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A Dual Inhibitory Mechanism Restricts *msl-2* mRNA Translation for Dosage Compensation in *Drosophila*

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Summary

***Drosophila* MSL-2 is the limiting component of the dosage compensation complex. Female flies must inhibit *msl-2* mRNA translation for survival, and this inhibition is mediated by Sex-lethal (SXL) binding to sites in both the 5′ and the 3′ untranslated regions (UTRs). Here, we uncover the mechanism by which SXL achieves tight control of translation initiation. SXL binding to the 3′UTR regulatory region inhibits the recruitment of 43S ribosomal preinitiation complexes to the mRNA. Ribosomal complexes escaping this block and binding to the 5′ end of the mRNA are challenged by SXL bound to the 5′UTR, which interferes with scanning to the downstream initiation codon of the mRNA. This failsafe mechanism thus forms the molecular basis of a critical step in dosage compensation. The results also elucidate a two step principle of translational control via multiple regulatory sites within an mRNA.**

Introduction

Translational control of gene expression is crucial for a large number of processes in development, such as spermatogenesis, axis formation, neurogenesis, and X chromosome dosage compensation (Wickens et al., 2000; Kuersten and Goodwin, 2003). In contrast to the control of global mRNA translation, which is usually achieved by the modulation of the activity of general translation factors (Sonenberg and Dever, 2003), the temporal or spatial regulation of the translation of specific mRNAs is typically governed by interactions between RNA binding proteins and regulatory sequences within the 5′ and/or the 3′ untranslated regions (UTRs) of the mRNA (Wilkie et al., 2003). These interactions are usually inhibitory and interfere with translation initiation (Gebauer and Hentze, 2004), which is frequently the rate-limiting step of translation.

Translation initiation begins with the formation of a 43S preinitiation complex, composed of the small 40S ribosomal subunit, a set of eukaryotic initiation factors (eIFs) and methionine-charged initiator tRNA. In the cap-dependent mode of translation initiation, this complex is attracted by the 5′ m^7GpppN cap structure through the cap binding complex eIF4F (Gingras et al., 1999). After binding to the 5′ end of the mRNA, the 43S preinitiation complex scans along the 5′UTR until it reaches the initiation codon, where it forms a stable 48S initiation complex (Kozak, 1989). Finally, hydrolysis of two GTP molecules bound to the initiation factors eIF2 and eIF5B allows the large 60S ribosomal subunit to join the 48S initiation complex and form a translation-competent 80S ribosome (Pestova et al., 2000a).

Since recruitment of the 43S preinitiation complex to the 5′ end of the mRNA is commonly the rate-limiting step in the initiation pathway (Mathews et al., 2000), it is not surprising that many translational regulators, including both 5′ and 3′UTR binding proteins, target this early step (Gebauer and Hentze, 2004). For example, iron-regulatory proteins (IRP) bind to the iron-responsive element (IRE) in the 5′UTR of *ferritin* mRNA and block the recruitment of the 43S preinitiation complex to the 5′ cap-associated eIF4F complex by steric hindrance (Gray and Hentze, 1994; Muckenthaler et al., 1998). Other regulators including *Drosophila* Cup and Bicoid as well as *Xenopus* Maskin act through the 3′UTR of their target mRNAs and block 43S complex recruitment by interfering with eIF4F complex assembly at the cap structure (Richter and Sonenberg, 2005). However, regulation can also occur at postrecruitment steps: GCN4 expression is regulated in yeast by controlling the frequency at which translation reinitiates following translation termination at an upstream open reading frame (uORF) (Hinnebusch, 1997). Elongating ribosomes stalled in the presence of arginine at the uORF of *CPA1* mRNA impose a secondary block onto the scanning of 43S complexes across this uORF toward the CPA1 translation initiation codon (Gaba et al., 2001). Another example of postrecruitment regulation is the translational control of *15-lipoxygenase* mRNA during early erythroid differentiation. In this case, joining of the 60S ribosomal subunit to the 48S initiation complex is inhibited by heterogeneous nuclear ribonucleoprotein (hnRNP) K and hnRNP E1 bound to the *15-lipoxygenase* 3′UTR (Ostareck et al., 2001). While 43S complex recruitment and 60S subunit joining have clearly been established as targets for translational control, it is an intriguing open question whether the scanning of bound 43S complexes to reach the initiation codon constitutes a primary target for translational regulators.

In *Drosophila*, translational control of *male-specific lethal 2* (*msl-2*) mRNA is essential for the survival of female flies (Kelley et al., 1995; Bashaw and Baker, 1997; Kelley et al., 1997). The MSL-2 protein is a critical component of the dosage compensation complex (DCC) that promotes hypertranscription of the single X chromosome in male flies and thus ensures similar transcript levels in both sexes (Bashaw and Baker, 1995; Gilfillan et al., 2004). In females, translation of the *msl-2* mRNA is inhibited by the female-specific RNA binding protein Sex-lethal (SXL), thus preventing the

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formation of the DCC (Bashaw and Baker, 1997; Kelley et al., 1997; Gebauer et al., 1998). SXL binds to poly(U) stretches present in an intron in the *msl-2* 5′UTR, which is thereby retained in the mRNA. Subsequently, SXL proteins bound to sites in both the 5′ and the 3′UTR cooperate to inhibit translation in vivo and in vitro. While low expression of MSL-2 is tolerated in transgenic female flies, two copies of *msl-2* transgenes lacking appropriate SXL binding sites cause female-specific developmental and viability defects. In contrast to most other translational regulators, which act from either the 3′ or the 5′UTR, efficient inhibition of translation requires SXL binding to both UTRs (Bashaw and Baker, 1997; Kelley et al., 1997; Gebauer et al., 1999). Using a cell-free translation system derived from *Drosophila* embryos, we have recently shown that SXL binding to both UTRs targets translation initiation by inhibiting the stable association of 40S ribosomal subunits with the mRNA (Gebauer et al., 2003). These results raised the question of whether SXL controls the recruitment of the 43S preinitiation complex to the 5′ end of the mRNA and/or the subsequent scanning along the 5′UTR. Furthermore, it is unclear whether both UTRs work together to establish an inhibitory mRNP that interferes with a single initiation step or whether the two UTRs act separately, possibly targeting two distinct steps of initiation. Here, we show that SXL acts as a bifunctional translational regulator. Our data reveal that SXL bound to the 3′UTR of *msl-2* mRNA interferes with the initial recruitment of the 43S preinitiation complex to the mRNA, while 5′UTR bound SXL stalls residual scanning 43S complexes upstream of its binding site. This "failsafe" mechanism explains how tight control of MSL-2 expression is achieved to ensure the viability of female flies.

Results

5′ and 3′UTR Bound SXL Proteins Interfere with Translation Initiation Prior to 48S Initiation Complex Formation

The *msl-2* mRNA contains two SXL binding sites within the 5′UTR and four binding sites within the 3′UTR (sites A–F, Figure 1A). Furthermore, the *msl-2* 5′UTR harbors five uORFs. Using a *Drosophila* embryo in vitro translation system that faithfully recapitulates the critical features of SXL-mediated translational repression of the *msl-2* mRNA, it was previously shown that inactivation of the upstream initiation codons neither affected the translation rate of the *msl-2* ORF nor influenced translational regulation mediated by SXL binding to the mRNA (Gebauer et al., 2003). Further investigations also demonstrated that SXL binding site B in the 5′UTR and sites E and F in the 3′UTR are sufficient for effective inhibition of translation (Gebauer et al., 2003). SXL bound to these three sites inhibits the stable association of 40S ribosomal subunits with the mRNA (Gebauer et al., 2003). To investigate whether the inhibition of 48S initiation complex formation is the result of a joint function of both 5′ and 3′UTR bound SXL or whether it can be independently achieved by SXL bound to either UTR, we generated constructs containing mutations of either site B or sites E and F.

Translation of the wild-type mRNA (BSEF, for site *B* short ORF sites *EF*, Figure 1A) is effectively repressed by increasing amounts of recombinant SXL, while translation of an mRNA derivative lacking all SXL binding sites remains unaffected (Figure 1B, BSEF and BmS(EF)m panels, respectively; see also Gebauer et al., 2003). Translation of the 5′ and 3′ mutant mRNAs is significantly inhibited by SXL (Figure 1B, BmSEF and BS(EF)m panels), but the translational repression of these mutant mRNAs is less efficient than that of the wild-type BSEF mRNA. This reflects the necessity of simultaneous binding of SXL to both UTRs for tight translational repression in vivo and in vitro (Bashaw and Baker, 1997; Kelley et al., 1997; Gebauer et al., 1999). To exclude that binding of SXL to the B site 67 nucleotides downstream of the 5′ end of the mRNA unspecifically disturbs translation initiation by sterically interfering with 43S complex recruitment (Stripecke et al., 1994), we replaced *Drosophila* SXL by mRBD, a *Musca domestica* SXL derivative. *Musca* SXL does not function in X chromosome dosage compensation (Meise et al., 1998) and mRBD does not repress *msl-2* or BS(EF)m mRNA translation in vitro (Figure 1B, right panel; Grskovic et al., 2003), although it binds to the mRNA with at least as high affinity (Figure 1C). Thus, translational repression via the B site is not merely caused by high affinity protein binding.

To investigate the regulatory mechanism of translation inhibition exerted by SXL bound to either UTR, we performed translation reactions in the presence of the nonhydrolysable GTP-analog GMP-PNP and resolved the initiation complexes by sucrose gradient centrifugation. Because GTP hydrolysis is essential for 60S ribosomal subunit joining, treatment with GMP-PNP stalls 43S complexes at the initiator AUG codon of the mRNA (Anthony and Merrick, 1992; Pestova et al., 2000b). Under these conditions, 48S complexes are readily detected in the absence of SXL (Figure 2, blue lines). As expected, binding of SXL to both UTRs of the indicator mRNA strongly reduces the formation of 48S initiation complexes (Figure 2A, red line; Gebauer et al., 2003). This reduction is specific because the initiation profile of a control mRNA lacking SXL binding sites remains unchanged in the presence of SXL (Figure 2D). Reduction of 48S initiation complex formation is also observed, albeit less efficiently, when SXL is restricted to bind either to the 5′ or to the 3′UTR of the mRNA (Figures 2B and 2C, red lines). This shows that 5′ and 3′UTR bound SXL can independently interfere with translation initiation prior to 48S complex formation.

Interestingly, the repressed mRNPs redistribute to different positions in the gradient depending on whether SXL acts from the 5′ or the 3′UTR. Inhibition mediated by the 5′UTR shifts the repressed mRNP into a light fraction (peak in fraction number 16, Figure 2C). By contrast, translational repression via the 3′UTR binding sites is associated with mRNAs that sediment as unusually heavy RNP particles between fractions 11 and 16 (compare red lines in Figures 2B and 2C and Figures S1B and S1C in the Supplemental Data available with this article online). The efficiently repressed BSEF mRNPs redistribute in a fashion that reflects the integral of the two single-site mutants (Figures 2A and S1A).

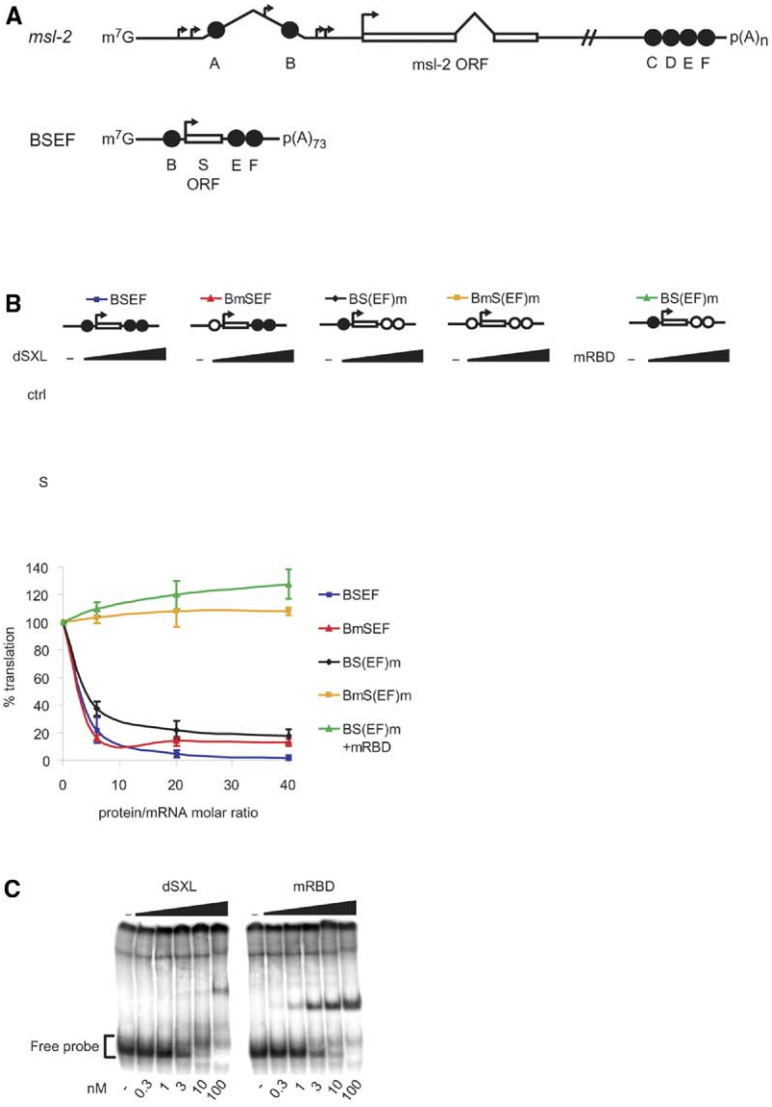

Figure 1. Inhibition of Translation by 5′UTR and 3′UTR Bound SXL

(A) Schematic representation of the wild-type *msl-2* mRNA (3992 nt) and the minimal reporter BSEF mRNA (375 nt). The SXL binding sites are denoted A to F (black ovals). BSEF mRNA was derived by fusing 69 nt of *msl-2* 5′UTR containing site B and 46 nt of the *msl-2* 3′UTR including sites E and F to a synthetic Flag-tag containing short open reading frame (sORF) (Gebauer et al., 2003).

(B) BSEF (blue line), BmSEF (red line), BS(EF)m (black line), and BmS(EF)m (orange line) mRNAs were translated in *Drosophila* embryo extract in the presence of increasing amounts of GST-SXL (64 kDa) or GST-mRBD (45 kDa) (BS(EF)m, green line). CAT mRNA was cotranslated as an internal control. ^{35}S-methionine-labeled translation products were selected by immunoprecipitation with α-Flag and α-CAT antibodies, resolved on a 15% denaturing polyacrylamide gel (upper panels) and quantified by phosphorimaging (lower panel). The Flag-sORF values were corrected for CAT translation levels. Translation values were plotted as a percentage of the translation activity obtained in the absence of recombinant protein against the molar ratio of protein to mRNA. The results from four independent experiments are shown with an indication of experimental variability. Black ovals represent SXL binding sites; white ovals denote mutations thereof.

(C) RNA binding of SXL and mRBD to the 5′UTR B site. A gel mobility-shift experiment was performed with increasing concentrations of recombinant GST-SXL or GST-mRBD and a ^{32}P-labeled probe comprising the first 93 nucleotides of the BSEF-uAUG mRNA (see Figure 3), including the wild-type site B.

As predicted from the data in Figure 1B, mRBD does not interfere with 48S initiation complex formation on BSEF and BS(EF)m mRNAs (Figures 2A and 2C), indicating that SXL inhibits BSEF mRNA translation by the bona fide mechanism. We conclude that binding of SXL exclusively to the 5′ or the 3′UTR suffices to interfere with translation initiation prior to stable 48S initiation complex formation and that the efficiency of inhibition is strongly increased when both UTRs function simultaneously.

5′ and 3′UTR Bound SXL Targets Two Distinct Initiation Steps

The formation of a stable 48S initiation complex involves binding of the 43S preinitiation complex to the 5′ end of the mRNA and subsequent scanning of the 5′UTR to the initiation codon (Pestova et al., 1998). In principle, both steps could be targeted by SXL. To distinguish between a 43S recruitment block and a scanning block, we introduced an AUG initiation codon upstream of the SXL binding site B in the 5′UTR of the mRNA and in-frame with the normal AUG initiation codon (Figure 3). We reasoned that translation from both the upstream and the normal AUG should be equally inhibited if SXL targets the initial recruitment of the 43S complex (Figure 3A). Alternatively, if SXL blocks ribosomal scanning, translation from the normal downstream AUG should be repressed, while translation from the upstream AUG may remain unaffected if the processive elongating 80S ribosome can negotiate the SXL block for scanning 43S complexes. We tested the ability of SXL to inhibit translation from both AUGs in a transcript containing wild-type SXL binding sites, which we dubbed BSEF-uAUG.

Figure 3B shows that SXL represses translation from both AUGs to a different extent (left panel, note the weaker inhibition of uAUG-S compared to AUG-S). While inhibition of translation from the normal AUG (AUG-S) is as efficient as that observed for the equivalent single AUG of BSEF mRNA, repression of translation from the

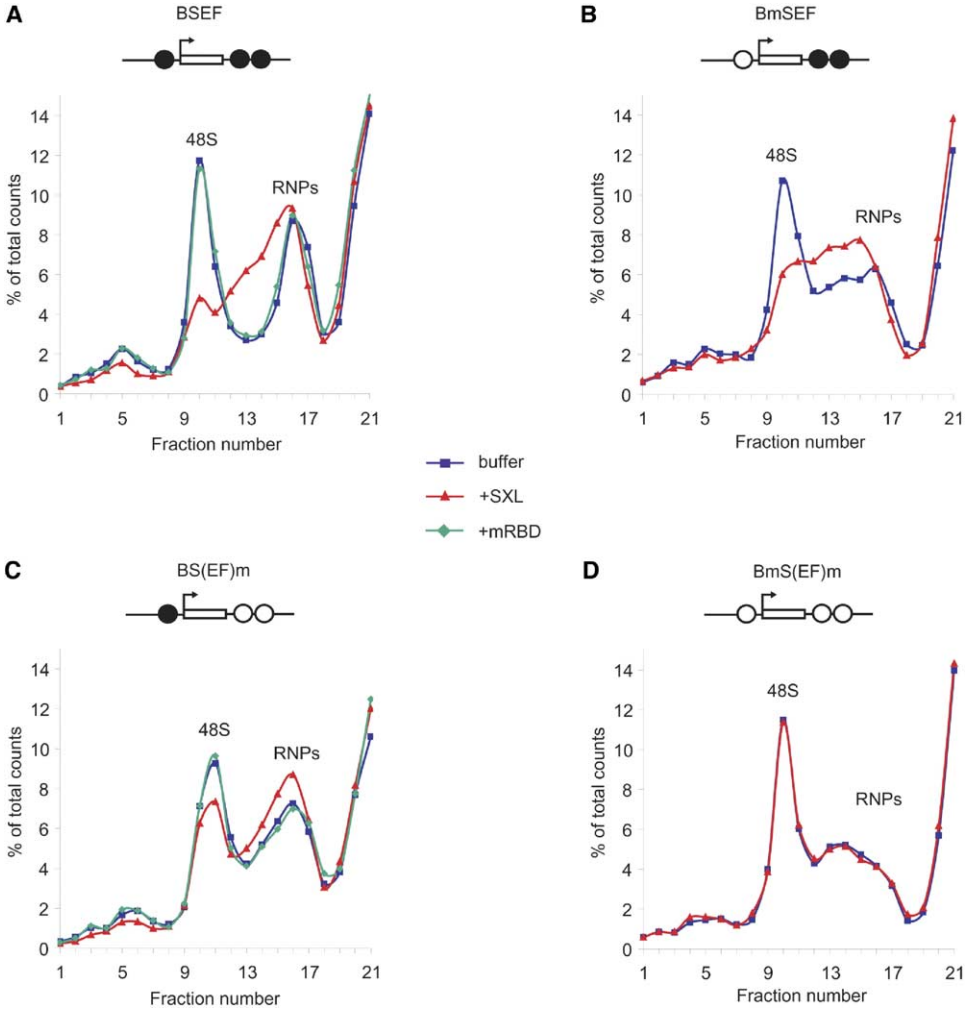

Figure 2. SXL Bound to Either UTR Inhibits 48S Preinitiation Complex Formation

In vitro translation reactions containing ^{32}P-radiolabeled BSEF (A), BmSEF (B), BS(EF)m (C), or BmS(EF)m (D) mRNAs were performed in the presence of cycloheximide and GMP-PNP and supplemented by either protein buffer (blue line), SXL (red line), or mRBD (green line). The reactions were loaded on 5%–25% linear sucrose density gradients and complexes were resolved by centrifugation. Fractions were taken from the bottom of the gradient and analyzed by scintillation counting. Radioactivity is expressed as percentage of total recovered counts, plotted against the fraction number. For averaged graphs of three independent experiments, see Figure S1.

upstream AUG (uAUG-S) is far less effective but comparable to that of BmSEF mRNA (compare left panel of Figure 3B with Figure 1B and the respective quantifications). By contrast, translation from both AUGs remains unaffected in a transcript containing mutated SXL binding sites (Figure 3B, BmS(EF)m-uAUG panel). These results reflect the predictions made for the involvement of a scanning block in that translational repression by SXL is partially derepressed when translation initiates upstream of the B site. However, we noted that translation from the uAUG is still significantly inhibited by SXL. This may suggest that SXL can also inhibit translation elongation in the context of the uAUG constructs. Alternatively, it could reflect SXL control of a step in addition to scanning when operating from both its 5′ and 3′UTR binding sites.

To distinguish between these two alternatives, we next examined whether SXL binding to either region contributes to a single regulatory mechanism or whether 5′ and 3′UTR bound SXL proteins act through different mechanisms targeting distinct initiation steps. We analyzed the effect of SXL on translation of the upstream AUG containing mRNAs in the context of mutated 5′ or 3′ SXL binding sites. The inhibition patterns of the two mutant mRNAs are remarkably distinct (Figure 3B, BmSEF-uAUG and BS(EF)m-uAUG panels). SXL acting only from the E and F sites in the 3′UTR inhibits translation from both AUGs precisely to the same extent, indicating that SXL interferes with the translation initiation pathway prior to scanning of 43S complexes (Figure 3B, BmSEF-uAUG panel). On the other hand, SXL bound only to the 5′UTR inhibits translation from the normal AUG, but completely fails to interfere with translation from the upstream AUG. This result strongly implicates a scanning block for translational inhibition mediated by the 5′ B site (Figure 3B, BS(EF)m-uAUG

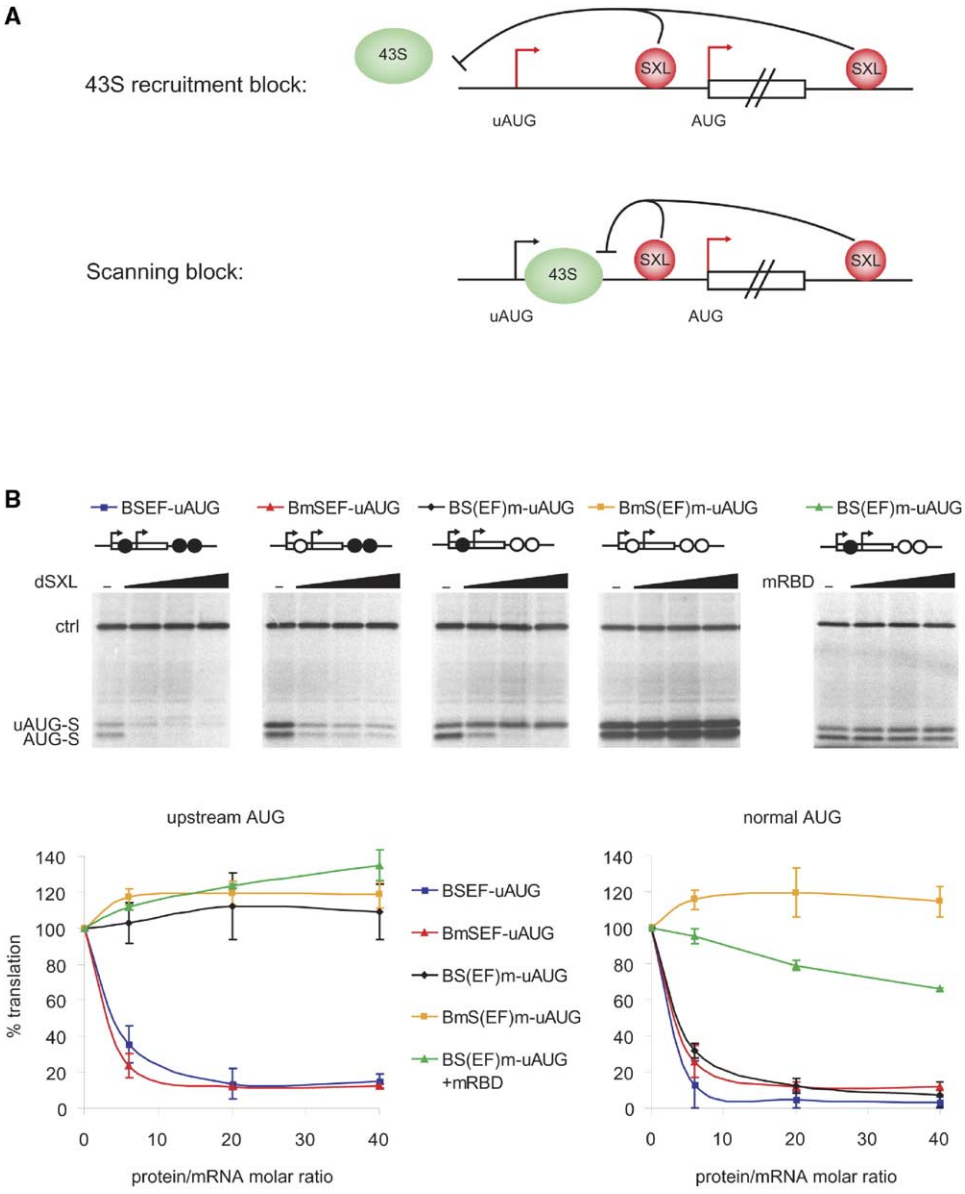

Figure 3. SXL Bound to the 5′ and 3′UTR, Respectively, Acts via Distinct Mechanisms

(A) Schematic representation of the experimental rationale. An AUG initiation codon was introduced upstream of SXL binding site B (uAUG). If translation is inhibited by blocking 43S complex recruitment (upper scenario), translation from both AUGs is affected similarly. If SXL inhibits scanning to the (downstream) AUG initiation codon, initiation at the uAUG is predicted to be less sensitive to SXL than is initiation at the normal AUG. Red arrows indicate AUGs at which translation initiation is predicted to be sensitive to SXL-mediated inhibition; black arrows indicate AUGs at which translation initiation remains unaffected in the presence of SXL.

(B) BSEF-uAUG (blue line), BmSEF-uAUG (red line), BS(EF)m-uAUG (black line), and BmS(EF)m-uAUG (orange line) mRNAs were translated in the presence of increasing amounts of SXL or mRBD (BS(EF)m-uAUG, green line), and the reactions were analyzed as described in Figure 1B. Representative experiments are shown on the upper panels. The quantified translation values from the upstream AUG are presented in the lower left panel, and values from the normal AUG are shown on the lower right panel. An average of three independent experiments is shown, except for the BSEF-uAUG mRNA, where only two experiments were averaged because low signal-to-noise ratios did not allow reliable quantification in some experiments. Error bars indicate experimental variability.

panel). Although mRBD as a control slightly inhibits translation from the normal AUG of the BS(EF)m-uAUG mRNA, this inhibition appears not to be very significant compared to SXL-mediated repression of the same mRNA. We also observed a slight enhancement of translation from the upstream AUG by mRBD (Figure 3B, right panel and the respective quantifications). This effect might reflect a minor change of the relative usage of the two AUG codons due to mRBD binding. Similar effects have been described for hairpin structures between AUGs (Kozak, 1990).

We conclude that the different inhibition patterns of

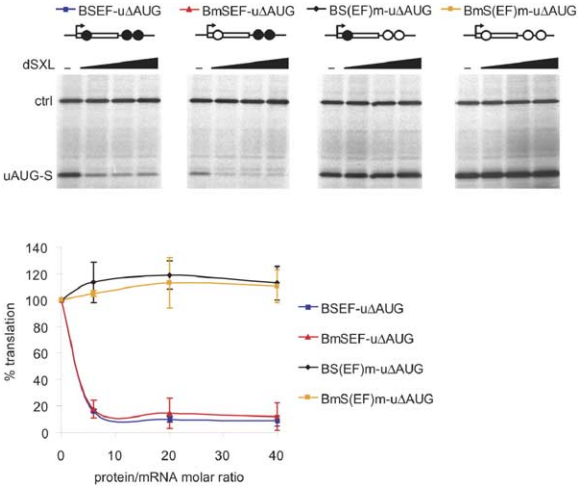

Figure 4. Confirmation of the Bifunctional Mode of Action of SXL with a Single Initiation Codon Reporter

BSEF-uΔAUG (blue line), BmSEF-uΔAUG (red line), BS(EF)m-uΔAUG (black line), and BmS(EF)m-uΔAUG (orange line) mRNAs, containing exclusively the upstream AUG were translated in the presence of increasing amounts of SXL and analyzed as described in Figure 1B. The results from three independent experiments are shown with an indication of experimental variability.

the 5′ and 3′ mutant mRNAs indicate distinct contributions of the two UTRs to the inhibitory mechanism. Recalling the rationale behind the upstream AUG-containing constructs, SXL binding to the B site elicits an inhibition pattern that perfectly meets the predictions of a scanning block. Since mRBD is not able to substitute for SXL, this block is not a simple sterical one. By contrast, 3′UTR-mediated repression acts even earlier on the initiation pathway as reflected by an inhibitory pattern typical for a block of 43S complex recruitment.

Inhibition of Both Ribosome Recruitment and Scanning Ensures Tight *msl-2* mRNA Regulation by SXL

Translation initiation on an mRNA containing two initiation codons in a similar context raises the possibility that the presence of one would affect the function of the other. To eliminate this possible concern, we mutated the normal ATG initiation codon in the constructs bearing the upstream ATG to an ATT sequence. This mutation permitted us to investigate the effect of SXL on translation initiation upstream of the 5′ B site independent of a potentially interfering downstream initiation event. The resulting mRNA (BSEF-uΔAUG) is repressed with the same efficiency as a transcript in which the 5′ B site has been inactivated (Figure 4, compare BSEF-uΔAUG with BmSEF-uΔAUG; also compare these mRNAs with BmSEF in Figure 1B). Conversely, mutation of the 3′UTR SXL binding sites in this context completely abrogates translational inhibition (Figure 4, BS(EF)m-uΔAUG panel), indicating that repression occurs exclusively via the 3′UTR when translation initiates upstream of the B site.

Furthermore, we analyzed the translation initiation complexes formed on BSEF-uΔAUG mRNA and derivatives lacking 5′ or 3′UTR SXL binding sites by sucrose density gradient analysis (Figure 5). As expected, SXL does not affect 48S initiation complex formation on the control mRNA lacking SXL binding sites (Figure 5D). In contrast to the BSEF and BmSEF mRNAs, where mutation of the B site partially derepressed 48S complex formation (compare Figures 2A and 2B), initiation on the upstream AUG of the BSEF-uΔAUG and BmSEF-uΔAUG mRNAs is affected almost identically, demonstrating that site B fails to contribute to inhibition in this context (compare Figures 5A and 5B). Importantly, SXL bound only to site B completely fails to affect 48S initiation complex formation on the upstream AUG (Figure 5C). These results provide strong functional evidence for a scanning block imposed by SXL bound to the 5′UTR without interfering with the initial 43S complex recruitment.

To obtain direct physical evidence for the regulatory mechanisms implicated in the respective functions of the 5′ and 3′UTR, we established a toeprint assay to identify translation complexes that bind to the mRNA in the *Drosophila* embryo extract. To this end, translation complexes were allowed to assemble on mRNAs bearing the 5′ or 3′UTR regulatory sites in the presence or absence of SXL. Annealing of a primer to the ORF and subsequent extension by reverse transcription identifies cDNA products arrested at the leading edge of bound ribosomal complexes. Ribosomal complexes at the initiation codon of the mRNA have been reported to yield toeprints 15–17 nucleotides 3′ of the AUG (Anthony and Merrick, 1992), whereas 43S complexes recruited to the 5′ end of the mRNA have been identified by a toeprint mapping 21–24 nucleotides downstream from the cap structure (Pestova et al., 1998). As expected, a strong toeprint is detected on BS(EF)m mRNA just downstream of the AUG codon in the absence of SXL (Figure 6A, lane 7). In the same lane, recruitment to the 5′ end of the mRNA can also be identified. Both complexes are specific and require ongoing translation initiation, because they are profoundly inhibited by (1) addition of cap analog before (Figure 6A, lane 3) but not after (Figure 6A, lane 5) the translation initiation reaction, (2) incubation on ice instead of 25°C (Figure 6A, lane 1), and (3) incubation in buffer instead of extract (Figure 6A, lane 10). Binding of SXL to site B yields a toeprint of somewhat variable intensity at the appropriate position on the naked RNA (Figure 6A, lane 11) as well as after incubation in translation extract (Figure 6A, lanes 2, 4, 6, 8, lane 9 for mRBD). As predicted by the functional data, SXL strongly inhibits the formation of a ribosomal toeprint at the AUG codon of BS(EF)m mRNA (Figure 6A, lanes 6 and 8, see also lane 4). The inhibition of this toeprint is accompanied by the appearance of a strong, new toeprint upstream of the SXL binding site (Figure 6A, lanes 6 and 8). By all criteria, this toeprint is specific to site B-mediated inhibition, including the lack of this toeprint from the reaction containing mRBD instead of SXL (Figure 6A, lane 9). These results provide direct physical evidence for a stalled complex.

Our model of the scanning block mechanism implies that SXL bound to the 5′UTR blocks scanning 43S complexes whereas translating 80S ribosomes traverse the SXL binding site unhampered. To directly test this mechanistic model, we investigated the ability of SXL

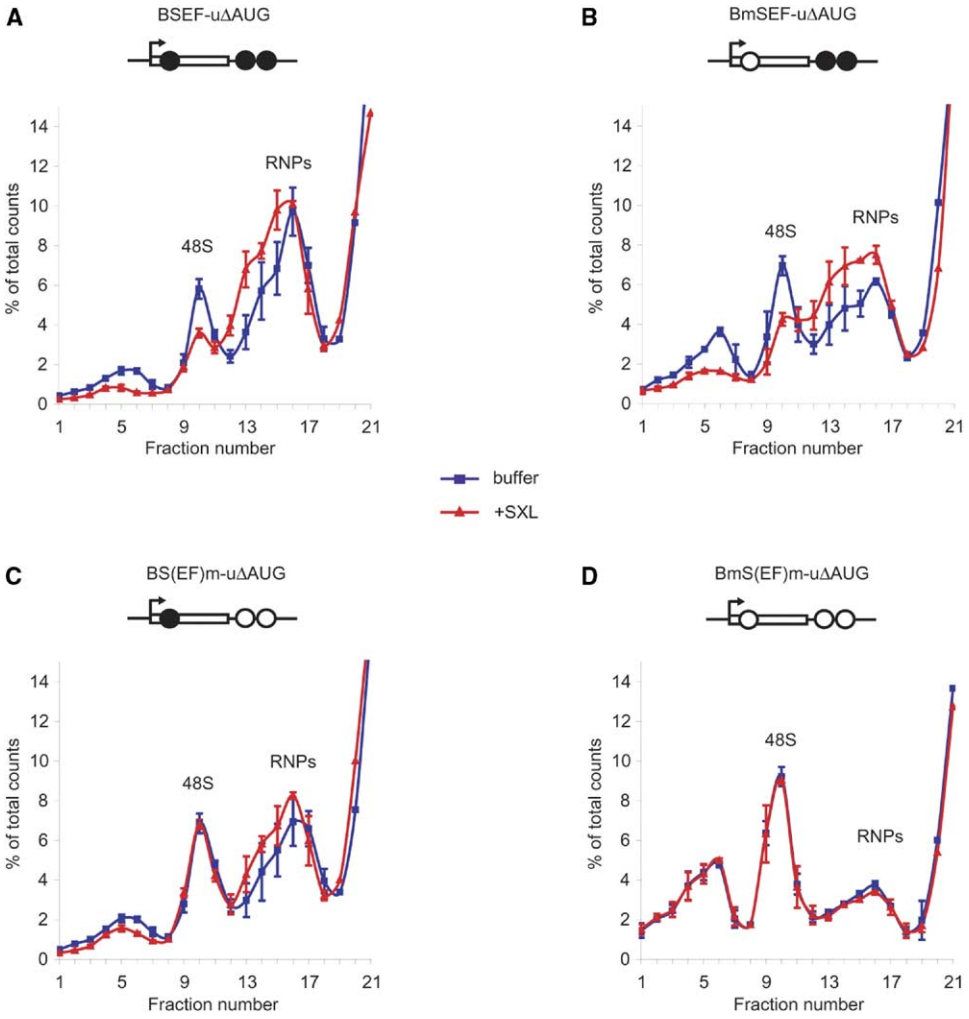

Figure 5. 3′UTR but Not 5′UTR Bound SXL Inhibits 48S Initiation Complex Formation on an Initiation Codon Upstream of the B Site

In vitro translation reactions containing ^{32}P-labeled BSEF-uΔAUG (A), BmSEF-uΔAUG (B), BS(EF)m-uΔAUG (C), or BmS(EF)m-uΔAUG (D) mRNAs were performed in the presence of cycloheximide and GMP-PNP, with (red line) or without (blue line) added SXL. Reactions were analyzed by sucrose density gradient centrifugation as described in Figure 2. For each mRNA, an averaged graph of three independent experiments is shown together with an indication of experimental variability.

bound to the B site to stall translating 80S ribosomes that initiate on the uAUG of BS(EF)m-uΔAUG mRNA by toeprinting analysis (Figure 6B). In contrast to the inhibition of 80S complex formation at the initiation codon of BS(EF)m mRNA accompanied by stalling of 43S complexes upstream (Figure 6B, lanes 9 and 10, see also Figure 6A), SXL binding to the B site does not affect 80S complex formation at the uAUG of BS(EF)m-uΔAUG mRNA (Figure 6B, red stars, lanes 3 and 4) in the presence of cycloheximide. When cycloheximide is omitted to allow translation elongation, the toeprints corresponding to stalled 43S complexes on the BS(EF)m mRNA are also present, although the signal is less strong. In contrast, such a toeprint is clearly absent when SXL is left out from the reaction (Figure 6B, lane 8) or, importantly, when 80S complexes negotiate the B site in BS(EF)m-uΔAUG (Figure 6B, lane 6). In agreement with the fact that SXL binding to the B site does not inhibit translation from the uAUG (Figures 3 and 4), this result shows that the mechanism exerted by SXL bound to site B inhibits 43S scanning while being unable to hinder translating 80S ribosomes.

Analysis of the 3′UTR regulatory mechanism by toeprinting on BmSEF mRNA shows a different picture, as predicted by the functional data. While toeprint formation at the AUG codon is also inhibited by SXL (Figure 6C, compare lanes 5 and 7 with lanes 6 and 8), no SXL toeprints or complexes stalled within the 5′UTR can be detected. While SXL binding to site B caused a small increase in the signal of recruited 43S complexes at the 5′ end, which may indicate "queuing" behind the stalled scanning complex (Figure 6A), SXL binding to the 3′UTR effectively inhibits this toeprint (Figure 6C, lanes 6 and 8). Importantly, SXL does not cause this effect on BmS(EF)m mRNA that lacks the 3′UTR binding sites (Figure 6C, lane 10).

Taken together, the toeprint experiments provide direct physical evidence for a scanning block imposed

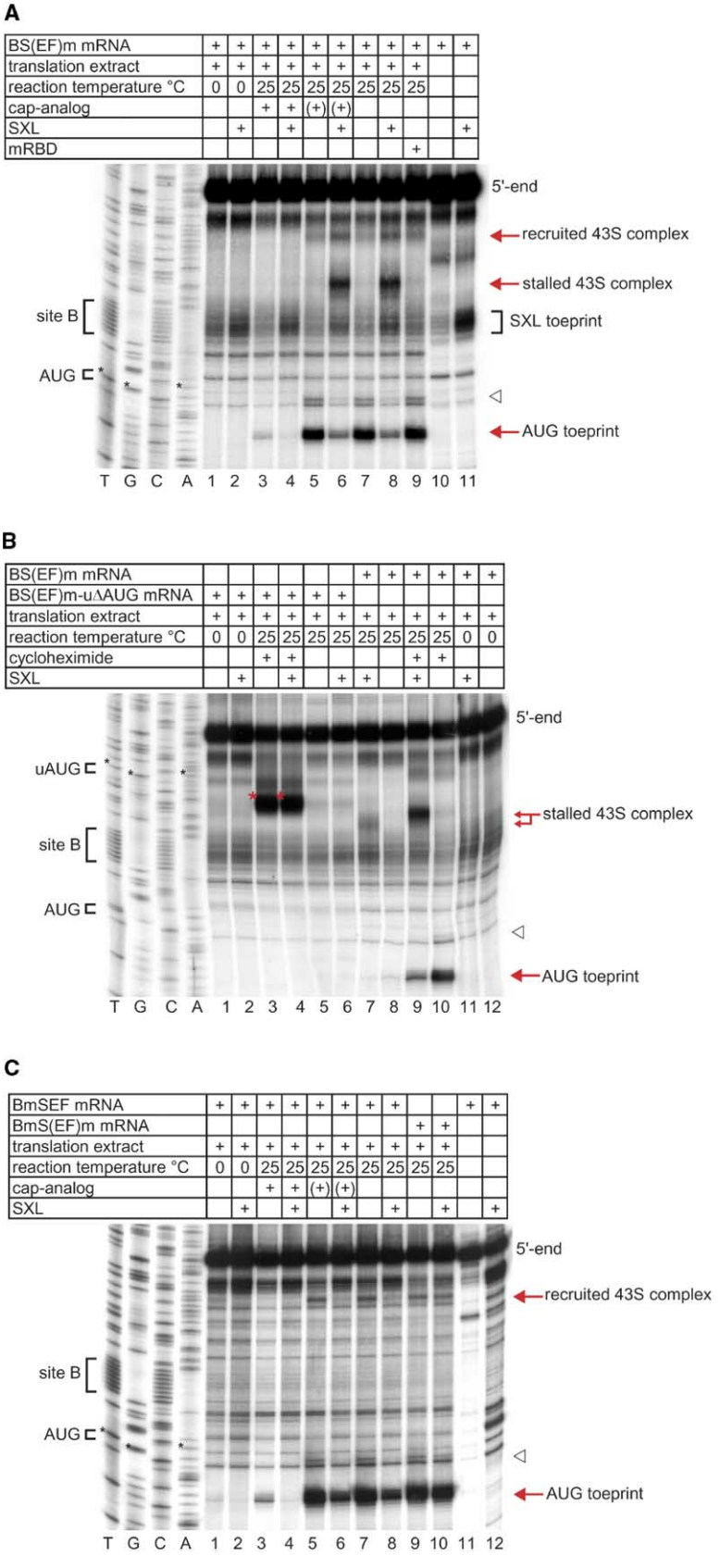

Figure 6. Physical Evidence for the Proposed Regulatory Mechanisms by Toeprinting

(A) Ribosomal complexes were allowed to assemble on the mRNA at 0°C (lanes 1 and 2) or at 25°C (lanes 3–9) in in vitro translation reactions containing cycloheximide and either protein buffer (lanes 1, 3, 5, and 7), SXL (lanes 2, 4, 6, and 8), or mRBD (lane 9). Translation initiation was blocked by adding cap analog to the in vitro translation reactions in lanes 3 and 4. In lanes 5 and 6, cap analog was added after ribosomal complex assembly on the mRNA (marked with (+) in the legend). The products of the reverse transcriptase control reaction on the BS(EF)m mRNA in buffer in the presence or absence of SXL are shown in lane 10 and 11. 80S ribosomal complexes at the initiation codon are detected as toeprints resulting from a stop of the reverse transcriptase reaction 16–18 nucleotides 3′ from the AUG of the mRNA. The toeprint marked with an open arrowhead indicates the presence of ribosomal complexes bound to a GUG codon, which resides 18 nucleotides upstream of this toeprint. The toeprint ~26 nucleotides downstream of the 5′ end is the result of 43S complexes bound to the 5′ end of the mRNA. Lanes T, G, C, and A represent the sequence of BmS(EF)m mRNA, obtained from the primer used in the toeprint assay.

(B) Ribosomal toeprint assay as described for (A) using BS(EF)m-uΔAUG (lanes 1–6) or BS(EF)m mRNA (lanes 7–12). Red stars in lanes 3 and 4 indicate toeprints of 80S complexes bound at the uAUG of BS(EF)m-uΔAUG mRNA. Cycloheximide was omitted from the reactions in lanes 1, 2, 5–8, 11, and 12 to allow translation elongation beyond the uAUG. Under these conditions, the toeprint corresponding to stalled 43S complexes on the BS(EF)m mRNA reproducibly migrates slightly faster than in the presence of cycloheximide. Importantly, all specificity criteria are fully met.

(C) Ribosomal toeprint assay as described for (A) using BmSEF (lanes 1–8, 11, and 12) or BmS(EF)m mRNA (lanes 9 and 10). A representative example of three independent experiments is shown.

by 5′UTR bound SXL. To the best of our knowledge, a physiological example of scanning being regulated by an RNA binding protein has not been reported before. Furthermore, the inhibition of the 5′ end proximal toeprint by SXL bound to the E and F sites (Figure 6C), in combination with the fact that 3′UTR-mediated inhibition by SXL is independent of the 5′ B site and the position of the AUG (Figures 3–5), demonstrates that SXL bound to the 3′UTR interferes with the initial recruitment of the small ribosomal subunit. Therefore, the molecular basis of the efficient inhibition of *msl-2* translation by SXL rests on an integrated mechanism with a block of two consecutive initiation steps, mediated separately by the 5′ and the 3′UTRs of the *msl-2* mRNA.

Discussion

We have investigated how SXL silences translation of the *msl-2* mRNA. In female flies, this regulation prevents the formation of the dosage compensation complex and thus deleterious hypertranscription of the two female X chromosomes. Our results define the molecular basis of this critical regulatory step. They also reveal a mechanism of translational control that is based on the integration of two separable components. Individually, each of the two components provides insights into functional properties of 5′ and 3′ regulatory complexes to interfere with translation initiation, and each of these two components appears to be mechanistically unprecedented.

SXL Controls Dosage Compensation as a Bifunctional Translational Regulator

In male flies, MSL-1 and MSL-2 mediate the assembly of the DCC on the single X chromosome, which is thought to spread along the entire chromosome promoting an ~2-fold increase in transcription levels (Gilfillan et al., 2004). The absence of MSL-2 in females does not allow DCC formation, while transgenic female flies expressing MSL-2 assemble the complex, showing that MSL-2 is the limiting subunit of the DCC (Bashaw and Baker, 1995; Kelley et al., 1995; Zhou et al., 1995). Although the *msl-2* transcript levels in females are reduced to 20%–30% of those in male flies (Zhou et al., 1995; Bashaw and Baker, 1997; Kelley et al., 1997), translational control mediated by SXL is crucial to block MSL-2 expression.

SXL bound to either UTR of *msl-2* mRNA inhibits translation by interfering with initiation prior to 48S complex formation (Figures 1 and 2; Gebauer et al., 2003). Furthermore, this work shows that the two regulatory regions independently interfere with translation initiation by different means (Figures 3–5). Earlier evidence suggested that the roles of SXL bound to the 5′UTR and the 3′UTR, respectively, are noninterchangeable: first, the 5′UTR SXL binding site B cannot substitute for sites E and F when introduced into the 3′UTR (Gebauer et al., 2003). Second, UV-crosslinking experiments identified at least one protein that is recruited by SXL specifically to the 3′UTR (Grskovic et al., 2003). The crosslinks require the sequences that flank the E and F sites (see Figure 7), and RNA competition experiments functionally implicated this 3′UTR binding protein in SXL-mediated translational control; by contrast, crosslinks to the 5′UTR are limited to SXL itself (Grskovic et al., 2003). The simplest interpretation of this earlier work was that both UTRs engage in a functional and perhaps physical interaction to block the stable engagement of the small ribosomal subunit with the mRNA at a single step.

This work now reveals that such a simple assumption is incorrect. Rather than forming a single repressor complex that targets one step in the initiation pathway, SXL acts as a bifunctional regulator: 3′UTR bound SXL inhibits translation from cap-proximal and cap-distal AUGs identically well (Figure 3), while 5′UTR bound SXL can only function when it binds upstream of the initiation codon (Figures 3–5). Based on these data and direct physical evidence provided by the toeprint experiments (Figure 6), we conclude that SXL bound to the 3′UTR impedes the initial recruitment of 43S complexes to the mRNA while 5′UTR bound SXL stalls scanning 43S complexes upstream of its binding site.

How does SXL binding to the B site achieve a scanning block? Apparently, SXL hinders the transit of 43S complexes across site B (Figure 6A). Steric hindrance of ribosomal scanning has been proposed for IRP/IRE complexes that were introduced ~100 nucleotides downstream from the cap structure of a reporter mRNA (Paraskeva et al., 1999). However, scanning inhibition by SXL bound to the B site does not appear to follow a simple steric mechanism, i.e., to be imposed solely by high affinity mRNA binding. mRBD fails to repress translation or stall scanning 43S complexes, although it binds to site B with an affinity as high as that of SXL (Figures 1–3 and 6). Furthermore, tethering of a λ peptide-SXL fusion protein to a BoxB element replacing SXL binding site B in the 5′UTR of *msl-2* mRNA does not inhibit translation despite the high affinity of the λ peptide-BoxB interaction (Grskovic et al., 2003).

Therefore, we propose that SXL regulates scanning either by altering the 5′UTR secondary structure and/or promoting the formation of a higher-order assembly on the B site. Such a complex could then act as a (steric) roadblock to scanning, without being able to halt elongating 80S ribosomes (Figure 6B). Alternatively, SXL and potential interacting proteins may specifically interfere with the function of a translation initiation factor or the small ribosomal subunit which is required for scanning but not for translation elongation. Interestingly, site B is composed of 16 uridine residues that could be bound by a SXL dimer (Wang et al., 1997). Since SXL engages in protein-protein interactions through its RNA binding domains (Deshpande et al., 1996; Sakashita and Sakamoto, 1996; Wang et al., 1997; Samuels et al., 1998; Dong and Bell, 1999), SXL dimerization and additional factors recruited by SXL may promote the formation of a higher-order repressor complex that blocks scanning, in as much as a stalled elongating ribosome can function as a block to scanning (Gaba et al., 2001).

How does SXL bound to the 3′UTR inhibit 43S preinitiation complex recruitment? The recruitment of the 43S complex represents a previously identified target of 3′UTR binding translational regulators. For example,

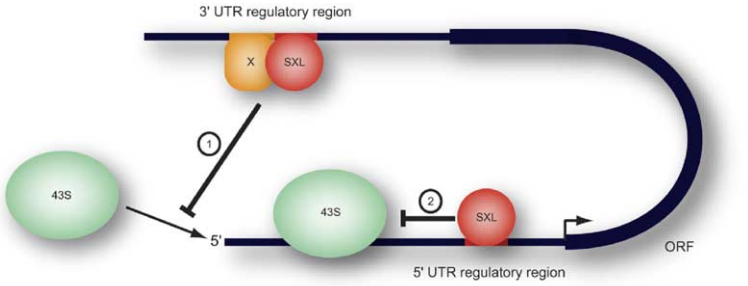

Figure 7. A Failsafe Mechanism Explains the SXL-Mediated Silencing of *msl-2* mRNA Translation

The cartoon illustrates the two early translation initiation steps, 43S complex recruitment, and subsequent scanning of the 5′UTR. Note that translational repression of the *msl-2* mRNA by the 3′UTR repressor complex involves an additional factor X that has been shown to be recruited by SXL depending on the sequences flanking sites E and F but remains to be identified (Grskovic et al., 2003). The 3′UTR corepressor complex blocks 43S preinitiation complex recruitment independently of 5′UTR bound SXL. Those 43S complexes that escape this block are subsequently challenged by SXL bound to the 5′UTR, which inhibits ribosomal scanning.

Maskin, Cup, and Bicoid block the recruitment of the 43S complex by interfering with the assembly of eIF4F at the cap structure (Richter and Sonenberg, 2005). However, SXL-mediated inhibition is independent of the cap structure (Gebauer et al., 2003), implying that the 43S complex recruitment block imposed by SXL is different from that mediated by these regulators.

We noticed that 3′UTR-mediated translational inhibition by SXL involves the accumulation of the repressed mRNA within unusually heavy RNP particles (see Figures 2, 5, and S1). Interestingly, SXL has previously been found in large, RNase-sensitive complexes sedimenting faster than bulk mRNPs in sucrose density gradient experiments (Samuels et al., 1994). An attractive possibility is that SXL in association with the 3′UTR corepressor (X in Figure 7) nucleates a large repressor complex or that the 3′UTR repressor complex promotes the multimerization of mRNPs leading to the formation of mRNP clusters. Multimerization of mRNPs has been observed during the localization of translationally silent *bicoid* mRNA to the anterior pole of the *Drosophila* oocyte (Ferrandon et al., 1994, 1997). Clustered mRNAs may be less accessible to the translation initiation machinery, providing a possible mechanism of 3′UTR-mediated inhibition of 43S complex recruitment independent of mRNA-specific 3′ to 5′ end communication.

A Failsafe Mechanism of Translational Control

What are the biological advantages of the duality of translational control by SXL? Such an integrated failsafe mechanism allows efficient repression of translation in situations where the leakiness of a single mechanism could be deleterious for the cell and/or the organism. Indeed, forced expression of the MSL-2 protein in female flies enables the loading of the dosage compensation complex onto the two X chromosomes and causes lethality (Bashaw and Baker, 1995; Kelley et al., 1995; Zhou et al., 1995). Therefore, the translational repression of female *msl-2* mRNA must be robust, which is achieved by the combination of the two mechanisms that cooperate to prevent 43S complexes from reaching the initiation codon.

Oskar (*osk*) mRNA translation in *Drosophila* oocytes also appears to be regulated at multiple steps. The synthesis of the posterior determinant Osk must be strictly restricted to the posterior pole of the embryo. This is achieved by the posterior accumulation of *osk* mRNA and the translational repression of unlocalized *osk* mRNA (Ephrussi et al., 1991; Kim-Ha et al., 1991; Ephrussi and Lehmann, 1992; Rongo et al., 1995). The protein Bruno binds to Bruno-response elements (BREs) in the 3′UTR of the *osk* mRNA and is important for inhibition of translation (Kim-Ha et al., 1995; Castagnetti et al., 2000). The fact that Bruno interacts with the repressor Cup suggested that the responsible mechanism targets translation initiation (Wilhelm et al., 2003; Nakamura et al., 2004), although this has not been shown directly. A recent report identified a significant fraction of unlocalized *osk* mRNA in association with ribosomes, indicating that translation of the *osk* mRNA may be regulated (in addition) at a postinitiation step (Braat et al., 2004). Similar observations implicating multiple levels of translational control have also been made for the spatially and temporally controlled *nanos* mRNA in *Drosophila* embryos. In this case, both mechanisms may not be simultaneously active at all stages of development (Clark et al., 2000; Forrest et al., 2004; Nelson et al., 2004). Translational failsafe mechanisms like the one described here may become recognized as a more widespread principle of robust control over protein synthesis.

Experimental Procedures

Plasmids

The BSEF, BS(EF)m, and BmS(EF)m plasmids have been described (Gebauer et al., 2003). To obtain BmSEF, a SacI/HpaI fragment containing the B site in the BSEF plasmid was replaced by a similar fragment originating from the BmS(EF)m plasmid.

To generate the plasmids BSEF-uAUG, BmSEF-uAUG, BS(EF)m-uAUG, and BmS(EF)m-uAUG, the sequence ATTA (positions 31–34) in the 5′UTR of the above described plasmids was changed to ATGG by oligonucleotide-directed mutagenesis. In these plasmids, the ATG at position 100–102 was then changed to ATT by oligonucleotide-directed mutagenesis to obtain BSEF-uΔAUG, BmSEF-uΔAUG, BS(EF)m-uΔAUG, and BmS(EF)m-uΔAUG.

In Vitro Transcription and Translation

RNAs were synthesized as described (Gebauer et al., 1999). All mRNAs contained a 5′ m^7GpppG cap and a poly(A) tail of 73 nucleotides. The 5′ B probe was transcribed from the BSEF-uAUG plasmid linearized with SmaI.

Drosophila embryo extracts were prepared and cell-free transla-

tion reactions carried out as previously described (Gebauer et al., 1999). In vitro translation reactions were performed with each 65 fmol of the template mRNA and the control CAT mRNA in a final volume of 10 μl. GST-SXL or GST-mRBD was added at a 0×, 6×, 20×, or 40× molar excess with respect to the template mRNA. Translation products were immunopreciptitated with monoclonal α-Flag (Sigma) and monoclonal α-CAT antibodies and separated on a 15% denaturing polyacrylamide gel. Translation efficiencies were quantified by 2D densiometry using a phosphorimager.

Recombinant Protein

Proteins were expressed in *E. coli* as GST fusions and purified as described previously (Grskovic et al., 2003). GST-mRBD was dialyzed against 20 mM HEPES (pH 8.0), 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.01% NP40, and GST-SXL against the same buffer containing 100 mM KAc.

Sucrose Density Gradient Analysis

Translation initiation intermediates on radiolabeled mRNAs were assembled in 50 μl in vitro translation reactions, containing 2 mM magnesium acetate, 1 mM cycloheximide, and 1 mM GMP-PNP. Where indicated, GST-SXL or GST-mRBD protein was added at 17× molar excess with respect to the mRNA. In Figure 2, reactions containing 0.23 pmol mRNA were incubated for 10 min at 25°C. In Figure 5, 0.69 pmol mRNA were used and the reactions were incubated for 30 min at 25°C. The reactions were then diluted 1:1 with sucrose gradient buffer (24 mM HEPES [pH 7.4], 100 mM KAc, 2 mM MgAc, 1 mM DTT) and loaded on top of a linear 5%–25% sucrose gradient (4.5 ml). After centrifugation at 45,000 rpm for 83 min at 4°C in an SW55Ti rotor, fractions were collected from the bottom of the gradient and analyzed by scintillation counting.

Gel Mobility Shift Assays

In vitro transcribed ^{32}P-labeled RNA containing 93 nt (positions 1–93) of the BSEF-uAUG 5′UTR was incubated with increasing amounts of recombinant protein as described previously (Grskovic et al., 2003). RNA-protein complexes were separated on a 4% nondenaturing polyacrylamide gel at 4°C.

Toeprint Assay

After incubation of the mRNA at 95°C for 30 s, translation initiation intermediates were assembled in 5 μl in vitro translation reactions, containing 60 mM KAc, 2 mM MgAc, 1 mM cycloheximide and 0.23 pmol mRNA. In Figures 6A and 6B, GST-SXL or GST-mRBD protein were added at a 20× molar ratio; in Figure 6C, GST-SXL was added at a 60× molar ratio with respect to the mRNA. Where indicated, 4 mM m^7GpppG cap analog was added. The reactions were incubated on ice or at 25°C for 10 min and subsequently diluted 20-fold with buffer (50 mM Tris [pH 7.5], 60 mM KCl, 6 mM $MgCl_2$, 5 mM DTT, 0.5 mM cycloheximide, 0.5 mM dCTP, dGTP, dTTP, 0.005 mM dATP and 0.2 units/μl RNasin), followed by incubation at 50°C for 105 s. This incubation was essential for achieving ribosomal toeprints. 25 pmol of the toe-1 primer, complementary to nucleotides 152–173 downstream of the AUG initiation codon were added and annealed at 37°C for 1 min. Finally, reactions were supplemented with 5 μCi of ^{32}P-dATP (~3000 Ci/mmol) and 200 units Superscript II (Invitrogen) reverse transcriptase and incubated for 15 min at 37°C. The reactions were adjusted to a total volume of 150 μl and 0.2% SDS and 10 mM EDTA (pH 8.0). Reverse transcription products were purified by phenol/chloroform extraction, precipitated in ethanol/NH_4Ac and loaded onto a 6% denaturing sequencing gel. A dideoxynucleotide sequencing ladder was obtained using the toe-1 primer and the BmS(EF)m (Figures 6A and 6C) or BmS(EF)m-uΔAUG (Figure 6B) plasmid DNA.

Supplemental Data

Supplemental Data include one figure and can be found with this article online at http://www.cell.com/cgi/content/full/122/4/529/DC1/.

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