the reverse of what we observed for Sxl protein binding to these two RNAs. Moreover, the U_7(5′A) and U_6(3′A) variants, while functioning essentially as well as PIP4 WT in splicing (51), were substantially affected, in opposite ways, for Sxl binding (Table 1). Thus, as predicted, Sxl binding preferences are apparently subtly different from those of other factors recognizing splice acceptor sites.

Possible protein-protein interactions in Sxl binding. Several observations suggest the occurrence of protein-protein interactions as part of Sxl's in vitro activity. All of the RNAs employed in the band shift experiments were subject to secondary shifts at higher protein concentrations with both the MBP-Sxl fusion and unfused Sxl. In some cases, for example, SxlMut and U_0, higher-order complexes were observed even when no singly bound complexes were detected on native gels. We believe this result is most consistent with additional interactions between proteins stabilizing the higher-order complexes on these weakly binding RNAs, although we have not attempted to quantitate this effect. In addition, UV cross-linked dimeric bacterial Sxl was observed by Sakamoto et al. (53), and noncovalently complexed Sxl dimers have been proposed to form during in vitro translation (58). These observations similarly suggest that Sxl may interact strongly with itself. However, it must be noted that Sxl protein-protein interactions were not specifically observed in another study of Sxl RNA binding (66).

Besides these qualitative observations, quantitative analysis of binding to the TraWT and TraMut RNAs was also consistent with, although not definitive of, cooperative interactions stabilizing protein binding to a second site (probably the U_5 tract). Further experiments are in progress to attempt to measure cooperativity with paired strong binding sites.

Cooperativity in RRM proteins is not a novel suggestion. Protein-protein interactions, as well as specific RNA binding, are important in snRNP assembly (7, 59). Cooperative binding of the hnRNP A1 protein to single-strand nucleic acid has also clearly been demonstrated, and the C-terminal glycine-rich repeat domain of A1 was specifically implicated in protein-protein interactions (17). Sxl contains no such domain, but other sequences outside the two RRMs could be involved in cooperativity. We are currently testing this in vitro and with transgenic flies carrying truncated versions of *Sxl* proteins. Alternatively, the Sxl RRMs themselves may interact intermolecularly.

There is good reason to believe that cooperativity is relevant to the in vivo functioning of Sxl. For *tra*, the upstream U_5 tract at the regulated acceptor site is highly conserved in *tra* genes of distantly related *Drosophila* species (45), consistent with functional significance of interactions besides the previously documented U_8 Sxl binding. Moreover, we and others (66) observed residual in vitro binding activity of TraMut RNA, although all regulation by *Sxl* in vivo was lost when this mutant acceptor site was reconstructed in the complete *tra* gene (62). Regulation of *tra* may thus depend on cooperative binding of multiple Sxl protein molecules.

Apart from the single U_8 tract at the *Sxl* male exon acceptor site, most of the other long poly(U) tracts in the introns surrounding the *Sxl* male exon occur as double poly(U) tracts separated by short stretches of non-poly(U) (13). Moreover, in *Sxl* of *D. virilis*, multiple poly(U) tracts also occur near the male exon. These may be even more important than those in *D. melanogaster*, as the male exon acceptor site of *D. virilis* itself lacks a long poly(U) tract (13). Sakamoto et al. showed by UV cross-linking that the intronic compound poly(U) tracts of *D. melanogaster* bound Sxl in vitro, although the number of Sxl molecules bound per tract could not be determined (53). Since these intronic poly(U) stretches have been clearly implicated in full *Sxl* autoregulation, for both *tra* and *Sxl*, cooperativity may then be an important feature of Sxl's site selection.

These in vitro RNA-binding data leave unexplored the individual roles of the two RRMs in Sxl. For both poly(A)-

binding protein and U2AF, multiple RRMs are involved in generating the sequence preferences these proteins evince (11, 70). The two Sxl RRMs may similarly both be required for strong poly(U) binding. Evolutionary comparison has not in this respect proved helpful, as the *D. virilis Sxl* gene is essentially identical to *D. melanogaster Sxl* at the amino acid level throughout the RNA-binding regions (13).

Polytene chromosome localization of Sxl protein. The polytene chromosome-staining assay demonstrated that Sxl proteins associate with RNA of the *Sxl* regulatory target gene. Protein was also targeted to RNA from a trans gene containing only 7 kb of *Sxl* genomic sequences including the regulated splicing region. This strongly supports the long-standing prediction of a direct physical interaction between *Sxl* gene products and nascent transcripts undergoing RNA processing.

In spite of this specificity in binding to *Sxl* gene sequences, Sxl protein was also observed to accumulate at a large number of other sites on female polytene chromosomes (see reference 23 also). It seems unlikely that many of these represent true regulatory targets for *Sxl* action. Other than gametogenesis-specific genes, only a few genes have been shown to have sex-specific functions, including *tra*, *tra-2*, *dsx*, and the set of four male-specific lethals. Of these, only *tra* is a regulatory target of *Sxl*, although one of the male lethals (*msl-2*) remains to be molecularly cloned and is a potential target in the dosage compensation pathway. There are not, therefore, a significant number of genetically defined somatic sex-specific genes that could be novel Sxl targets.

If the many ectopic sites of Sxl localization do not reflect regulated genes, then what is their nature? Most probably, these are transcribed genes that happen to have long poly(U) tracts either in intervening sequences or in 5′ or 3′ noncoding regions. For example, strong staining of the X-chromosomal 3C region may represent Sxl binding to *Notch* (*N*) locus transcripts. Although there is no evidence of regulation of *Notch* by *Sxl*, *N* does have a number of long poly(U) tracts (68). The fact that anti-Sxl antibody labels approximately 100 chromosomal sites does not imply that Sxl protein is bound with equal affinity to these sites. The limitations of the assay in distinguishing binding events of different affinities are not known. Indeed, the greater retention of Sxl protein at the *Sxl* locus than at other loci during early heat shock supports the hypothesis that not all chromosomal sites are equally tightly bound by Sxl.

It is probable, therefore, that Sxl binding to some or most RNAs is without regulatory consequence. This must be taken into account in any model of *Sxl* action. Until recently, the hypothesis prevailed that Sxl proteins act directly by binding to splice acceptor sites containing poly(U), thereby competing with U2AF binding and preventing splice site utilization (66). If this model were generally true, then binding of Sxl to poly(U) tracts in other regions of primary transcripts, such as introns or 3′ nontranslated regions, would have little genetic effect. However, as noted above, results from several laboratories concur that *Sxl* is capable of altering splicing patterns on target genes lacking poly(U) tract acceptor sites and indeed can mediate splice site choice, even when the presumptive Sxl binding sites lie far from the actual regulated splice junctions. How other genes avoid *Sxl* regulation when Sxl proteins associate with their transcripts remains to be elucidated. Possibly there are cooperative and noncooperative binding modes leading to different effects (or lack thereof) on the general splicing machinery.

Polytene chromosome localization has been examined for a number of other RNA-binding proteins, including known hnRNP packaging proteins. It is difficult to compare antibody staining results carried out in different laboratories under different conditions. However, our impression is that Sxl was localized to fewer sites than were other more general RNA-binding proteins such as hrps. The Sxl binding pattern was also more restricted than that of the presumptive snRNP protein snf.

Effects of heat shock on localization of Sxl and other RNA-processing components. In the course of assaying for targeting of Sxl proteins to a transgene driven by the hs83 promoter, the surprising result was noted that besides accumulating at the transgene insertion site, Sxl proteins also accumulated at the heat shock 93D locus following prolonged heat shock. This chromosomal site has previously been shown to accumulate a known hnRNP packaging protein upon heat shock (P11, equivalent to hrp36) (19, 36). To our knowledge, Sxl is the first example of a regulatory RNA-processing factor shown to accumulate at 93D. The hs93D locus is abundantly transcribed during heat shock, although it possesses no long open reading frame (21). The transcript also lacks long poly(U) tracts (20, 31, 52), suggesting that Sxl may not be directly binding to 93D transcripts but instead may be interacting with other proteins. Ultrastructural analyses of 93D puffs following heat shock show large granules containing P11 (hrp36) protein, suggesting that nuclear RNA-binding proteins may be assembled into large RNP aggregates during heat shock (19). The 93D locus may thus, among other functions, serve as a storage site for the pre-mRNA packaging and processing machinery under stress conditions.

Surprisingly, the snf protein was not restricted to puff 93D following heat shock. Under these conditions, snf was observed at numerous heat shock loci. Localization of snRNPs to hsp70 loci has previously been reported (35). These results are unexpected, given that many heat shock transcripts are unspliced; in fact, the 93D ω transcripts are among the few spliced heat shock RNAs. snRNPs may simply be scanning the heavily transcribed loci. In any case, it is clear that hnRNP and snRNP components can assort independently after heat shock.

Sxl RNP complexes in nuclei. Sxl proteins were observed in large RNP aggregates when extracted from embryonic nuclei under low-salt-concentration conditions. The literature on hnRNP complexes sedimented following sonication of nuclei is complex. The observed size of such complexes depends on numerous factors, including salt, stress of the cells, and especially the amount of endogenous RNase activity. Therefore, it is difficult to compare the absolute sizes of complexes prepared in different laboratories. However, the Sxl-containing RNP aggregates observed in this work sedimented much more rapidly than those containing the general packaging proteins. These sedimented in the uppermost half of the gradients, in approximate agreement with the observations of Raychaudhuri et al. (48).

Several reasons can be suggested for the fast sedimentation of Sxl complexes relative to that of hnRNPs. One possibility is that Sxl protein protects RNA from endogenous RNase activity during extraction. Or, perhaps Sxl complexes contain additional components such as hnRNPs or snRNPs. A third possibility is that while bound to RNA, Sxl proteins form large complexes with each other, a possibility supported by the observed aggregation behavior of purified bacterial Sxl and MBP-Sxl fusion proteins at high concentrations.

No free pool of Sxl protein was observed in untreated nuclear extracts. Taken together with the polytene chromosome-staining data, this suggests that most Sxl in the nucleus is bound to RNA, probably mostly in interactions with unregulated genes. It would be interesting to examine the distribution of *Sxl* transcripts across the sucrose gradients to see whether it

is consistent with the sedimentation profile of the bulk of Sxl protein. Such an experiment might clarify the question of whether Sxl proteins formed physically similar structures on both regulated and nonregulated target RNAs.

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