Translational control of dosage compensation in *Drosophila* by Sex-lethal: cooperative silencing via the 5' and 3' UTRs of *msl-2* mRNA is independent of the poly(A) tail

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Translational repression of male-specific-lethal 2 (*msl-2*) mRNA by Sex-lethal (SXL) controls dosage compensation in *Drosophila*. *In vivo* regulation involves cooperativity between SXL-binding sites in the 5' and 3' untranslated regions (UTRs). To investigate the mechanism of *msl-2* translational control, we have developed a novel cell-free translation system from *Drosophila* embryos that recapitulates the critical features of mRNA translation in eukaryotes: cap and poly(A) tail dependence. Importantly, tight regulation of *msl-2* translation in this system requires cooperation between the SXL-binding sites in both the 5' and 3' UTRs, as seen *in vivo*. However, in contrast to numerous other developmentally regulated mRNAs, the regulation of *msl-2* mRNA occurs by a poly(A) tail-independent mechanism. The approach described here allows mechanistic analysis of translational control in early *Drosophila* development and has revealed insights into the regulation of dosage compensation by SXL.

Keywords: *Drosophila*/in vitro translation/*msl-2*/poly(A) tail/Sex-lethal

Introduction

In *Drosophila*, dosage compensation occurs by hypertranscription of the genes of the single male X chromosome. This is mediated by a heteromeric complex formed by the male-specific-lethal (*msl*) gene products MSL-1, MSL-2, MSL-3 and MLE (reviewed in Baker *et al.*, 1994; Kelley and Kuroda, 1995). In females, formation of the MSL complex cannot occur due to the lack of MSL-2. Expression of *msl-2* is inhibited by the female-specific RNA-binding protein Sex-lethal (SXL) in a concerted mechanism that requires, first, inhibition of *msl-2* pre-mRNA splicing in the nucleus, and then repression of *msl-2* mRNA translation in the cytoplasm (Bashaw and Baker, 1997; Kelley *et al.*, 1997; Gebauer *et al.*, 1998). To inhibit splicing, two SXL-binding sites located near the splice donor and splice acceptor regions within a 5' untranslated region (UTR) intron of *msl-2* pre-mRNA are required. These sites consist of U₁₁ and U₁₆, and SXL

binds to them with high affinity and specificity (Gebauer *et al.*, 1998). In transgenic flies, translational repression requires not only these two sites, but also a cluster of four additional sites with the sequence U₇₋₉ located in the 3' UTR (Bashaw and Baker, 1997; Kelley *et al.*, 1997). The control of translation appears to be the primordial mode for the regulation of dosage compensation by SXL, because the *msl-2* mRNA of *Drosophila virilis* does not contain an intron in the 5' UTR but retains SXL-binding sites (Bashaw and Baker, 1997). SXL is a highly conserved protein in Diptera (Bopp *et al.*, 1996; Meise *et al.*, 1998) that binds RNA via two RRM (RNA recognition motif)-type RNA-binding domains (RBDs) (Handa *et al.*, 1999). In addition, it includes a glycine/asparagine (GN)-rich N-terminal domain that has been shown to mediate cooperative interactions between SXL molecules for RNA binding (Wang and Bell, 1994), suggesting that it could represent a protein-protein interaction domain.

The translational control of *msl-2* expression by SXL is one of many examples of translational regulation during development in worms, flies, frogs and mammals. Recent work has identified some of the regulatory proteins that bind to the 5' or 3' UTRs of the regulated mRNAs (e.g. Kim-Ha *et al.*, 1995; Dubnau and Struhl, 1996; Rivera-Pomar *et al.*, 1996; Wharton *et al.*, 1998; Jan *et al.*, 1999). Many of these interactions are inhibitory, but the mechanisms by which they function are essentially unknown (Wickens *et al.*, 1996; Gray and Wickens, 1998). One exception is that of iron regulatory protein (IRP) binding to the iron-responsive element (IRE) in the 5' UTR of ferritin mRNAs. Monomeric IRP-1 or IRP-2 binds to a cap-proximal IRE, and inhibits the binding of the 43S pre-initiation complex (a complex of the small ribosomal subunit including the initiator tRNA and translation initiation factors) to the mRNA (Gray and Hentze, 1994; Muckenthaler *et al.*, 1998). This steric blockage requires the positioning of the IRE close to the cap (Goossen and Hentze, 1992; Paraskeva *et al.*, 1999). Little mechanistic insight is currently available for regulators that bind to the 3' UTR.

Translational control via the 3' UTR highlights a functional link between the mRNA ends for translation. Recent work showed that the terminal structures of the mRNA, the cap and the poly(A) tail, indeed interact via the factors eIF4F and poly(A)-binding protein (PABP) that bind to them (Wells *et al.*, 1998; reviewed in Sachs *et al.*, 1997). mRNA circularization is thus emerging as a current concept to explain how the cap and the poly(A) tail synergistically stimulate translation and how 3' UTR-binding proteins may regulate translation initiation (Sachs *et al.*, 1997; Gingras *et al.*, 1999; Preiss and Hentze, 1999).

The mechanism of translational regulation by SXL is even more complex in that it involves both the 5' and 3' UTRs of the message. The developmental control of

oskar mRNA translation represents another example of regulation involving 5' and 3' UTR sequences (Gunkel *et al.*, 1998). However, studies of translational control during development in *Drosophila* and other model systems have been hampered by the lack of *in vitro* systems that accurately recapitulate the events of regulated translation *in vivo*. We describe the generation of a novel cell-free system from *Drosophila* embryos that recapitulates the translational synergism between the cap and the poly(A) tail. This system also faithfully reflects the inhibition of *msl-2* mRNA translation by SXL: the SXL-binding sites in both the 5' and 3' UTRs of *msl-2* mRNA cooperate for translational repression in the *Drosophila* system, in contrast to rabbit reticulocyte lysates (RRL). Using this system, we have gained insight into the regulation of dosage compensation by SXL.

Results

Partial reconstitution of *msl-2* mRNA regulation by SXL in rabbit reticulocyte lysates

In flies, complete inhibition of *msl-2* mRNA translation by SXL requires SXL-binding sites located in both the 5' and 3' UTRs of the message (Bashaw and Baker, 1997; Kelley *et al.*, 1997). To investigate the mechanism of translational repression by SXL, *msl-2* translation was assessed *in vitro* using RRL. The translation of a chimeric mRNA consisting of the luciferase-coding region precisely flanked by the 5' and 3' UTRs of *msl-2* mRNA [wild type (WT) mLm RNA, Figure 1A; WT and mutated SXL-binding sites are represented by filled and empty ovals, respectively] was analyzed in the presence of increasing amounts of recombinant SXL. CAT mRNA lacking *msl-2* sequences was co-translated as an internal control (see below). As shown in Figure 1B (filled squares), the translation of WT RNA was specifically inhibited by SXL in a dose-dependent manner. To further test whether the RRL system faithfully recapitulates the regulatory features found *in vivo*, the translation of two additional mRNAs containing mutated SXL-binding sites at either the 5' (5'A+Bmut mLm RNA) or the 3' (3'mut mLm RNA) UTRs was examined, so that regulation via the 5' or 3' UTR SXL-binding sites could be measured independently. Translation of 3'mut RNA was inhibited with a dose-response similar to the WT RNA (Figure 1B, open circles), indicating that the 5' UTR sites suffice for maximal inhibition in this system. Moreover, only a modest translational inhibition was observed for 5'A+Bmut RNA at high SXL concentrations (filled triangles), showing that inhibition via the 3' UTR was not observed in RRL. The relative biological roles of the 5' and 3' UTRs in the translational regulation of *msl-2* mRNA are thus imbalanced with respect to the situation *in vivo*. The results indicate that the RRL system recapitulates *msl-2* mRNA translational control only partially, and is hence of limited value.

A poly(A) tail-dependent cell-free translation system from *Drosophila* embryos

Translational regulation of *msl-2* mRNA by SXL operates in *Drosophila* embryos from the blastoderm stage (Franke *et al.*, 1996). We reasoned that a *Drosophila* embryo extract should in principle be able to recapitulate all

Fig. 1. (A) mRNAs used in this study. Chimeric mRNAs containing the full-length 5' and 3' UTRs of the *msl-2* mRNA fused to the luciferase coding region (mLm RNAs) were used in wild type (WT) or mutant (mut) versions. WT RNA contained intact SXL-binding sites in the 5' and 3' UTRs (solid ovals). Mutant versions of this construct containing individual (5'A mut, 5'B mut) or combined (5'A+B mut) substitutions of the 5' UTR SXL-binding sites (indicated by open ovals), or mutations in the 3' UTR sites (3'mut) were obtained. The mRNAs coding for luciferase (luc) and chloramphenicol-acetyl transferase (CAT) have been described previously (Iizuka *et al.*, 1994; Preiss and Hentze, 1998). The maps are drawn to scale. (B) Translation inhibition of mLm RNAs by SXL in RRL. WT mLm RNA (WT, ■) or mutant versions containing functional sites only in the 5' UTR (3'mut, ○) or 3' UTR (5'A+Bmut, ▲) were translated in RRL in the presence of increasing amounts of recombinant SXL. The translation efficiency was determined by measuring luc activity. CAT mRNA was co-translated as an internal control, and CAT synthesis was quantified by ELISA. The luc values were corrected for CAT expression, and are plotted as percentages of luc activity obtained in the absence of SXL on a logarithmic scale against the molar ratio SXL:RNA. The absolute values in the absence of SXL were similar for all mLm variant mRNAs at $\sim 6 \times 10^3$ arbitrary light units/ μ l reaction. These data represent the average of two experiments. The experimental error for the different data points was 8–15% of the plotted values.

aspects of translational control by SXL. A previous report describing a translation-competent extract from *Drosophila* embryos was used as a starting point (Scott *et al.*, 1979). Upon optimization of both extract preparation (Figure 2; see Materials and methods for a detailed protocol) and translation assays (Table I; Materials and methods), a system was obtained that was able to translate

Fig. 2. Outline of the preparation of translation extracts from *Drosophila* embryos. For a detailed explanation and discussion, see Materials and methods and Table I.

Table I. Optimization of translation in *Drosophila* embryo extracts

Component	Range tested	Optimal ^a	RNA
Creatine phosphate	0–168 mM	17–23 mM	luc ^b
Creatine kinase	0–1.68 mg/ml	0.08 mg/ml	luc
KOAc	0–200 mM	30 mM	c-luc-a ^{c,d}
Mg(OAc) ₂	0–3 mM	0.4 mM	c-luc-a ^c
Spermidine	0–1.8 mM	0.1 mM	c-luc-a ^c
Amino acids	0–100 μM	60 μM	c-luc-a
DTT	0–4 mM	0 mM	c-luc-a ^c
tRNA ^e	0–150 μg/ml	100 μg/ml	luc
Temperature	18–37°C	25°C	c-luc-a
Incubation time	0–3 h	90 min	c-luc-a

^aOptimal is defined as maximal luc activity when all other parameters are kept constant.

^bluc mRNA, which was uncapped and non-adenylated, was purchased from Promega.

^cShould be optimized for each mRNA template.

^dc-luc-a mRNA, containing an m⁷GpppG cap and a 98-residue poly(A) tail, was synthesized from the pT3Luc(pA) plasmid (Iizuka *et al.*, 1994).

^eCalf-liver tRNA was purchased from Sigma.

exogenously added mRNAs reproducibly with high efficiency.

Figure 3 shows pertinent features of the translation of mRNA variants with different end modifications. Specifically, we wanted to know whether this *in vitro* system would recapitulate the poly(A) tail dependence that is a hallmark of translation *in vivo*. Figure 3A depicts the translation profiles of Luc mRNAs (Figure 1A) containing no end modifications (–) or any of the following modifications: a m⁷GpppG cap (c), a poly(A) tail of 98 residues (a) or both a cap and a poly(A) tail (c-a). The mRNA containing both end modifications is translated many fold better than those containing either one of the modifications or none at all. The physical stabilities of the various trace-labeled transcripts were assessed by RNA re-extraction and phosphorimaging, showing that all the capped mRNAs were approximately equally stable (Figure 3B, panels c and c-a), while the uncapped mRNAs were less stable (Figure 3B, panels – and a). Whereas these data indicate

that the *Drosophila* embryo translation system is strongly poly(A) dependent, the lower stabilities of the uncapped mRNAs precluded the interpretation that this poly(A) dependence reflects synergism between the mRNA ends for translation.

To address this important aspect further, we synthesized mRNAs with an ApppG cap, which fails to bind eIF4E and thus fails to stimulate translation. The stabilities of these RNAs were indeed similar to their m⁷GpppG-capped counterparts (Figure 3B, compare panels c* and c*-a with c and c-a, respectively). In contrast, their translation profiles mirrored those of their uncapped counterparts (Figure 3A, compare c* and c*-a with – and a, respectively). Similar results were obtained using a set of CAT instead of Luc mRNAs (data not shown). These findings demonstrate that the *Drosophila* cell-free system is competent to recapitulate the translational synergism between the cap and the poly(A) tail. To our knowledge, this is the first *in vitro* system from a multicellular eukaryote able to recapitulate this property of translation that is normally observed *in vivo*. The degree of synergism (defined as the translational efficiency of the c-a RNA divided by the sum of the individual efficiencies of the c and a RNAs) displays quantitative variation (ranging between 5- and 15-fold) depending on the mRNA template. As is the case for many developmentally regulated mRNAs *in vivo* (Richter, 1999), capped CAT mRNAs with increasing lengths of their poly(A) tails display increased translational efficiencies in the embryo extract (Figure 3C). More than 31 adenosine residues are required for this effect (Figure 3C).

Specific inhibition of *msl-2* mRNA translation by SXL faithfully recapitulated *in vitro*

The WT (mLm) mRNA used in Figure 1B was translated in *Drosophila* embryo extracts in the presence of increasing amounts of recombinant SXL. CAT mRNA was co-translated as an internal control to account for small variations in translation unrelated to SXL inhibition. Luc values were obtained, corrected for the CAT values and expressed as the percentage of the activity obtained in the absence of SXL, which was set to 100%. These values were plotted against the molar excess of SXL to RNA. The results show that, while translation of CAT was unaffected, translation of WT RNA was efficiently repressed by SXL (Figure 4A). The efficiency of repression in *Drosophila* extracts was ~10-fold higher than in RRL (compare Figure 1B with 4A, note the difference in the x-axes). Importantly, translational inhibition was observed both via the 5' and the 3' UTR of *msl-2* mRNA, and maximal translational repression required the presence of both UTRs (Figure 4A). The differences in Luc activity observed for the various RNAs were not related to different RNA stabilities in the extract (data not shown).

Differential contribution of the 5' UTR SXL-binding sites to translational repression

To determine the relative contribution of the two SXL-binding sites within the 5' UTR, each of the two sites was mutated separately (Figure 1A, RNAs 5'Amut and 5'Bmut) and translated in the presence of increasing amounts of SXL. 5'Amut mRNA is inhibited almost as effectively as the WT RNA (Figure 4B), whereas 5'Bmut

Fig. 3. Translational synergism between the cap and the poly(A) tail in the *Drosophila* embryo cell-free system. (A) Translation time-course of trace-labeled luc mRNAs containing the following end modifications: c, m⁷GpppG cap (□); a, 98-residue poly(A) tail (△); c-a, m⁷GpppG cap and poly(A) tail (■); -, no end modification (▲); c*, ApppG cap (○); c*-a, ApppG cap and poly(A) tail (●). Four-microliter aliquots were taken at 0, 10, 20, 40, 60, 90 and 120 min, and were used to measure the luc activity. (B) Physical stabilities of the RNAs used in (A). Total RNA was extracted from 10 μ l aliquots, and separated in a 1% denaturing agarose gel; the gel was dried and exposed in the phosphorimager. Forty nanograms of trace-labeled CAT mRNA were added as an extraction control. (C) The length of the poly(A) tail determines translational efficiency in the *Drosophila* extracts. Capped CAT mRNAs with poly(A) tails ranging from 0 to 98 adenosines were added to *Drosophila* translation reactions as detailed in Materials and methods. The graph depicts averaged results from three independent experiments. CAT protein levels were measured by ELISA and are given relative to the value for the c-a 98 mRNA, which was set to 1.0.

mRNA is as poorly regulated as 5'A+Bmut mRNA. Although site A may contribute to inhibition via the 5' UTR, site B is thus more critical for regulation *in vitro*. Therefore, cooperation between site 5'B and the 3' UTR sites confers *msl-2* translational repression.

Translational control of *msl-2* mRNA is independent of the poly(A) tail

As shown above, SXL inhibits *msl-2* mRNA translation by binding to both the 5' and 3' UTRs of the message. An intuitive explanation for SXL action is that it may interfere with the synergism between the cap and the poly(A) tail for translation. To test this possibility directly, we compared SXL-mediated translational inhibition of WT mLm RNA either containing or lacking a poly(A) tail. Preliminary control experiments revealed that the *Drosophila* extracts display endogenous polyadenylation activity (data not shown; see Figure 6B). Therefore, untreated or cordycepin-treated mRNAs were used as templates for non-adenylated RNAs. Cordycepin (3' deoxy-adenosine) prevents the 3' addition of further residues to the RNA molecule, thereby blocking polyadenylation and ensuring that non-adenylated input RNAs remain non-adenylated during the translation reaction (see Figure 6B). Figure 5 shows that WT RNAs lacking a poly(A) tail

were as effectively regulated by SXL as their counterparts that bear poly(A) tails of 73 residues. These results were confirmed with 3'mut or 5'A+Bmut mRNAs (data not shown). Thus, the mechanism of translational repression by SXL is independent of the poly(A) tail and, therefore, the poly(A) tail does not appear to act as the primary target for SXL function.

The results shown in Figure 5, and the observation that the degree of synergism between the cap and the poly(A) tail is influenced by the particular mRNA substrate (see above), led us to examine this synergism for *msl-2* mRNA translation. The translation of trace-labeled WT mLm RNA either containing or lacking a poly(A) tail was directly compared in the *Drosophila* embryo extract, including the control of cordycepin-treated non-adenylated RNA. Furthermore, the RNA was quantified after translation, revealing only minor variations (Figure 6B). The results in Figure 6A show that the poly(A) tail stimulated WT RNA translation only ~2.3-fold, in contrast to the large effect on luc mRNA (Figure 3A). To verify the polyadenylation status of the various RNAs after translation, we used a poly(A)-test (PAT) assay (see Materials and methods; Sallés and Strickland, 1995). Because of the nature of this reaction, RNAs that lack a poly(A) tail are not amplified, and therefore only the fraction of

Fig. 4. Translation of *msl-2* chimeric mRNAs in the *Drosophila* extract. (A) The *Drosophila* cell-free system recapitulates the inhibition of *msl-2* mRNA translation by SXL. Translation of WT (■), 3'mut (○) and 5'A+Bmut (▲) mRNAs was tested in the absence or presence of increasing concentrations of recombinant SXL. CAT mRNA was co-translated as an internal control. The data were processed and represented as described for Figure 1B. The data obtained for CAT are also shown (□). The absolute values obtained for the different mRNAs in the absence of SXL were similar, and ranged in the order of $1\text{--}4 \times 10^4$ arbitrary light units/ μl reaction. This graph represents the average of five experiments. The experimental error for the different data points was 5.6–14% of the plotted values. (B) Relative contribution of the 5' UTR SXL-binding sites to inhibition. WT (■), 5'Amut (●), 5'Bmut (□) and 5'A+Bmut (▲) RNAs were translated in the *Drosophila* cell-free system as indicated above. CAT mRNA was included as an internal control. The data were obtained and processed as described for Figure 1B. The average of two experiments is shown. The absolute values in the absence of SXL were $\sim 1 \times 10^4$ arbitrary light units/ μl reaction. The experimental error for the different data points was 4.8–15.7% of the plotted values.

polyadenylated RNA is visible. The results show that the polyadenylated mRNA (+) did indeed contain a poly(A) of 73 residues and was not deadenylated during the translation reaction (Figure 6B). The non-adenylated input RNA (–) received a poly(A) tail of ~ 30 residues, indicating that the *Drosophila* extracts contain some endogenous polyadenylation activity. As expected, only a minor fraction of the RNA that was treated with cordycepin received a poly(A) tail. This cordycepin-treated RNA was translated with a similar efficiency as the non-adenylated untreated RNA (Figure 6A), confirming that a poly(A) tail of 30 residues, as we had previously observed (Figure 3C),

Fig. 5. Inhibition of *msl-2* mRNA translation by SXL is independent of the poly(A) tail. Capped WT mRNAs were translated in *Drosophila* embryo extracts in the absence or presence of increasing amounts of SXL. The RNAs were modified at their 3' ends to contain either a poly(A) tail of 73 residues (+, ■), no poly(A) tail (–, △), or no poly(A) tail but cordycepin (co, ●). CAT mRNA was co-translated as an internal control. Luc and CAT values were obtained and processed as described for Figure 1B. The absolute values in the absence of SXL ranged between 1×10^4 and 2.5×10^4 arbitrary light units/ μl reaction. Similar data were obtained for 5'A+Bmut and 3'mut RNAs.

does not significantly increase translation. Similar results were obtained when 5'A+Bmut and 3'mut RNAs were used. Taken together, the results show that the poly(A) tail only marginally stimulates translation of WT mRNA.

The RBD region of SXL suffices for translational regulation

It has been suggested that SXL function could be mediated by interactions with itself and/or other proteins via the GN-rich N-terminal domain (Wang and Bell, 1994; Meise *et al.*, 1998). This domain has also been implicated in cooperative interactions between SXL molecules for RNA binding (Wang and Bell, 1994). To determine which regions of SXL are important for translational inhibition of *msl-2* mRNA, we analyzed the ability of two SXL derivatives to repress WT RNA translation. In particular, we analyzed the function of an SXL derivative containing the RBDs but lacking the N- and C-termini (Figure 7A, RBD) (Granadino *et al.*, 1997). As a specificity control, an SXL mutant lacking one of the RBDs and the C-terminus ($\Delta R2$ of Figure 7A) (Gebauer *et al.*, 1998), which fails to bind RNA, was also tested. The results (Figure 7B) show that the $\Delta R2$ mutant fails to repress translation, and that SXL needs to bind to the target mRNA for inhibition. Interestingly, the RBD variant regulates translation efficiently (see the dose–response curve with filled triangles). Similar observations were made with 5'A+Bmut and 3'mut RNAs (data not shown). These data indicate that the N- and C-terminal domains of SXL are dispensable for operating the inhibitory mechanism. The small difference between the RBD and the FL protein (Figure 7B) was reproducible, but more detailed analysis will be required to determine whether the regions missing from RBD contribute significantly to maximal translational repression.

Discussion

In this study we describe the establishment of a novel cell-free translation system from *Drosophila* embryos and

Fig. 6. The poly(A) tail only marginally stimulates translation of *msl-2* mRNA. **(A)** Translation efficiency in the *Drosophila* extracts of capped WT mLm RNAs containing a 73-base poly(A) tail (+), no poly(A) tail (-), or no poly(A) tail but the cordycepin modification (co). CAT mRNA was co-translated as an internal control and used to correct for small variations in translation. A fraction of the translation reaction was used to extract the total RNA and assess the amount of RNA after translation. The translation efficiency of each mRNA was determined by dividing the luc activity obtained after translation by the amount of recovered RNA. **(B)** PAT assay to determine the polyadenylation state of the RNAs used in (A) after translation. A fraction of the total RNA extracted in (A) was loaded directly on a 1% denaturing agarose gel (input), and the remainder was used for the PAT assay. The products amplified in this reaction were separated in a 6% denaturing acrylamide gel and run alongside a 100-bp DNA ladder. The estimated size of the different poly(A) tails is shown on the left.

its use in examining the translational regulation of *msl-2* mRNA by SXL. This system translates exogenously added mRNAs with high efficiency and retains critical features of translation *in vivo*, including the synergism between the mRNA cap and poly(A) tail (Gallie, 1991; Iizuka *et al.*, 1994).

The data presented in Figure 3A show that an mRNA

Fig. 7. The N-terminal domain of SXL is not required for translation inhibition. **(A)** Domain organization of SXL. Schematic representation of the SXL protein showing the GN-rich N-terminal domain (GN), two RNP-type RBDs (R1 and R2), and the C-terminal tail (C). The amino acid boundaries of these domains are shown in the upper part of the figure. The amino acids included in the RBD and ΔR2 mutants are also shown. **(B)** Comparison of the abilities of full-length SXL protein (FL, ■) and deletion mutants comprising amino acids 1–227 (ΔR2, ●), or 94–322 (RBD, ▲), to inhibit translation of WT mLm RNA. Translation was assessed as in Figure 1B. The data represent the average of two experiments. The experimental error for the different data points was 4.4–14.3% of the plotted values.

with both a cap and an A₉₈ tail is translated with ~20-fold higher efficiency than those which bear any of the terminal modifications alone. This increase in translational efficiency is not a result of increased mRNA stability (Figure 3B). Interestingly, the poly(A) must be longer than 31 adenosine residues to stimulate translation in the *Drosophila* cell-free system (Figure 3C), as has been shown previously in yeast (Preiss *et al.*, 1998). Because binding of a single PABP molecule protects ~25 adenosines (Sachs *et al.*, 1987), these results may reflect a requirement for at least two PABP molecules bound to the poly(A) to elicit synergism.

An unexpected observation is the relative lack of synergism between the cap and the poly(A) tail of *msl-2* mRNA. Figure 6 shows that polyadenylated mLm RNAs are translated with only a 2.3-fold higher efficiency than the corresponding non-adenylated RNAs. Although this stimulation may be biologically relevant, it is small by comparison with other mRNAs that we tested. Future work with other *Drosophila* mRNAs will have to show whether the relative poly(A) tail independence of translation is a more general feature of subfamilies of *Drosophila* mRNAs or an exceptional property of mRNAs bearing the *msl-2* 5' and 3' UTRs. Tanguay and Gallie (1996) proposed that translational stimulation by the poly(A) tail

is affected by the length of the 3' UTR. They showed that increasing lengths of the 3' UTR decreased the stimulatory effect of the poly(A) tail. However, deletion of the *msl-2* 3' UTR did not increase the stimulatory effect of the poly(A) tail of Luc constructs bearing the *msl-2* 5' UTR (F.Gebauer and M.W.Hentze, unpublished observations). This suggests that the *msl-2* 5' UTR mediates efficient ribosome recruitment, which can only be marginally stimulated by a poly(A) tail.

In contrast to RRL, the cell-free system from *Drosophila* embryos faithfully recapitulates the regulation of *msl-2* mRNA translation by SXL. As originally established *in vivo* (Bashaw and Baker, 1997; Kelley *et al.*, 1997), SXL-binding sites located in both the 5' and 3' UTRs of *msl-2* mRNA are required for optimal translational repression in the *Drosophila in vitro* system (Figure 4A), whereas translational regulation via the *msl-2* 3' UTR is non-functional in RRL, and repression via the 5' UTR alone is equivalent to repression via both UTRs (Figure 1B). These important differences suggest that a *Drosophila*-specific co-factor may be required for inhibition of *msl-2* mRNA translation via the 3' UTR, particularly since other 3' UTR-mediated translational control events are faithfully reflected by the RRL (Ostareck *et al.*, 1997). Alternatively, the differences between the two translation systems may reflect species-specific features of the general translation apparatus. Interestingly, SXL-mediated inhibition is ~10 times more efficient in the *Drosophila* extract than in RRL (compare inhibition of WT mLm RNA in Figures 1B and 4A). Although the *Drosophila* extracts contain endogenous SXL, we believe that this does not contribute significantly to repression, because *msl-2*-derived reporter mRNAs with WT or mutated SXL-binding sites (Figure 1A) are translated equally well in the absence of added recombinant SXL (data not shown; see also figure legends). We rather think that the higher efficiency of SXL in the *Drosophila* embryo extract may be a further indication of the existence of a *Drosophila* co-repressor and/or the requirement of SXL to engage in specific interactions that cannot occur equally well in RRL.

In this context, it is remarkable that the RBDs of SXL are sufficient for translational regulation *in vitro* (Figure 7), and that the first 38 amino acids of the protein, which are required to mediate cooperative interactions between SXL molecules for RNA binding (Wang and Bell, 1994), are dispensable. This is consistent with previous results obtained *in vivo* (Yanowitz *et al.*, 1999) and suggests that cooperative interactions for RNA binding are not required for translational inhibition. The available data hence support the hypothesis that co-repressor interactions may well be mediated by the SXL RBDs. Indeed, it has recently been claimed that when bound to RNA the RBDs of SXL can interact with other proteins (Samuels *et al.*, 1998). SXL is well conserved in Diptera but SXL protein from *Musca domestica* nonetheless fails to inhibit *msl-2* expression when overexpressed in *Drosophila melanogaster* (Meise *et al.*, 1998). We suggest that the functional differences reside in a limited set of amino acids within the RBDs.

Of the two SXL-binding sites located in the 5' UTR, the downstream one is far more important for translational repression (Figure 4B). This is an intriguing observation since SXL binds with similar affinities to both sites

(Gebauer *et al.*, 1998). This result suggests that the mechanism of translational repression by SXL differs fundamentally from that of IRP, which requires binding sites located close to the cap structure (Goossen and Hentze, 1992; Paraskeva *et al.*, 1999) and does not involve 3' UTR regulatory sites.

Translational regulation of *msl-2* mRNA *in vitro* primarily rests on the cooperation between SXL-binding site B in the 5' UTR and the sites located in the 3' UTR. The presence of functional sites in only one of the UTRs results in a 2-fold inhibition by SXL, while the presence of sites in both UTRs achieves >10-fold inhibition (Figure 4). This suggests that SXL action from the 5' and 3' UTR sites cooperates to prevent translation. One possible scenario is that SXL-driven interactions between the 5' and 3' UTRs package the *msl-2* mRNP into a conformation that renders it poorly accessible to the translational machinery. An alternative explanation is that SXL inhibits different processes at the 5' and 3' UTRs independently, but that these processes cooperate for translation. This model would not pose any a priori requirement for interactions between SXL molecules bound to the two ends of *msl-2* mRNA.

A major result of this work is the realization that SXL action is independent of the presence of the poly(A) tail, at least *in vitro* (Figure 5). The cordycepin-treated mRNA contains a small fraction of messages with a poly(A) tail of 30 residues (Figure 6B, co). These do not appear to contribute significantly either to overall translation or to regulation by SXL, because the non-treated mRNA, which contains a higher proportion of A₃₀ molecules (Figure 6B, -), is translated and repressed by SXL with comparable efficiency (Figures 6A and 5, respectively). Importantly, the *Drosophila* embryo system is principally poly(A) dependent (see above). Since it so closely reflects the known aspects of *msl-2* regulation *in vivo*, we confidently predict poly(A) independence as an important feature of how SXL regulates dosage compensation in the fly. The *Drosophila* cell-free translation system described here now allows the study of an important aspect of developmental biology in the test tube. Future experiments using this system should help to shed further light on the mechanism by which SXL inhibits translation. Equally importantly, it provides a novel approach to dissect other examples of translational regulation during *Drosophila* embryogenesis, and to study the mechanism by which the poly(A) tail promotes translation in multicellular eukaryotes.

Materials and methods

Plasmids

The 626-nucleotide 5' UTR of *msl-2* mRNA (Gebauer *et al.*, 1998) was cloned into the *SmaI*–*Bam*HI sites of BSK-A, a Bluescript vector containing a poly(dA) of 73 residues (Gebauer *et al.*, 1994). The coding region of the firefly luciferase was subsequently cloned into the *Bam*HI site of the resulting plasmid to yield the construct mL-A. The region preceding the initiator ATG was designed to match the *Drosophila* consensus sequence (CCACCATG), and was similar to that of the endogenous *msl-2* mRNA (CCATCATG). The *msl-2* 3' UTR (1047 bp) was originally obtained by PCR amplification from genomic DNA, and modified to introduce *Bam*HI–*Bgl*II sites at its termini. This insert was cloned into the *Bgl*II site of mL-A to yield plasmid mLm WT. A derivative lacking a portion of the 5' UTR that includes the SXL-binding sites was obtained by digestion of mLm WT with *SacI*, and was called Lm-A. 5' UTR fragments containing individual or combined mutations of the SXL-binding sites were then introduced into the *SacI* site of

Lm-A to yield plasmids 5'Amut, 5'Bmut and 5'A+Bmut. The mutations consisted of the substitution of T11 (site A) by TTCTCTCTCT, or T16 (site B) by TTCTCTCTCTCTCT (Gebauer *et al.*, 1998). To obtain mLm 3'mut, the 3' UTR of *msl-2* was cloned into the PCRII-TOPO vector (Invitrogen) and the SXL-binding sites mutated to the sequence GTTAACCTAAGAATGCGGCCGC by PCR-directed mutagenesis. The mutated 3' UTR was then cloned into the *Bgl*II site of mL-A. All constructs were confirmed by DNA sequencing.

The Luc plasmid was described by Iizuka *et al.* (1994). The CAT construct used for the tail length series (IRE.CAT; Preiss *et al.*, 1998) and that used as an internal control (OT.CAT; Preiss and Hentze, 1998) were described previously.

In vitro transcription

Capped RNA was synthesized as described (Gray and Hentze, 1994). The RNAs were trace-labeled with [α - 32 P]UTP to facilitate assessment of their concentration and integrity. All RNAs used in the same experiment were synthesized in parallel.

Extracts from *Drosophila* embryos: preparation and in vitro translation

Extracts were prepared based on a protocol by Scott *et al.* (1979) with the following critical modifications. Overnight embryos were collected in a pile of sieves, washed with tap water and transferred to a cylinder containing freshly prepared EW buffer (0.7% NaCl, 0.04% Triton X-100). Embryos were washed twice with EW buffer by exchanging the buffer in the cylinder and letting the embryos settle by gravity. Embryos were dechorionated for 3 min in 260 ml of EW buffer supplemented with 3% sodium hypochlorite final, at 25°C, under vigorous agitation provided by a magnetic stirrer. Dechorionated embryos were transferred quickly to the sieves, and washed extensively (5–10 min) with a strong stream of tap water. Embryos were transferred to a measuring cylinder set on ice containing freshly prepared DE buffer [10 mM HEPES pH 7.4, 5 mM dithiothreitol (DTT)]. Floating (dead) embryos were eliminated by suction. Embryos were washed twice with 100 ml of DE buffer as described above, and resuspended in one volume equivalent (with respect to the settled embryos) of DEI buffer (10 mM HEPES pH 7.4, 5 mM DTT, 1× COMPLETE-Protease Inhibitors from Boehringer Mannheim). The embryo suspension was homogenized at 4°C by 20 strokes of a Potter-Elvehjem homogenizer at ~1500 r.p.m. The homogenate was spun by centrifugation at 40 000 g for 20 min at 4°C. Centrifugation in a microfuge at 13 000 r.p.m. also worked well for small volumes. The clear aqueous interphase was taken by puncturing the tube with a syringe, glycerol was added to 10% final concentration, and the extract was aliquoted, frozen in liquid nitrogen and stored at –70°C. Extracts were prepared from 90 min, 3 h, 6 h and overnight embryos with similar results.

In vitro translation of mLm RNAs in the *Drosophila* embryo extracts was typically performed in a final volume of 12.5 µl containing 60 µM amino acids, 16.8 mM creatine phosphate, 80 ng/µl creatine kinase, 24 mM HEPES pH 7.4, 0.6 mM Mg(OAc)₂, 80 mM KOAc, 100 ng/µl calf-liver tRNA and 40% embryo extract. The embryo extract was not treated with micrococcal nuclease and, thus, contained the full complement of endogenous mRNAs. The mLm template mRNA was added in the linear range of translation, at a final concentration of 3.2 ng/µl. Unless indicated otherwise, the template RNAs contained an m⁷GpppG cap and a poly(A) tail of 73 residues. Capped CAT mRNA, containing a poly(A) of 98 residues, was added at a concentration of 0.8 ng/µl as an internal control. The reaction was incubated at 25°C for 90 min. For the experiments shown in Figure 2, similar conditions were used except that Luc mRNAs were translated in 0.4 mM Mg(OAc)₂, 30 mM KOAc and 0.1 mM spermidine, and CAT mRNAs were translated in 0.4 mM Mg(OAc)₂, 40 mM KOAc, 0.6 mM spermidine and 1.2 mM DTT.

To study translational regulation by SXL, increasing amounts of recombinant SXL were added at a volume of 1 µl to the extract before including the RNA template. Recombinant SXL was kindly provided by J. Valcárcel and purified as described previously (Gebauer *et al.*, 1998).

Luciferase activity was determined according to Brasier *et al.* (1989) or by using the Luciferase Assay System from Promega. The CAT protein product was quantified using a CAT-ELISA kit from Boehringer Mannheim.

Translation in rabbit reticulocyte lysates

In vitro translation in nuclease-treated RRL was performed in a final volume of 12.5 µl containing 40% RRL supplemented with 64 µM amino acids, 20 U RNasin, 40 mM KOAc and 0.1 mM Mg(OAc)₂. mLm RNAs were added at a concentration of 3.2 ng/µl. As an internal

control, 0.8 ng/µl of CAT mRNA were also added. The reactions were incubated for 1 h at 30°C.

Translation inhibition and determination of Luc and CAT activities were performed as described above.

Poly(A) test assay

PAT assays were performed essentially as described by Sallés and Strickland (1995). An oligonucleotide with the sequence GCACGTGAA-CCTAGGATTAAG was used to specifically amplify the *msl-2* 3' end.

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