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Drosophila Sex-lethal protein mediates polyadenylation switching in the female germline

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The *Drosophila* master sex-switch protein Sex-lethal (SXL) regulates the splicing and/or translation of three known targets to mediate somatic sexual differentiation. Genetic studies suggest that additional target(s) of SXL exist, particularly in the female germline. Surprisingly, our detailed molecular characterization of a new potential target of SXL, *enhancer of rudimentary* (*e(r)*), reveals that SXL regulates *e(r)* by a novel mechanism—polyadenylation switching—specifically in the female germline. SXL binds to multiple SXL-binding sites, which include the GU-rich poly(A) enhancer, and competes for the binding of CstF64 *in vitro*. The SXL-binding sites are able to confer sex-specific poly(A) switching onto an otherwise non-responsive polyadenylation signal *in vivo*. The sex-specific poly(A) switching of *e(r)* provides a means for translational regulation in germ cells. We present a model for the SXL-dependent poly(A) site choice in the female germline.
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Introduction

The choice of sexual identity is a fundamental biological process. Highly varied molecular mechanisms control sexual differentiation in well-studied metazoans (Ryner and Swain, 1995). In *Drosophila melanogaster* somatic cells, a hierarchy of alternative splicing events controls various aspects of sexual differentiation. The key sex determining genes (*Sex-lethal* (*Sxl*), *transformer* (*tra*), and *double-sex* (*dsx*)) are spliced differently in male (XY) and female (XX) flies (Schutt and Nothiger, 2000).

The master sex-switch protein SXL (Schutt and Nothiger, 2000) is an RNA-binding protein (Perez-Canadillas and Varani, 2001). SXL is absent in male flies and present in females. It blocks splice sites in three known pre-mRNAs by binding to adjacent uridine-rich sequences or Py-tracts, leading to exon skipping in *Sxl*, 3′ splice site switching in *tra*, and intron retention in *msl2* (Black, 2003; Forch and Valcarcel, 2003). Moreover, SXL binds to uridine-rich sequence(s) in untranslated regions (UTRs) of the *Sxl* and *msl2* mRNAs (Forch and Valcarcel, 2003), and represses translation. In female somatic cells, SXL allows synthesis of the TRA protein to mediate sexual differentiation and courtship behavior, and prevents synthesis of the MSL2 protein to allow proper dosage compensation (Schutt and Nothiger, 2000; Forch and Valcarcel, 2003).

In addition to its somatic functions, SXL also controls female germline development (Schutt and Nothiger, 2000). Absence of SXL in the female germline results in mitotic and meiotic defects, resulting in ovarian tumors or multicellular cysts of small undifferentiated cells (Schupbach, 1985; Salz *et al*, 1987; Steinmann-Zwicky *et al*, 1989) and in defects in chromosome pairing and meiotic recombination (Bopp *et al*, 1999). However, the mechanism of sex determination and differentiation in the germline is more complex and differs from the well-defined mechanism in somatic cells in several key aspects such as the nature of the sex-determining signal, the roles of *Sxl*, *tra*, and *dsx*, and dependence on an inductive signal from the soma (Mahowald and Wei, 1994; Steinmann-Zwicky, 1992; Schutt and Nothiger, 2000).

Several independent genetic studies suggest that additional targets of SXL exist, particularly in the female germline (Samuels *et al*, 1994; Kelley *et al*, 1995; Hager and Cline, 1997; Schutt and Nothiger, 2000; Fujii and Amrein, 2002; Vied *et al*, 2003). Whereas all known somatic targets of SXL are regulated at the level of splicing and/or translation, here we provide the first evidence that SXL regulates a new target, the *enhancer of rudimentary* (*e(r)*) mRNA, by polyadenylation switching in the female germline. Embryonic lethality of both sexes from RNA interference could explain why *e(r)* escaped previous genetic screens. Finally, we show functional significance of this regulation, and present a model for this sex-specific poly(A) switching.

Results

Use of alternative poly(A) sites accounts for the sex-specific e(r) isoforms

As all known examples of SXL regulation involve its binding to uridine-rich sequences, we searched the entire *Drosophila* genome for potential high-affinity SXL-binding sites. The SXL consensus (UUUUUGUU(G/U)U(G/U)UUU(G/U)UU) used here for the search was described previously (Singh *et al*, 1995); a search using a shorter SXL-binding site (U_8) (Sakashita and Sakamoto, 1994; Samuels *et al*, 1994) yielded a significantly larger and experimentally unmanageable number of hits in the genome and therefore the search was not pursued further. Seven of the candidates showed sex-specific mRNA isoforms (A Rahn and R Singh, unpublished results; details for the search algorithm will be published separately). Here, we report the detailed characterization of one of the seven candidates, *e(r)* (Wojcik *et al*, 1994). The GADFLY

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annotation database showed two *e(r)* transcripts (CT5770 and CT29800) resulting from an alternatively spliced exon and two alternative polyadenylation sites (Figure 1A). We found multiple potential SXL-binding sites in *e(r)*: one adjacent to the 3′ splice site of exon 2 (Figure 1A(i)) and three downstream of the proximal polyadenylation site (Figure 1A(ii); 1, 2, and 3).

To determine whether alternative splicing of exon 2 was the basis for the sex-specific isoform of *e(r)*, we performed RNase protection analysis. We found that exon 2 was alter-

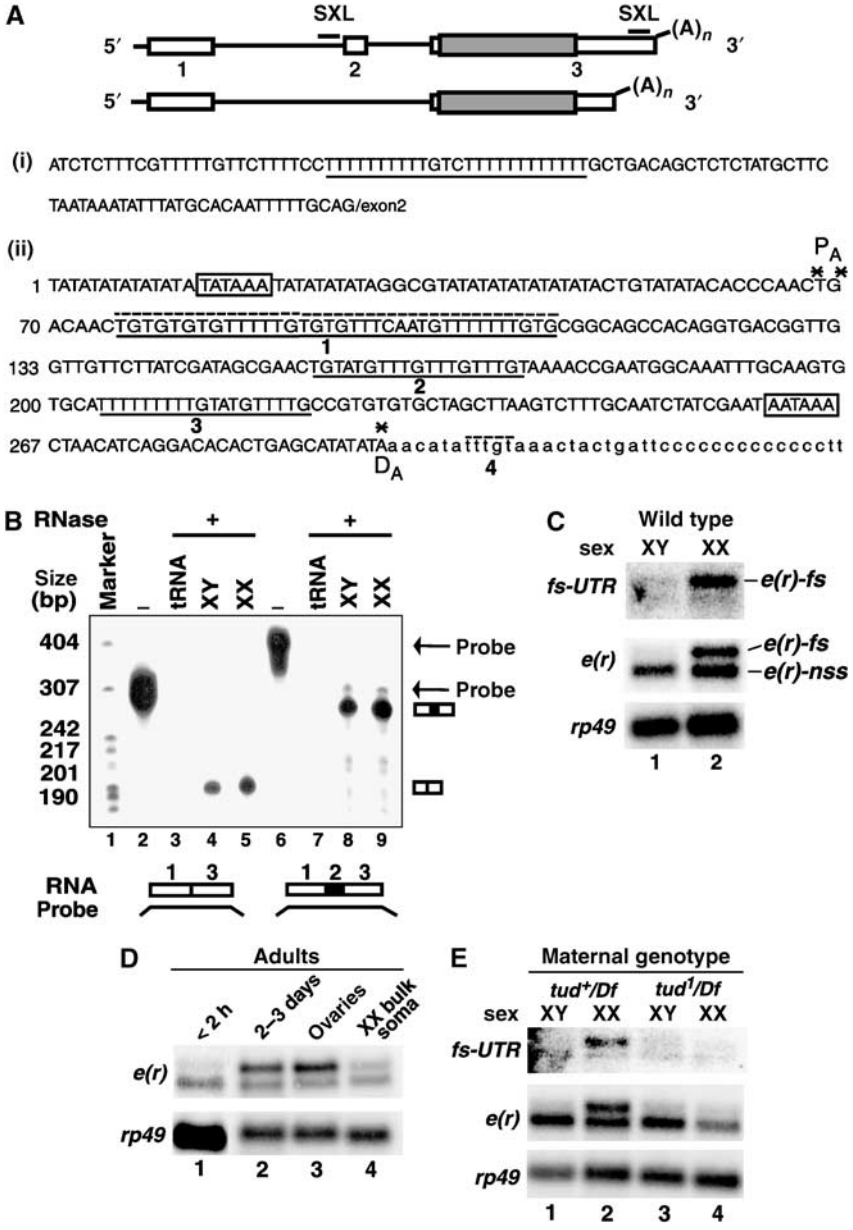

Figure 1 Female germline-specific poly(A) site switching accounts for the sex-specific isoforms of *e(r)*. (**A**) (Top) Schematics of the two known transcripts of *e(r)*, which differ with respect to inclusion/exclusion of exon 2 and use of alternative polyadenylation sites (Wojcik *et al*, 1994). Lines, introns; boxes, exons; shaded boxes, coding regions; (A)n, poly(A) tail; and SXL, potential SXL-binding sites. (i) Sequence of the 3′ end of intron 1. The potential SXL-binding site is underlined. (ii) Sequence of a portion of the 3′UTR region of *e(r)*. Boxed nucleotides, alternative polyadenylation signals; asterisks, cleavage/polyadenylation sites; underlined nucleotides, potential SXL-binding sites (1, 2, and 3); dashed line above the sequence, potential GU-rich enhancer/CstF-64-binding sites (1 and 4). Lowercase residues downstream of the distal (D_A) poly(A) site were sequenced in this study. (**B**) Exon 2 is alternately spliced, but not in a sex-specific manner. RNase protection was performed using labeled probes, shown at the bottom, corresponding to either exons 1 and 3 or 1, 2, and 3. Positions of the intact probes and relevant protected fragments are shown on the right. (**C**) The female-specific *e(r)-fs* transcript uses the downstream poly(A) site. A Northern blot with poly(A)$^+$ RNA from male and female flies (lanes 1 and 2) was probed with either the *e(r)* cDNA or the fs-UTR probe corresponding to the sequence between the two poly(A) sites (P_A and D_A), represented by asterisks in (A(ii)). For this and subsequent figures, XY and XX indicate chromosomal sex. *rp49* is a loading control. (**D**) The *e(r)-fs* transcript is primarily expressed in the ovaries of mature but not newly eclosed females. Northern blot of RNA isolated from newly eclosed (<2 h) adult XX flies (lane 1), 2- to 3-day-old adult XX flies (lane 2), isolated ovaries (lane 3), and the bulk somatic tissue (whole flies minus ovaries) of XX flies (lane 4). (**E**) (Top) Loss of the female germline results in the disappearance of the *e(r)-fs* transcript (lane 4). The poly(A) + RNA was isolated from the progeny of *tud* mothers, and probed with the same probes as in panel C.

natively spliced (Figure 1B, lanes 4 and 5 versus 8 and 9), but was not spliced in a sex-specific manner (Figure 1B, lane 4 versus 5 and lane 8 versus 9). Next, we probed an RNA blot with the fs-UTR probe (Figure 1C), which corresponds to the sequence between the two polyadenylation sites of *e(r)* and includes potential SXL sites (Figure 1A(ii)). This probe hybridized to the longer isoform that was present in females (Figure 1C, lane 2, top panel), but not to the shorter isoform present in both sexes (lanes 1 and 2, middle panel). Hereafter, the shorter isoform will be referred to as *e(r)-non-sex-specific* (*e(r)-nss*) and the longer isoform will be referred to as *e(r)-female-specific* (*e(r)-fs*). Presence of non-sex-specific and female-specific isoforms of *e(r)* is reminiscent of the known SXL target, *tra*, although it is the sex-specific splicing regulation that contributes to the synthesis of the two isoforms of *tra* (Sosnowski *et al*, 1989). We conclude that use of two poly(A) sites, rather than alternative splicing, accounts for the sex-specific size difference of the *e(r)* transcripts.

e(r)-fs is restricted to the female germline

SXL controls sexual differentiation in both somatic and germ cells. To test whether *e(r)-fs* is synthesized in somatic cells or the germline, we dissected ovaries from the bulk somatic tissue of wild-type (wt; w^{1118}) females, and analyzed *e(r)* expression by Northern analysis. Figure 1D shows that ovaries (lane 3), rather than the bulk somatic tissue (whole fly minus ovaries) (lane 4), were the primary source of the *e(r)-fs* transcript. However, the *e(r)-fs* transcript was barely detectable in newly eclosed (<2 h) females, which contain up to stage seven egg chambers (Figure 1D, lane 1). These data show that the *e(r)-fs* transcript is expressed specifically in the female germline during later stages of oogenesis.

As a complementary approach, we analyzed *e(r)* expression in the progeny of *tudor* (tud^1/Df) flies, which lack a germline and thus are sterile (Boswell and Mahowald, 1985). Indeed, the *e(r)-fs* transcript was absent from the female progeny of tud^1/Df flies (Figure 1E, lane 4 versus 2), demonstrating that *e(r)-fs* is a female germline-specific transcript.

SXL is necessary for the synthesis of e(r)-fs in the female germline

As the *e(r)* gene was identified as a potential target of SXL and showed a female germline-specific isoform, we tested whether synthesis of the *e(r)-fs* transcript *in vivo* was dependent on *Sxl* function, which is important both in somatic cells and in the germline (Schutt and Nothiger, 2000). To analyze the involvement of SXL specifically in the germline, we used two viable, female-sterile mutations of the *sans fille (snf)* gene (snf^{1621} and snf^{148}), which disrupt *Sxl* function in the female germline but not in the soma (Salz, 1992; Nagengast *et al*, 2003). We found that female flies homozygous for either the snf^{1621} (Figure 2, lane 1) or snf^{148} (lane 3) alleles failed to synthesize the *e(r)-fs* transcript, indicating that loss of SXL expression by the *snf* mutations disrupted synthesis of *e(r)-fs* in the germline.

In addition, we examined *e(r)* expression in Sxl^{f4} and Sxl^{f5} homozygous females. We found that the female-sterile Sxl^{f4} and Sxl^{f5} alleles, which prevent proper nuclear localization of SXL in the germline but permit normal somatic function (Bopp *et al*, 1993), also resulted in a loss of the *e(r)-fs* transcript (Figure 2, lanes 7 and 8). As a complementary approach, we expressed SXL in the *snf* mutant backgrounds

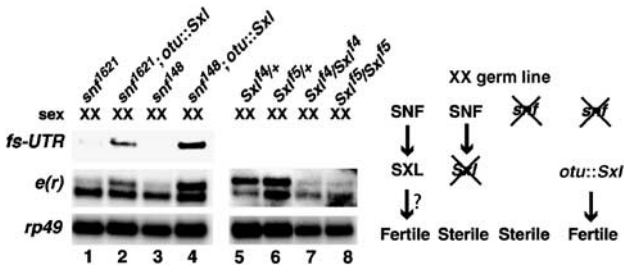

Figure 2 SXL function in germ cells is necessary for the sex-specific poly(A) switching of *e(r)*. (Left panel) XX flies homozygous for the snf^{1621} or snf^{148} alleles, which disrupt SXL synthesis in the germline, do not express *e(r)-fs* (lanes 1 and 3). Expression of the SXL cDNA in *snf* mutant backgrounds under the control of the *otu* promoter restores the synthesis of the *e(r)-fs* transcript (lanes 2 and 4). Sxl^{f4} and Sxl^{f5} homozygotes show a loss of *e(r)-fs* expression (lanes 7 and 8). (Right panel) Schematics of SXL-related events and their disruption by specific mutations in the female germline. Disruption of SXL synthesis/function by mutations in *Sxl* or *snf* leads to female sterility, whereas expression of the *Sxl* cDNA under the control of the germline-specific *otu* promoter (*otu*::*Sxl*) restores fertility in *snf* flies. Lanes 5–8 were not analyzed with the fs-UTR probe.

using a germline-specific *ovarian tumor* (*otu*) promoter (Hager and Cline, 1997), which is known to restore female fertility in these lines (Nagengast *et al*, 2003). We found that the germline-specific expression of SXL in *snf* flies indeed restored female fertility (Nagengast *et al*, 2003; data not shown) and the expression of the *e(r)-fs* transcript (Figure 2, lanes 2 and 4). As controls, ovaries isolated from snf^{1621} flies contained only the *e(r)-nss* transcript (data not shown), indicating that tumorous ovaries transcribe *e(r)*, process it to produce *e(r)-nss*, but fail to switch it to *e(r)-fs*. Furthermore, our analysis with the suppressor, $Su(Sxl^{f4})46$ (Bopp *et al*, 1999), of the female-sterile Sxl^{f4} mutation showed that it rescued the poly(A) switching of *e(r)* (Supplementary Figure S1, lane 3 versus 2). Based on what we know about the suppressor (see details in Supplementary data), these results are consistent with our model that SXL regulates *e(r)*, and support the idea that the suppressor acts by influencing an event(s) upstream of SXL, to restore its activity and/or localization. Therefore, our combined results show that SXL function in the female germline is required for the generation of the *e(r)-fs* transcript, and that the *Sxl* cDNA alone is sufficient to restore the expression of the *e(r)-fs* isoform in the *snf* mutant germlines, which lack SXL function and express only the *e(r)-nss* transcript.

SXL-binding site is important for poly(A) site choice in vivo

We asked what sequence features were important for sex-specific poly(A) site choice: specifically, whether the potential SXL-binding site(s) was important for the default use of the proximal poly(A) site in males, for poly(A) switching in females, or both; whether the order (or relative positions) of the two poly(A) sites was important for the observed sex-specific pattern; and whether the female-specific poly(A) switching required repression of the proximal site or activation of the distal site. To address these issues, we developed an *in vivo* reporter system in which various combinations of the wt or mutant 3′UTRs of *e(r)* were joined downstream of the coding region of a heterologous (EGFP) sequence (to distinguish it from the endogenous *e(r)* transcript) and placed under the control of the *e(r)* promoter for proper expression.

These constructs were introduced into flies using standard *P*-element-mediated transformation, and their polyadenylation patterns in male and female flies were analyzed by Northern analysis using an EGFP probe.

First, whereas nontransgenic lines gave no signal (Figure 3, lanes 1 and 2), transgenic lines with the wt *e(r)* 3′UTR (wt construct) showed a shorter non-sex-specific transcript (Figure 3, lanes 3 and 4) and a longer female-specific transcript (Figure 3, lane 4). Thus, our reporter faithfully recapitulates the sex-specific poly(A) site choice observed for the endogenous *e(r)* transcript. Second, mutation of the first potential SXL-binding site (Figure 1A(ii)), which also corresponds to a GU-rich enhancer element for the proximal poly(A) site (Zhao *et al*, 1999), activated the distal (D_A) site in males (Figure 3, lane 7); males lack SXL and do not normally use the distal site (D_A) for the wt 3′UTR. The poly(A) pattern was identical in males and females (Figure 3, lane 8). Another mutation (mutant2; see Figure 4A) of this GU-rich element only partially activated the D_A site in males, and showed little, if any, further switching in females (Figure 3, lanes 9 and 10). These results show that the SXL-binding site/GU-rich enhancer element is important both for the default choice of the proximal poly(A) site in males and for sex-specific switching in females. Third, we switched the order of the proximal (P_A) and distal (D_A) polyadenylation sites. For DP transcripts, males exclusively used the D_A site (now located upstream) (Figure 3, lane 5). Moreover, DP females showed no switching to the P_A site (now located downstream) (Figure 3, lane 6). We can draw the following conclusions from these experiments: (i) The distal site is competent to support efficient polyadenylation in (DP) males, which argues against the possibility that poly(A) switching requires a female germline-specific activator (or activation) of the distal site. (ii) The natural order of the two poly(A) sites plays an important role in their sex-specific usage. (iii) *Cis* competition with the proximal poly(A) site contributes to why the distal site is normally not used.

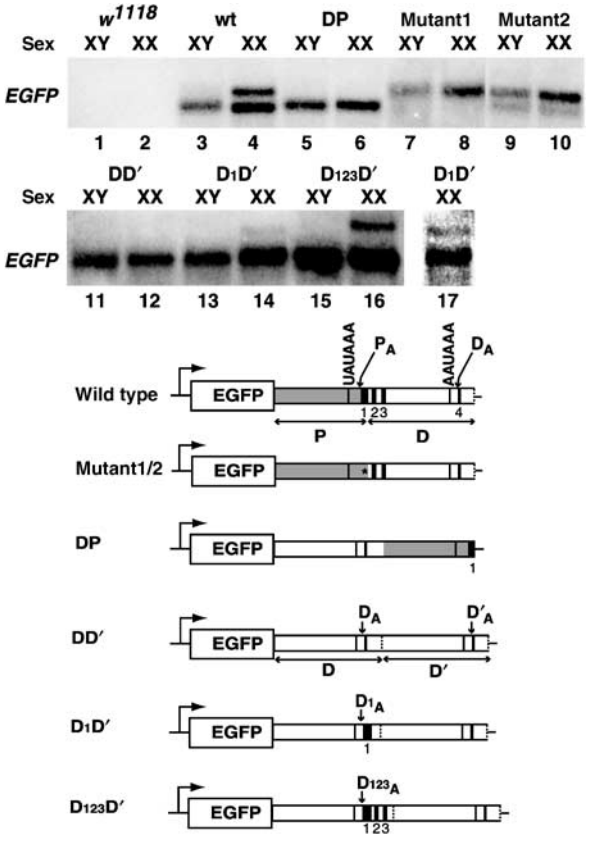

Figure 3 The SXL-binding site is important for the sex-specific poly(A) switching of *e(r)*. (Top) The order of the two poly(A) sites and the proximal GU-rich element are important for default polyadenylation in males. Northern analysis was performed using an EGFP probe on three independent transgenic lines for each construct. w^{1118} is the parental line without the EGFP transgene. (Bottom) Schematics of reporter constructs with the *e(r)* promoter (site of transcription is indicated by an arrow upstream of EGFP) and different 3′UTRs of *e(r)* used to generate various transgenic lines. P and D represent the proximal (gray box) and distal (white box) portions of the 3′UTR, respectively. Locations of the proximal (P_A), distal (D_A), duplicated distal (D_A'), and modified distal (D_{1A} and D_{123A}) poly(A) sites are shown. The GU-rich elements (1, 2, 3, and 4) are as in Figure 1A(ii). In the DP construct, the SXL-binding site is downstream of the P sequence and is indicated by the black box labeled 1. The asterisk indicates mutation of the proximal GU-rich element. The wt sequence of the proximal GU-rich element (Figure 1A(ii), 1) was changed to TGTTTTAGCACACACCACAC GAATTCACACAACAC in mutant1 or to TGTGTGTGTTGAATTCGTG in mutant2. Lane 17 is an overexposure of lane 14.

The above experiments suggested that the SXL-binding site adjacent to P_A was important for sex-specific poly(A) switching. To complement results from the mutagenesis of the GU-rich element, we asked whether the GU-rich sequence(s) downstream of P_A was able to confer female-specific switching onto an otherwise nonresponsive site *in vivo*. Therefore, we duplicated the distal site (DD′), and analyzed it for sex-specific splicing. As would be expected from the DP construct, the first distal site (D_A) was used in males (lane 11). Furthermore, there was no activation of the second distal site (D_A') in females (lane 12), indicating that D_A is nonresponsive to female-specific switching. However, insertion of three GU-rich elements (or the SXL-binding site(s)) (1, 2, and 3; see Figure 1A(ii)) downstream of D ($D_{123}D'$) supported efficient poly(A) switching in the female germline (lane 16), but not in males (lane 15). Incorporation of the first GU-rich element alone (D_1D') caused a smaller but reproducibly detectable poly(A) switching (lanes 14 and 17). Lack of poly(A) switching in DP and DD′ females served as a negative control for the specificity of sex-specific poly(A) switching. We conclude that GU-rich elements downstream of the proximal poly(A) site confer female-specific poly(A) site repression onto an otherwise nonresponsive site, causing sex-specific poly(A) switching.

SXL competes for the binding of CstF-64 to the proximal GU-rich element in vitro

The experiments described above show that both SXL and the putative SXL-binding site(s) are important for the female germline-specific poly(A) switching in *e(r)*. Next, we investigated the molecular mechanism for the poly(A) switching of *e(r)*. The cleavage stimulatory factor 64 (CstF-64), a component of the CstF complex of the poly(A) machinery, is known to directly bind to GU-rich enhancer elements and stimulate polyadenylation by facilitating the binding of the cleavage and polyadenylation specificity factor to the AAUAAA signal (Zhao *et al*, 1999). Therefore, we reasoned that SXL could compete with CstF-64 for binding to the proximal GU-rich element in *e(r)*, resulting in the activation of the distal poly(A) site in females.

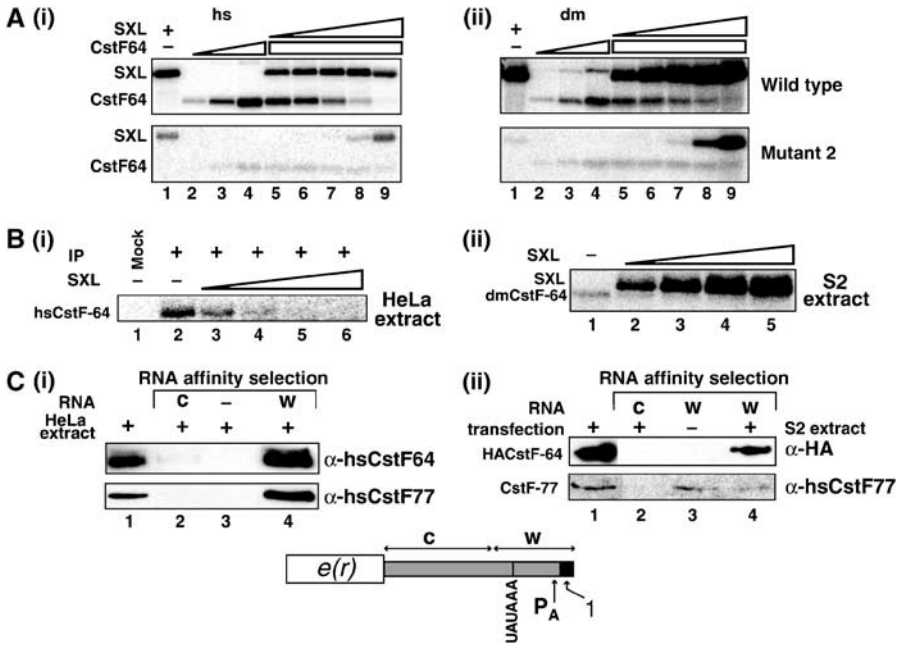

Figure 4 SXL competes with CstF-64 for binding to the proximal GU-rich element *in vitro*. (**A**) (i) Radiolabeled RNA containing the proximal GU-rich element was crosslinked to specific proteins and analyzed on an SDS–polyacrylamide gel. The concentrations (ng/μl) for SXL and hsCstF-64 were as follows: SXL (i and ii): lane 1, 150.0; lane 5, 1.85; lane 6, 5.5; lane 7, 16.5; lane 8, 50.0; lane 9, 150.0; hsCstF-64 (i): lane 2, 13.8; lane 3, 41.6; lanes 4–9, 125.0; and dmCstF-64 (ii): lane 2, 16.6; lane 3, 50.0; lanes 4–9, 150.0. The mutant2 is as described in Figure 3. Longer exposure is shown because of the weaker signal from dmCstF-64. (**B**) SXL competes with the human CstF-64 (i) and the putative *Drosophila* CstF-64 (ii) in the nuclear extract for binding to the proximal GU-rich element. Identity of the human hsCstF-64 protein was confirmed by immunoprecipitation with anti-CstF64 antibodies. (**C**) RNA affinity selection confirms that the CstF complex assembles on the proximal GU-rich element. The proximal GU-rich element (w), but not a control RNA (c), pulls down both CstF-64 and CstF-77 from the HeLa (i) and S2 (ii) nuclear extracts. A schematic of the 3′UTR and the positions of the RNAs used for affinity selection are shown.

To test this hypothesis, we performed a competition assay. In an *in vitro* RNA-binding experiment using photochemical crosslinking to radiolabeled RNA and analysis on an SDS–polyacrylamide gel, we found that recombinant SXL and the RNA-binding domain of the human (hsCstF64) and *Drosophila* (dmCstF-64) CstF-64 proteins crosslinked to the proximal GU-rich element (Figure 4A(i) and (ii), lanes 1–4, wt). Most importantly, SXL competed *in vitro*, in a concentration-dependent manner, for the crosslinking of both the human (Figure 4A(i), lanes 5–9, wt) and the *Drosophila* (Figure 4A(ii), lanes 5–9, wt) CstF-64 to the proximal GU-rich element, indicated by the disappearance of the crosslinked CstF-64 band. However, both SXL and CstF-64 showed significantly reduced crosslinking to a GU-rich element mutant (mutant2), which is a reflection of their specificity for GU-rich sequences and is consistent with the known RNA-binding properties of these proteins (Singh *et al*, 1995; Takagaki and Manley, 1997). In addition, at these concentrations, SXL showed no detectable competition for the low-level binding of CstF-64 to the mutant sequence (Figure 4A(i) and (ii), lanes 5–9, mutant2). We conclude that the proximal GU-rich element is a specific, high-affinity SXL-binding site, and that SXL directly competes *in vitro* for the binding of CstF-64 to this element.

To confirm that the competition seen with recombinant SXL and CstF64 is also relevant in the context of crude nuclear extract and in the context of endogenous CstF complex, we performed the following experiments. First, SXL competed with the endogenous human CstF64 in HeLa nuclear extract (Figure 4B(i), lane 2 versus lanes 3–6). The identity of the human CstF64 was confirmed using immunoprecipitation with α-CstF64 antibodies (Figure 4B(i), lane 1 versus 2). In addition, SXL competed with the putative *Drosophila* CstF64 protein (the only crosslinked band was of the expected molecular mass), in the nuclear extract from S2 cells (Figure 4B(ii), lanes 2–5), although antibodies were unavailable to independently confirm the identity of the *Drosophila* ortholog. Second, to determine whether intact CstF complex assembled onto the proximal poly(A) site, we performed an RNA affinity selection, using RNAs corresponding to the proximal polyadenylation elements (w) or an upstream control sequence (c) immobilized onto agarose beads. CstF64 from nuclear extract prepared from HeLa cells (Figure 4C(i), lane 4) and from S2 cells transfected with HA-tagged CstF64 (Figure 4C(ii), lane 4) was specifically recruited to the appropriate RNA substrate (w; see Schematics). As negative controls, CstF64 was not pulled down by the control RNA (c) (Figure 4C(i) and (ii), lane 2), by beads only (i, lane 3), or from an extract prepared from untransfected S2 cells (ii, lane 3). Moreover, the affinity-selected CstF64 co-immunoprecipitated with CstF77 in both HeLa and S2 nuclear extracts (Figure 4C(i) and (ii), lane 4, bottom panel), indicating that intact CstF complex is recruited to the proximal GU-rich element; a weaker signal for the *Drosophila* extract (Figure 4C(ii), lane 4, bottom panel) reflects poor crossreactivity of anti-hsCstF77 antibodies to the *Drosophila* ortholog. We conclude that SXL competes with the CstF64 subunit of the intact CstF complex in nuclear extract for binding to the proximal GU-rich element.

We determined the binding affinities of both SXL and CstF64 for RNAs containing single and multiple potential

SXL-binding sites (sites 1 or 1–3). The binding affinity of SXL for site 1 ($K_d \sim 10$ nM) (Figure 5A, lanes 8–12) was comparable to that of the well-characterized high-affinity SXL-binding site in *tra* (lanes 2–6) (Valcarcel *et al*, 1993), within 2- to 3-fold experimental variation. SXL formed a single complex on the RNA substrate with a single binding site. However, on the RNA with three sites, we observed up to three or four supershifted RNA–protein complexes in an SXL concentration-dependent manner (lanes 14–19), suggesting that SXL can bind to each of these sites. However, CstF64 bound to both RNAs in a similar manner, with a binding affinity (K_d) of $\sim$350 nM for site 1 based on the disappearance of free RNA in a gel mobility shift (Takagaki and Manley, 1997) and on UV crosslinking approaches (Figure 5B(i) and (ii), lanes 2–7 and 9–14). This affinity is within the range ($\sim$100 nM to 1.5 μM) reported for CstF64-selected RNA aptamers (Takagaki and Manley, 1997). Thus, both SXL and CstF64 bind to the GU-rich elements with high affinity. Moreover, occupancy of multiple sites by SXL could at least in part explain why the construct with three SXL sites ($D_{123}D'$) is more efficient than that with a single site (D_1D') in conferring female-specific poly(A) switching in a heterologous context (Figure 3).

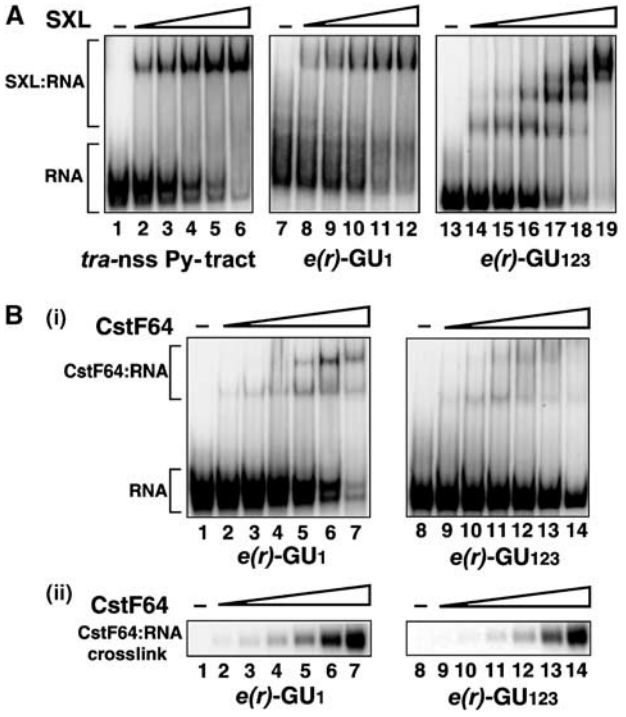

Figure 5 SXL binds to multiple binding sites in *e(r)*. Gel mobility shift analysis with SXL (**A**) and CstF64 (**B**, i) using RNAs containing either one (*e(r)*-GU_1) or three (*e(r)*-GU_{123}) SXL-binding sites, shown in Figure 1A(ii). The *transformer* non-sex-specific Py-tract (*tra-nss* Py-tract), a known SXL-binding site (Valcarcel *et al*, 1993), was used as a positive control. (B, ii) The same RNA-binding reaction as in panel (B, i) except that the RNA–protein complex was crosslinked with UV light and separated on a denaturing SDS–polyacrylamide gel. The concentrations (ng/μl) for SXL and hsCstF-64 were as follows. (A) SXL: lanes 1, 7, and 13, no protein; lanes 2, 8, and 15, 0.34; lanes 3, 9, and 16, 0.68; lanes 4, 10, and 17, 1.37; lanes 5, 8, and 18, 2.75; lanes 6, 12, and 19, 5.5; lane 14, 0.17; and (B, i and ii) hsCstF-64: lanes 1 and 8, no protein; lanes 2 and 9, 1.7; lanes 3 and 10, 5.1; lanes 4 and 11, 15.3; lanes 5 and 12, 46.0; lanes 6 and 13, 140.0; lanes 7 and 14, 420.0.

Poly(A) switching provides a means for translation regulation in the female germline

To determine the biological significance of the female germline-specific poly(A) switching of *e(r)* on gene expression, we fused portions of the 3′UTR of *e(r)* (proximal (P), distal (D), or both (P + D)) or of control 3′UTRs (*Adh* and *K10*) downstream of the coding region of an EGFP reporter that was expressed from the *e(r)* promoter, and introduced these constructs into flies. Expression of the EGFP reporter was determined by fluorescence microscopy in isolated ovaries. Nontransgenic flies showed barely detectable signal (Figure 6A, panel w^{1118}), whereas control 3′UTRs allowed efficient expression of the EGFP reporter (panels *Adh* and *K10*). The non-sex-specific portion of *e(r)* showed an extremely high level of reporter expression (panel P). However, the female-specific portion of *e(r)* showed significantly re-

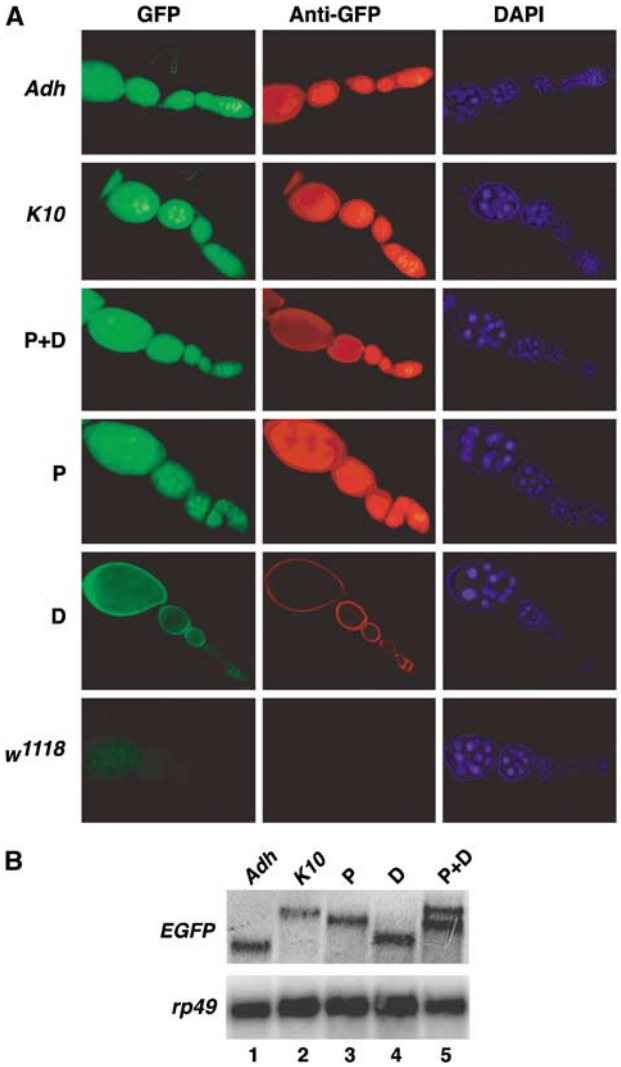

Figure 6 The female-specific portion of *e(r)* represses translation *in vivo*. (**A**) Different portions of the 3′UTR of *e(r)* (P, D, P + D) (see Materials and methods for details) and control 3′UTRs (*Adh*, *K10*) were placed downstream of an EGFP reporter under the control of the *e(r)* promoter, and analyzed for reporter translation *in vivo* using fluorescence microscopy (left panels) and immunostaining with anti-GFP antibodies (middle panels) in isolated ovaries. DAPI staining (right panels) is also shown. (**B**) Northern analysis of RNA from the transgenic lines.

duced reporter expression (panel D) in germ cells. There was a detectable signal in the surrounding follicle cells, which are of somatic origin, suggesting that this 3′UTR is otherwise competent to support translation. The entire 3′UTR (P + D) of *e(r)* showed reporter expression level much lower than that in the P line (panel P + D), consistent with the extent of poly(A) switching in this line (Figure 6B). These results were also confirmed using immunostaining with anti-GFP antibodies (middle panels). To exclude the possibility that the level of EGFP expression was due to differences in levels of RNA synthesis or stability, we performed Northern analysis. The level of EGFP poly(A)$^+$ RNA was comparable in P, D, and P + D flies (Figure 6B, lanes 3–5). Thus, we argue that the EGFP signal reflects primarily differences in reporter translation. We conclude that the female-specific portion of *e(r)* represses reporter translation *in vivo* in the female germline.

Discussion

In this study, we demonstrate that SXL regulates *e(r)* expression *in vivo* by a novel mechanism—polyadenylation switching—which allows translation regulation in the female germline.

Model for the sex-specific polyadenylation of e(r)

A model that explains the SXL-mediated regulation of *e(r)* must answer the following questions. First, what is the molecular basis for the default polyadenylation pattern in male flies? Second, how does SXL mediate poly(A) switching in females? Third, why does the poly(A) switching of *e(r)* occur primarily in the female germline?

Male-specific polyadenylation. We show that three key factors account for the default poly(A) pattern of *e(r)* in male flies: differences in the binding affinities of CstF-64 for the two alternative GU-rich elements; order of the two polyadenylation sites; and *cis* competition between the two poly(A) signals. First, the longer GU-rich enhancer element downstream of the proximal cleavage/polyadenylation site provides at least in part a basis for the increased apparent binding affinity for CstF-64, most likely by providing multiple registers for binding (Banerjee *et al*, 2003), and thus confers competitive advantage to the proximal poly(A) site.

Second, the order of the two poly(A) sites is also important. Contrary to our expectation that the proximal site is inherently stronger than the distal site, DP males (Figure 3, lane 5) exclusively use the D_A site. This demonstrates that the arrangement of the two sites is important for the default poly(A) site choice and excludes the possibility that the distal site is inherently weak. The usage of the D_A site is consistent with the known RNA-binding properties of CstF-64 (Takagaki and Manley, 1997) and sequences of natural poly(A) sites (MacDonald *et al*, 1994). Moreover, in this system, the promoter-proximal site is always used by default in males (Figure 3; P_A in wt, D_A in DP, and D_{123A} in $D_{123}D$). Both transcription and splicing influence polyadenylation (Niwa and Berget, 1991; Hirose and Manley, 2000; Vagner *et al*, 2000; Proudfoot *et al*, 2002). As our EGFP reporter, unlike the endogenous transcript, lacks an intron, coupling between the transcription and polyadenylation machineries best explains why the promoter-proximal site is always preferred in males.

Third, mutation of the SXL-binding site or the proximal GU-rich element compromises CstF64 binding and activates the distal site in males. This is what would be expected if the SXL-binding site is also a polyadenylation element (or the CstF64-binding site) and if SXL blocks the proximal poly(A) site, which is consistent with the SXL blockage model proposed for splicing regulation (Sosnowski *et al*, 1989; Inoue *et al*, 1990; Valcarcel *et al*, 1993). As CstF-64 binds directly to the GU-rich enhancer element to facilitate recruitment of the polyadenylation machinery to the poly(A) signal (Zhao *et al*, 1999), reduced affinity of CstF-64 to the mutant proximal GU-rich element provides a basis for the activation of the distal site in males. This shows that the longer GU-rich element associated with the proximal site is also important for the default poly(A) site choice. It is possible that the non-canonical poly(A) element (UAUAAA instead of AAUAAA) and its sequence context (Figure 1A(ii)) confer increased dependence on the long GU-rich element for polyadenylation at the proximal site. This feature could make the proximal GU-rich element and CstF-64 binding an attractive target for SXL regulation.

Female-specific poly(A) switching. The simplest explanation for our combined results is that in the female germline SXL competes for the binding of CstF-64 to the proximal GU-rich element and represses polyadenylation at the proximal site, leading to activation of the distal site (Figure 7); SXL does not bind to the distal GU-rich element (data not shown). The proximal GU-rich element is much longer than is necessary for polyadenylation or CstF-64 binding (MacDonald *et al*, 1994; Takagaki and Manley, 1997), most likely to confer competitive advantage (by providing multiple registers for CstF-64 binding; Banerjee *et al*, 2003) to the proximal site in males and to accommodate SXL binding in females. Moreover, SXL and CstF-64 have similar sequence preference

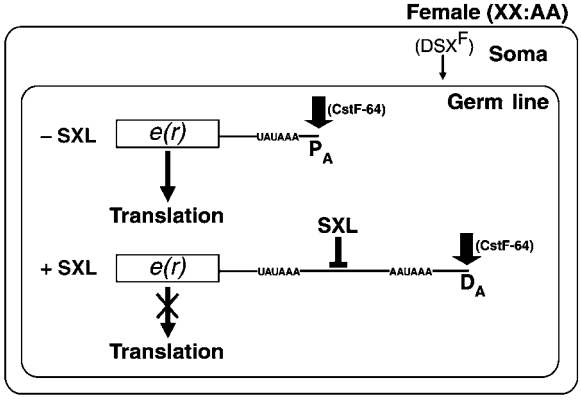

Figure 7 A model for sex-specific polyadenylation site switching of *e(r)* in the female germline. (−SXL), CstF-64 binds to the proximal GU-rich element and promotes assembly of the polyadenylation machinery preferentially at the proximal site, producing the *e(r)-nss* isoform. (+SXL), SXL competes with CstF-64 for binding to the proximal GU-rich element, leading to the activation of the distal poly(A) site. The *e(r)-nss* transcript is translated in the female germline, but *e(r)-fs* isoform is not; SXL may also be involved in translation repression. Thick arrows indicate the preferred (proximal (P_A) or distal (D_A)) cleavage/polyadenylation sites.

(GU-rich sequences devoid of cytidines) (Singh *et al*, 1995; Takagaki and Manley, 1997). Therefore, it is not surprising that we could not uncouple the sequence requirement of the proximal GU-rich element for polyadenylation in males and for poly(A) switching in females (Figure 3), as was performed using a three nucleotide U-to-C substitution for SXL-mediated splicing regulation of *tra* (Sosnowski *et al*, 1989; Inoue *et al*, 1990), which involves competition between SXL and U2AF (Valcarcel *et al*, 1993); U2AF prefers uridine- and cytidine-rich sequences devoid of guanosines (Singh *et al*, 1995, 2000). Specificity of SXL for the proximal but not the distal GU-rich element and our ability to convert a poly(A) site that is nonresponsive to poly(A) switching to a site that supports female-specific switching provide an explanation for why switching occurs in wt and $D_{123}D'$ females, but not in DP and DD′ females (Figure 3). Moreover, SXL-dependent poly(A) site switching of *e(r)* (Figure 3) as well as splice site switching of *Sxl* and *msl2* transcripts displays a need for multiple SXL-binding sites for efficient regulation (Forch and Valcarcel, 2003). The ability of SXL to discriminate between alternative GU-rich sequences is the crux of the sex-specific poly(A) switching. GU-rich sequences belong to a class of regulatory RNA motifs that are defined by base composition rather than an exact sequence. Such simple repetitive sequences offer several advantages for gene regulation: for example, binding affinity but not specificity for RNA-binding proteins can change as a function of the length of the sequence (Banerjee *et al*, 2003); two proteins (e.g., SXL and CstF-64) with different binding site length requirements can recognize the same sequence (Singh *et al*, 1995; Takagaki and Manley, 1997); a regulatory protein such as SXL can regulate multiple processes, polyadenylation and splicing, by antagonizing CstF-64 and U2AF65, respectively, because of the possibility of overlapping binding specificities; and single nucleotide changes are less disruptive.

It is possible that future development of an *in vitro* poly(A) switching assay may provide additional evidence for a direct role of SXL or formally exclude a less likely possibility of involvement of another factor that is regulated by SXL, is an RNA-binding protein, is female germline specific, and has RNA-binding properties identical to those of SXL. Nonetheless, several lines of evidence presented here (Figures 2–4) strongly argue that SXL has a direct role in female germline-specific poly(A) switching of *e(r)*.

Female germ-line specificity of poly(A) switching. Intriguingly, the female germline-specific *e(r)-fs* transcript is barely detectable in the female soma where SXL is present and functional. Our experiments have ruled out that female germline-specific activation of the distal site contributes to poly(A) switching. Several factors, which are consistent with the repression of the proximal site, could account for why SXL-dependent poly(A) switching is seen in a female germline-specific manner. For example, levels of SXL change during oogenesis (Vied and Horabin, 2001), female germline-specific SXL isoforms are known (Bopp *et al*, 1991; Samuels *et al*, 1991), and changes in the nuclear translocation of SXL are observed during oogenesis (Bopp *et al*, 1993). Finally, SXL may associate with a germline-specific cofactor for efficient poly(A) switching, as described for SXL-dependent translation repression of *msl2* in embryonic extract (Grskovic *et al*, 2003).

Comparison with other examples of poly(A) site switching

CstF-64 plays an important role in constitutive and regulated polyadenylation. The levels of CstF-64 change significantly during B-lymphocyte differentiation and an increase in the levels of CstF-64 is necessary and sufficient for the poly(A) site switching of the immunoglobulin heavy-chain (IgM) mRNA (Takagaki *et al*, 1996; Takagaki and Manley, 1998; Proudfoot *et al*, 2002). However, there are important differences between the IgM and *e(r)* systems. For example, in IgM, there are competing splicing and polyadenylation sites, the two poly(A) sites are separated by >2 kb, and changes in relative concentrations/activities of ubiquitously expressed GU-rich element-binding proteins (CstF64 and hnRNPs) contribute to poly(A) site choice (Takagaki *et al*, 1996; Takagaki and Manley, 1998; Zhao *et al*, 1999; Veraldi *et al*, 2001; Castelo-Branco *et al*, 2004). In contrast, the sex-specific poly(A) site choice for *e(r)* is unaffected by splicing, the two sites are separated by only 221 nucleotides, and the absence or presence of the female-specific protein SXL correlates with poly(A) site choice. Whereas poly(A) switching produces different protein isoforms (secreted versus membrane bound) of IgM, it affects the 3′UTR rather than the coding sequence of *e(r)* and influences protein synthesis. Finally, unlike most other examples of alternative polyadenylation, which often involve skipping of a weaker proximal poly(A) site to allow use of a stronger distal site by default (Perez Canadillas and Varani, 2003), a promoter-proximal site is always preferred by default in *e(r)*.

Biological significance

The poly(A) site switching described here provides a new mechanism for the retention of an SXL-binding site(s) (in 3′UTR) in a female germline-specific manner for translation repression, although it remains to be determined whether the elements that mediate translation regulation are similar to or different from those required for poly(A) switching. SXL-mediated alternative splicing (intron retention in the 5′UTR) of *msl2* is another mechanism for retention of SXL-binding sites for translation repression in somatic cells in females (Forch and Valcarcel, 2003). Thus, it would not be surprising if SXL were also involved in translation repression. Comparison of the two mRNAs should provide important insights into the molecular mechanism of translation regulation.

Although SXL regulates distinct steps along the gene expression pathway (splicing, translation, and now polyadenylation) and targets different factors (e.g., U2AF, TIA-1, and CstF-64) (Forch and Valcarcel, 2003), it uses a common underlying mechanism, that is, competition for the binding of factors that recognize GU- or U-rich sequences and play non-overlapping roles in the assembly of appropriate multi-ribonucleoprotein or -protein complexes. Thus, overlapping RNA-binding specificities and the possibility of combinatorial control from association with cofactors could allow SXL to specifically regulate distinct processes (splicing, translation, and polyadenylation) in different tissues. ER is a potential transcription repressor (Pogge von Strandmann *et al*, 2001), interacts with components of the transcription machinery (Amente *et al*, 2005), and may be subject to cell cycle regulation (Gelsthorpe *et al*, 1997). As loss of SXL in the female germline results in ovarian tumors, it is tempting to

speculate that *e(r)* may act as an anti-oncogene and provide the missing link in the pathway connecting SXL to germ cell proliferation/differentiation and oncogenesis. Loss of *e(r)* from microinjection of double-stranded RNA into embryos results in non-sex-specific embryonic lethality, which could at least in part explain why the sex-specific regulation of the *e(r)* transcript escaped previous genetic screens (see Supplementary data).

Conclusions

Identification of *e(r)* represents the first known example of SXL-dependent poly(A) switching, and opens up the possibility that other genes may also be regulated in this manner. Poly(A) switching provides a new mechanism to allow subsequent translation regulation. As *e(r)* is suggested to be a transcription regulator, it appears to be a potent downstream target of the sex determination pathway and may help unravel the mysterious role of SXL in the female germline.

Materials and methods

Materials

The *Sxl* stocks were obtained from the Bloomington Stock Center, the *snf* stocks from Dr Helen Salz, the suppressor stocks from Drs Jamila Horabin and Daniel Bopp, and the *mago* and *tudor* stocks from Dr Robert Boswell. Flies were raised on standard corn meal food at 25°C. The cDNA clones and ESTs were obtained from Research Genetics, CA, and the *e(r)* genomic DNA was obtained from Dr Stuart Tsubota. The 3′UTR *e(r)-fs* probe (fs-UTR) was generated by PCR from the EST clone LD36385 using primers A and B. Recombinant GST-fusion proteins were as described: SXL (Valcarcel *et al*, 1993); hsCstF-64 (Takagaki and Manley, 1997); *Drosophila* CstF-64 cDNA was PCR amplified from the EST clone RE27227 using primers C and D and cloned into pGEX-6P-1 vector. The anti-HA antibody (12CA5) was purchased from Roche, and the anti-hsCstF-64 and anti-hsCstF-77 antibodies were obtained from Dr Christine Milcarek and Dr David Bentley.

Plasmids, DNA templates, and primers

The EGFP reporter constructs, transcription templates, EGFP translation reporter constructs, and primers are described in Supplementary data.

RNase protection assay

The RNase protection was performed essentially as per the manufacturer's instructions for the RPA III kit (Ambion Inc.).

Northern analysis

Northern analysis was performed as previously described (Robida and Singh, 2003).

Generation of transgenic lines

Transgenes were introduced into the *Drosophila* genome by *P*-element-mediated transformation (Serano *et al*, 1994).

Gel shift, RNA–protein UV crosslinking assay, and immunoprecipitation

The gel mobility shift and RNA–protein UV crosslinking assays were performed as previously described (Valcarcel *et al*, 1993; Singh *et al*, 1995). For immunoprecipitation, anti-hsCstF64 antibodies or preimmune serum were crosslinked to protein A beads, incubated with the crosslinked RNA–protein complexes, washed, and separated on an SDS–PAGE gel (see Supplementary data).

RNA affinity selection and Western analysis

RNA was synthesized from relevant templates using T7 RNA polymerase, immobilized on adipic acid dihydrazine agarose beads, incubated with nuclear extract prepared from untransfected S2 cells, from S2 cells transfected with HA-CstF-64, or from HeLa cells, and subjected to Western analysis using appropriate antibodies (see Supplementary data).

Whole-mount fluorescence and immunological detection of reporter translation

Female flies were fed on yeast paste for 2–4 days after eclosion. Ovaries were dissected in PBST (1 × PBS, 0.1% Triton X-100), fixed for 15 min in PBST with 4% formaldehyde, stained with DAPI, and examined for EGFP signal by florescence microscopy and by immunostaining using a Zeiss 2.2.1 microscope (Mohr *et al*, 2001).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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