A shared enhancer controls a temporal switch between promoters during *Drosophila* primary sex determination

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***Sex-lethal* (*Sxl*), the master regulatory gene of *Drosophila* somatic sex determination, is stably maintained in an on or an off state by autoregulatory control of *Sxl* premRNA processing. Establishment of the correct *Sxl* splicing pattern requires the coordinate regulation of two *Sxl* promoters. The first of these promoters, *SxlPe*, responds to the female dose of two X chromosomes to produce a pulse of *Sxl* protein that acts on the premRNA products from the second promoter, *SxlPm,* to establish the splicing loop. *SxlPm* is active in both sexes throughout most of development, but nothing is known about how *SxlPm* is expressed during the transition from X signal assessment to maintenance splicing. We found that *SxlPm* is activated earlier in females than in males in a range of *Drosophila* species, and that its expression overlaps briefly with that of *SxlPe* during the syncytial blastoderm stage. Activation of *SxlPm* depends on the *scute*, *daughterless*, and *runt* transcription factors, which communicate X chromosome dose to *SxlPe*, but is independent of the X signal element *sisA* and the maternal co-repressor *groucho*. We show that DNA sequences regulating the response of *SxlPe* to the X chromosome dose also control the sex-differential response of *SxlPm*. We propose that co-expression of *Sxl* protein and its premRNA substrate facilitates the transition from transcriptional to splicing control, and that delayed activation of *SxlPm* in males buffers against the inappropriate activation of *Sxl* by fluctuations in the strength of the X chromosome signal.**

D. virilis | *D. yakuba* | X:A ratio | XSE | genetic switch

Cell fate decisions often are controlled by regulatory genes or pathways that respond to small quantitative differences in the concentrations of signaling molecules. The mechanisms that maintain such genes or pathways in stable states often differ from those that initially signal the responses. Such is the case in the somatic sex determination system of *Drosophila*. Throughout nearly all of the fly life cycle, sexual identity is maintained via a positive feedback mechanism that controls the splicing of the transcripts of the switch gene, *Sex-lethal* (*Sxl*), so that they encode active *Sxl* proteins in females and truncated, inactive proteins in males (1–3). This occurs because transcripts from the "maintenance" promoter, *SxlPm*, are processed into functional mRNA species only in the presence of *Sxl* protein (4–6). Whereas positive autoregulatory splicing of *SxlPm*-derived transcripts constitutes a self-sustaining loop, a different mode of regulation is required to initiate the process. The key event is the transient activation of the "establishment" promoter, *SxlPe,* in response to the female dose of two X chromosomes (7–9). Transcripts from *SxlPe*, unlike those from *SxlPm*, are spliced by default so as to produce functional *Sxl* mRNA and protein (7, 10, 11). Thus, the burst of *SxlPe* activity in XX embryos supplies the protein that initiates female-specific splicing from *SxlPm*. In contrast, the failure to activate *SxlPe* in XY embryos leaves the *SxlPm*-derived RNAs to be spliced in the nonfunctional male state.

Whereas the transient female-specific activation of *SxlPe* in response to X chromosome dose has been the target of much experimental scrutiny, little is known about the control of *SxlPm*. The standard view is that *SxlPm* is a "housekeeping" promoter active in both sexes from around the time of gastrulation through adulthood. Analysis of *Sxl* RNA by Northern blot or RNase protection assays (4, 7, 12, 13) has suggested a time lag of 1–2 h between the cessation of *SxlPe* activity in early nuclear cycle 14 and the onset of *SxlPm* expression (14), supporting the concept that the two promoters are expressed independently. On the other hand, Barbash and Cline (15) detected *SxlPm*-derived transcripts during cycle 14, and Keyes (16) noted that *SxlPm* appeared to be expressed earlier in XX embryos than in XY embryos, raising the possibility of a direct regulatory connection between *SxlPm* and *SxlPe*.

To gain insight into the switch between the two *Sxl* promoters, we analyzed the time course of *SxlPm* activity during and immediately after syncytial cycles 12, 13, and early 14, when the X chromosome dose is assessed at *SxlPe* (9, 17, 18). Remarkably, we found that the activities of the two promoters overlap briefly in female embryos and that there is a short, but distinct, delay in the appearance of *SxlPm* transcripts in males. This female-first pattern of maintenance promoter activity is conserved in *Drosophila* species, suggesting that *SxlPm* also responds to the number of X chromosomes. Here we show, using genetic and transgenic analyses, that the earlier onset of *SxlPm* in females is not simply a consequence of the female-specific activation of the downstream *SxlPe*, but that the two promoters share a common enhancer that responds to X chromosome dose. We report that some, but not all, of the X-linked signal element (XSE) proteins that regulate *SxlPe* (8, 9, 19) are needed for the earliest expression from *SxlPm*, demonstrating an unexpected complexity in the X-counting mechanism. We propose that the early overlapping activities of the two promoters facilitates the transition to stable splicing control, and that the association between X chromosome dose and *SxlPm* further amplifies the XSE signal and thus contributes to the robustness of the on-or-off control of *Sxl*.

Results

To define when *SxlPm* is activated, we developed an in situ hybridization assay using an intron-derived probe (Fig. 1) that enabled us to identify nascent *SxlPm*-derived transcripts as focused dots of staining in embryonic nuclei. Because *Sxl* is located on the

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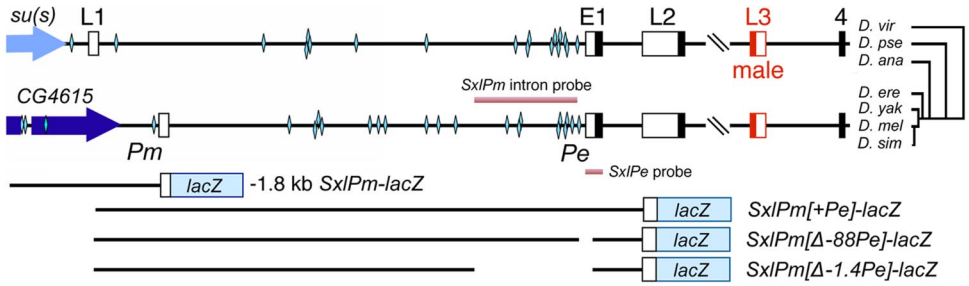

Fig. 1. Map of *Sxl* locus and *SxlPm-lacZ* fusions. (*Top*) Structure of *Sxl* exons L1, E1, L2, L3, and 4 in *D. pseudoobcura* and *D. melanogaster*. Exons 5–10 are omitted. *SxlPm* and *Pe* promoters are marked. Female splice patterns are E1 to 4 and L1 to L2 to 4. The male splice pattern is L1–L2–L3–4. Diamonds represent known or predicted *Sc*/*Da* binding sites. Filled portions of *Sxl* exons represent coding regions. A chromosomal rearrangement exchanged the ancestral upstream *su(s)* gene for *CG4615* after divergence of the *D. ananassae* and the *D. erecta*, *D. yakuba*, *D. melanogaster*, and *D. simulans* lineages. *D. virilis* diverged from the other species about 40 million years ago. (*Bottom*) *SxlPm-lacZ* transgenes. Genomic fragments extended 1.8 kb or 0.5 kb upstream of *Sxl* exon L1. Internal deletions from -88 to + 85 or -1,452 to + 85 bp relative to exon E1 removed the *SxlPe* promoter and regulatory sequences.

X chromosome, we could differentiate between male (XY) and female (XX) embryos based on the number of dots visible in the nuclei. Progression through cycle 14 was monitored using two different parameters: the ratio between the length and width of the surface nuclei and the extent of cell membrane invagination during the cellularization process (20, 21).

***Sxl* Maintenance Promoter Is Activated Earlier in Females than in Males.** Inspection of embryos after hybridization with *SxlPm*-specific probes revealed that *SxlPm* is expressed in both sexes from early in the cellularization cycle until the completion of embryonic development (Fig. 2*A* and data not shown). Analysis of early embryos showed that the initial expression of *SxlPm* was sexually dimorphic. Transcripts from *SxlPm* first appeared in females during nuclear cycle 13 [Fig. 2*A*; supporting information (SI) Table S1]. Initially, only about 15% of the female nuclei expressed *SxlPm*, and many nuclei expressed it from only one of the two X chromosomes, suggesting that activation of *SxlPm* is a stochastic process occurring independently on each X chromosome. During the first minutes of cycle 14, the proportion of expressing nuclei, as well as the number of nuclei expressing both alleles, increased, until by 10–15 min, every female nucleus expressed both copies of *SxlPm*. In the male embryos, expression from *SxlPm* was delayed by about 10 min relative to that in the female embryos (Fig. 2*A*; Table S1). Nascent transcripts from *SxlPm* were first seen in the XY embryos as very faint dots in scattered nuclei in early cycle 14. As cycle 14 progressed, the proportion of XY nuclei expressing *SxlPm* and the staining intensity of the nuclear dots increased, until by 20–30 min, every male nucleus was transcribed from *SxlPm*. Once *Sxl* was fully active, both sexes maintained expression from *SxlPm* throughout embryogenesis (data not shown).

Our findings demonstrate that *SxlPm* becomes active earlier than was previously estimated from Northern blot and RNase

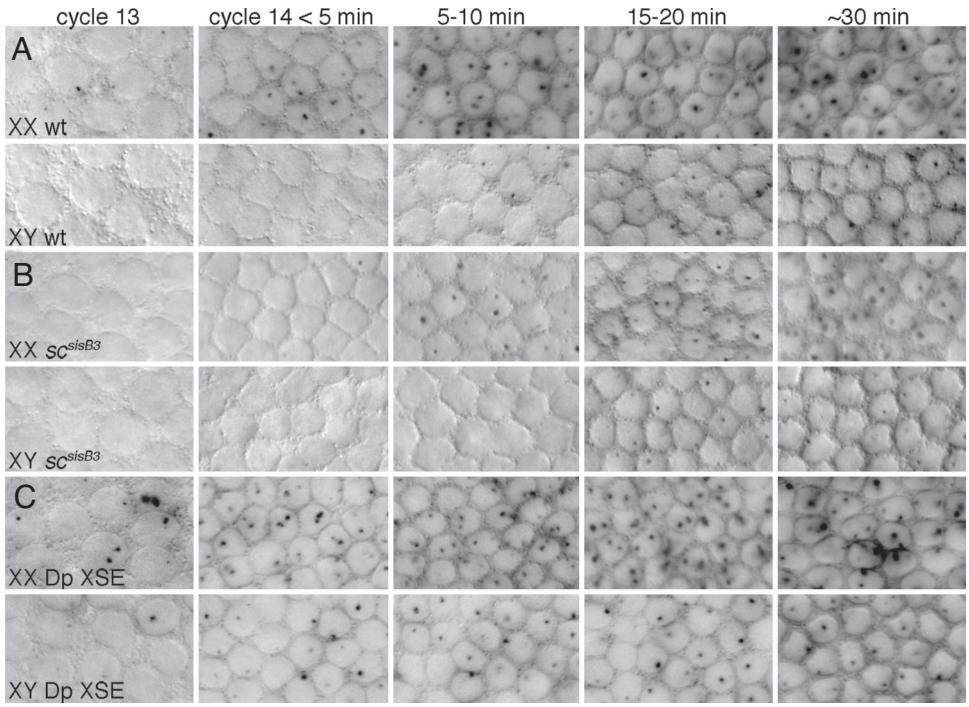

Fig. 2. Time course of nascent transcripts from *SxlPm* determined by in situ hybridizations with a probe specific for *SxlPm*-derived premRNA. Surface views of syncytial nuclei. (*A*) Wild-type XX and XY embryos. (*B*) sc^{sisB3} mutant XX and XY embryos. (*C*) XX and XY embryos with two extra copies of the XSEs sc^{+} and $sisA^{+}$. Embryos in (*B*) were progeny of: y sc^{sisB3}/y sc^{sisB3} Sxl^{M4} *sn* females and *y* sc^{sisB3}/Y males. Embryos in (*C*) were progeny of: w^{1118} females and *y w cm* Sxl^{f1} ct^{6}/Y; 2X P(mini-w^{+},$sisA^{+}$)and 2X P(mini-w^{+},sc^{sisB+})/CyO males.

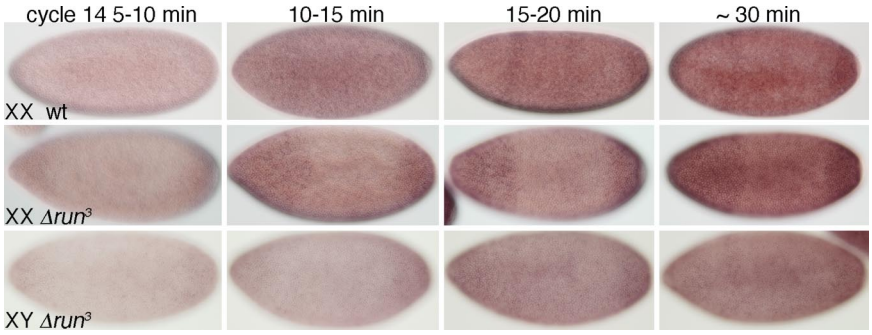

Fig. 3. The XSE *runt* controls expression from *SxlPm*, as determined by in situ hybridizations with a *SxlPm*-specific intron probe. (*Top*) Wild-type females at the indicated times in nuclear cycle 14. (*Middle*) Homozygous run^3 mutant XX progeny. (*Bottom*) Hemizygous run^3 XY embryos. Mutant XX embryos were progeny of *w f* run^3/FM7c females and *w f* run^3/Yy^+, mal^+ males. One-half of the XX embryos exhibited the abnormal staining pattern. The XY embryos were progeny of *w f* run^3/FM7c females and *FM7c*/Y males. Wild-type females were from w^{1118} parents.

protection analyses (12, 13). They also indicate that the periods of *SxlPe* and *SxlPm* expression overlap in females during the first 10–20 min of cycle 14 (9, 18, 22). To determine whether the sexually dimorphic pattern of *SxlPm* activation is conserved in other *Drosophila* species, we examined *D. virilis*, *D. yakuba,* and *D. simulans* (Fig. 1) using in situ hybridization. We found that all three species expressed *SxlPm* similarly to *D. melanogaster* (Fig. S1 and A.N.G. and J.W.E., unpublished data), suggesting that the female-first pattern of maintenance promoter activation is, like the female-specific activation of *SxlPe* (22), an ancient response to the number of X chromosomes.

The XSEs *scute* and *runt* and Maternal *daughterless* Regulate *SxlPm*. *SxlPe* is activated during nuclear cycle 12 and expressed through the first 10–20 min of cycle 14 in response to the two X chromosome dose of XSEs (9, 15, 18). To determine whether the same XSEs that control the on-or-off response of *SxlPe* also regulate *SxlPm*, we analyzed mutations in several XSEs and co-factors to determine whether they affected transcription from the maintenance promoter.

The XSE *scute* encodes a dose-sensitive bHLH transcription factor that dimerizes with the maternally supplied *daughterless* protein to directly activate *SxlPe* (23). We found that loss of zygotic *scute* (*sc*) or maternal *daughterless (da*) also affects *SxlPm*. In sc^{sisB3} and maternal da^1, mutant progeny expression of *SxlPm* was delayed in both sexes by about 5–10 min compared with wild type (Fig. 2*B* and Fig. S2*A*). In XX embryos, no expression was observed during cycle 13, and only a fraction of nuclei showed expression by 5 min in cycle 14. Thereafter the proportion of expressing nuclei increased, however, until by about 20 min in cycle 14, all female zygotic nuclei expressed *SxlPm* in a manner indistinguishable from wild type. Expression in sc^{sisB3} and da^1 males was similarly delayed. About one-half of the XY nuclei expressed *SxlPm* at 15 min into cycle 14, and all stably activated the maintenance promoter by 30 min into the cellularization process.

We observed similar results with the XSE *runt*, which is required to activate *SxlPe* in the central region of the embryo (24). In homozygous Δrun^3 females, the number of nuclei expressing *SxlPm*, and the staining intensity of the individual dots, decreased noticeably between 5 and 30 min in cycle 14 (Fig. 3). This caused a diminution of overall embryo staining intensity in central regions relative to the poles during early cycle 14. Similar effects were seen in Δrun^3 males, but the lower contrast resulting from the decreased expression level of their single *Sxl* allele made these effects more difficult to document photographically (Fig. 3).

Our findings demonstrate that Sc/Da and Runt regulate the onset of *SxlPm* expression in both sexes, but that none of these three proteins is required for maintenance promoter activity during the rest of embryonic development. This implies that at least two temporally distinct mechanisms control *SxlPm* activity: one mechanism regulating the sexually dimorphic onset of transcription in response to X chromosome dose and the other conferring constitutive expression throughout the remainder of the life cycle.

To test the notion that XSE dose specifies the timing of *SxlPm* activation, we investigated whether an increase in XSE copy number can cause earlier activation of the promoter (19). To increase the XSE dose, we crossed males heterozygous for a second chromosome carrying two transgenic copies each of sc^+ and $sisA^+$ with normal females (25, 26). This created a population consisting of XX embryos with four copies of sc^+ and $sisA^+$, XY embryos with three copies of sc^+ and $sisA^+$, and normal XX and XY embryos. We found that *SxlPm* was activated earlier in both sexes when the XSE dose was increased (Fig. 1*C*; Table S1). In females, the extra XSE genes caused about 70% of the nuclei to express *SxlPm* during cycle 13 and all XX nuclei to express *SxlPm* from the earliest stages of cycle 14. In males, the additional XSE copies caused nearly 30% of XY nuclei to express *SxlPm* ectopically during cycle 13 and nearly all to express *SxlPm* by 10 min into cycle 14.

Our findings demonstrate that the sexually dimorphic activation of *SxlPm* is controlled by some of the same determinants that signal the female-specific expression of *SxlPe.* They do not, however, allow us to distinguish whether the XSE proteins directly regulate *SxlPm* or whether their effects on *SxlPm* reflect indirect effects, due perhaps to local chromatin changes associated with the activation of the adjacent *SxlPe* (Fig. 1). As a first step toward answering the question of whether activation of *SxlPm* is linked in *cis* to that of *SxlPe,* we investigated whether we could identify conditions under which the activities of the two promoters could be uncoupled.

***SxlPe* Activity Is Not Needed for Proper Regulation of *SxlPm*.** Duplication of XSE activators leads to strong ectopic expression of *SxlPe* in male embryos. Reciprocally, elimination of the maternal co-repressor Groucho also causes strong ectopic *Sxl* expression in males by decreasing the threshold XSE concentrations necessary to activate *SxlPe* (17). We reasoned that if the initial female-specific response of *SxlPm* is coupled to the activation of the *SxlPe*, then a loss of *groucho* should result in premature expression from *SxlPm*. On the other hand, if Sc/Da and Runt activate the two promoters directly, then the sex-specific response of *SxlPm* may well be independent of *groucho*.

We found that embryos derived from mothers lacking *groucho* germline function expressed *SxlPm* in a wild-type pattern (Fig. S2*C*). Females first activated *SxlPm* in cycle 13

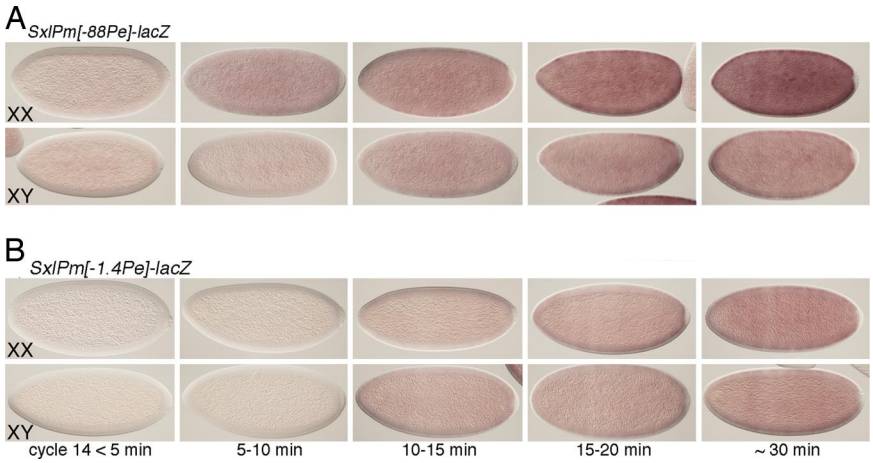

Fig. 4. A 1.4-kb deletion of *SxlPe* regulatory DNA equalizes *SxlPm* activity in the sexes. In situ hybridization was used to detect steady-state *lacZ* mRNA levels. (*A*) Embryos carried two copies of an autosomal *SxlPm[Δ-88Pe]-lacZ* transgene deleted for the *SxlPe* basal promoter region. (*B*) Embryos with two copies of an autosomal *SxlPm[Δ-1.4Pe]-lacZ* transgene deleted for *SxlPe* and its regulatory sequences to -1.4 kb. Sex was determined by fluorescent detection of endogenous *SxlPe*-derived transcripts. Times after the onset of cycle 14 are indicated. Four independent lines of each transgene were examined, with indistinguishable results.

and fully expressed the promoter by 10–15 min into cycle 14. Males initiated expression early in cycle 14 and fully expressed *SxlPm* some 20–30 min later. Our observations are consistent with direct regulation of the two promoters by the XSEs *scute* and *runt*, a conclusion further supported by our finding that the XSE *sisA* does not regulate *SxlPm*.

The X-linked *sisA* gene encodes a bZIP transcription factor needed for the female-specific activation of *SxlPe* in all somatic cells of the embryo (26, 27). When we analyzed the strong loss-of-function allele $sisA^1$, we found that neither homozygous $sisA^1$ females nor hemizygous $sisA^1$ males exhibited any delay in *SxlPm* activation or decreased staining of *SxlPm*-derived nascent transcripts compared with wild type (Fig. S2*B*). Taken together, our findings with *sisA* and *groucho* show that *SxlPe* activity can be blocked without affecting expression from *SxlPm*, and also that *SxlPe* activity can be induced ectopically without activating *SxlPm*. This strongly suggests that some, but not all, XSEs regulate the two promoters directly, but leaves open the question of whether the promoters share common enhancers or use independent *cis* regulatory elements.

***SxlPm* and *SxlPe* Share a Common Regulatory Element That Responds to X Chromosome Dose.** Our finding that *SxlPm* is activated earlier in XX embryos than in XY embryos in response to *scute*, *da*, and *runt* suggests that *SxlPm*, like *SxlPe*, responds directly to the number of X chromosomes present in the embryo. To determine whether the XSEs and other proteins regulate the two promoters through independent regulatory elements or whether they instead share a common enhancer, we analyzed the structure of *SxlPm* by creating a series of transgenes that fused different portions of the *Sxl* gene to a *lacZ* reporter.

We first assessed the function of the region upstream of the *SxlPm* transcription start site by fusing sequences from -1.8 kb to + 34 bp within exon L1 to *lacZ* (Fig. 1). We found that none of the four reporter lines tested expressed detectable *lacZ* mRNA in embryos, indicating that key regulatory elements necessary for *SxlPm* activity likely are located downstream of exon L1 (data not shown). Considering that the sequences upstream of *Sxl* are not conserved in all *Drosophila* species, having been exchanged by a chromosomal rearrangement some 10–15 million years ago, and that the 3′ ends of the upstream genes are located within about 200–500 bp of the *SxlPm* start site, we next tested a shorter upstream (−0.8 kb) and large downstream (+6.0 kb) segment encompassing the *SxlPe* regulatory elements for *SxlPm* function (Fig. 1). We found that these transgenes expressed *lacZ* mRNA in a manner consistent with normal *SxlPe* and *SxlPm* promoter activity (data not shown).

To analyze *SxlPm* independent of *SxlPe* activity, we created a modified version of the full-length transgene in which we removed a 171-bp segment that included the *SxlPe* basal promoter and part of the E1 exon (Fig. 1). This construct, designated *SxlPm[Δ-88Pe]-lacZ*, was expressed in a manner indistinguishable from the endogenous *SxlPm* promoter (Figs. 4*A* and S3*A*). Weak *lacZ* expression was detected in cycle 13 nuclei in XX embryos, and by 10–15 min into cycle 14, every nucleus appeared to express both copies of *SxlPm[Δ-88Pe]-lacZ*. Male embryos first expressed *SxlPm[Δ-88Pe]-lacZ* in cycle 14, with full activation occurring about 20 min later. Notably, XX embryos expressed *SxlPm[Δ-88Pe]-lacZ* mRNA at higher levels than XY embryos even when the transgenes were present in two copies in both sexes (Fig. 4*A*). This difference was maintained through cycle 14 and then gradually disappeared during gastrulation and germ band extension (data not shown). These results establish that all of the sequences necessary for normal expression of *SxlPm* lie between -0.8 and + 6.0 kb and confirm that a functional *SxlPe* is not required for the earlier onset of *SxlPm* activity in females.

Normal sex-specific regulation of *SxlPe* requires sequences an extension to −1.4 kb upstream of exon E1 (28). Within these sequences, two regions spanning from +20 to −400 bp and from −800 to −1,400 bp have been identified as being crucial for full *SxlPe* activity. To determine whether these *SxlPe* regulatory sequences also regulate *SxlPm*, we created a modified *SxlPm-lacZ* fusion carrying an internal deletion spanning the region from -1452 to +85 relative to the start site of exon E1 (Fig. 1). We found that the onset of expression from the *SxlPm[Δ-1.4Pe]-lacZ* transgenes was delayed relative to that from the *SxlPm[Δ-88Pe]* lines, and also that there was no longer any difference in the timing or level of expression between the sexes (Fig. 4 and Fig. S3). Expression from *SxlPm[Δ-1.4Pe]-lacZ* lines was first seen in a few nuclei in both XX and XY embryos about 10–15 min into cycle 14 (Fig. S3). The number of expressing nuclei increased thereafter, reaching a maximum 15–20 min later. Mature transcripts accumulated over time, but XX and XY embryos expressed equal *lacZ* mRNA levels at all times evaluated. We conclude that the 1.4-kb region that controls the female-specific expression of *SxlPe* also is required for the sex differential expression of *SxlPm*. We also note that the effects of

the *[Δ-1.4Pe]* mutation on *SxlPm* appeared to be stronger than those of *sc*, *da*, and *runt* mutations on the endogenous locus, because the mutations in the transacting regulators did not abolish male/female differences in *SxlPm* expression. This suggests that factors besides Sc/Da and Runt interact with the 1.4-kb region to control the initial activation of *SxlPm*.

Discussion

The *Drosophila* sex determination pathway elegantly illustrates the use of premRNA splicing control in development; however, the establishment of sex-specific splicing ultimately depends on the coordinated activities of two promoters for the master regulatory gene *Sxl*. In this work, we show that the switch from the initial assessment of X chromosome dose at *SxlPe* to the stable autoregulatory control of *Sxl* premRNA splicing exploits an unexpected level of transcriptional control of the *Sxl* maintenance promoter. We demonstrate that, contrary to the prevailing view, *SxlPm* responds to the X chromosome dose, and that it does so by sharing common X-signal elements and a common enhancer with *SxlPe*. The switch between *Sxl* promoters thus serves as a tractable model for exploring the logical circuitry and molecular mechanisms that control the fidelity of developmental switches and coordinate the uses of multiple promoters for a single gene (29).

Why Is SxlPm Regulated? A priori, a female embryo must do two things to establish and then remember its sex: It must produce a pulse of *Sxl* protein by transiently activating *SxlPe* in response to the XX signal, and it must activate *SxlPm,* so that its transcripts can be spliced to produce yet more *Sxl* protein. A male embryo needs only to keep *SxlPe* off so that no *Sxl* protein is present when *SxlPm* is active. The system would seem to impose no requirement for sexually dimorphic expression from *SxlPm* or even for a temporal overlap in transcription from the two promoters (14), yet both features are conserved across the breadth of *Drosophila* species. We suggest that the resolution of this paradox lies in recognizing that the transition to stable autoregulatory *Sxl* splicing requires the presence of substantial amounts of *Sxl* protein (4, 14) rather than being driven by trace quantities of *Sxl* protein (30, 31). Given this, we propose that overlapping expression from the two promoters ensures that XX cells rapidly engage autoregulatory *Sxl* splicing, whereas the delayed activation of *SxlPm* in XY cells buffers against improper *Sxl* activation due to random variations in regulatory protein concentrations. In effect, we suggest that robustness is conferred on the system by rapid reinforcement of correct decisions. In XX embryos, strong induction of *SxlPe*, coupled with early activation of *SxlPm*, ensures that high levels of *Sxl* protein and its premRNA substrate are present during the transition to splicing control. In XY embryos, chance fluctuations in XSE or inhibitor concentrations that caused low-level activation of *SxlPe* would not persist to activate *SxlPm*, thus preventing amplification of rare mistakes into the fully on state. We note that a logically similar, two-target control process operates in the primary sex determination of *Caenorhabditis elegans*. There, four XSE proteins exert primary control of the master regulator *xol-1* at the level of transcription, and a fifth XSE acts posttranscriptionally to ensure the fidelity of X chromosome counting (32, 33). The inclusion of multiple regulatory steps may prove to be a general mechanism for conferring robustness on dose-sensitive regulatory switches.

How Is *SxlPm* Regulated? *SxlPm* appears to be equally active in both sexes after the onset of gastrulation. Before that time, *SxlPm* is expressed in a graded fashion, becoming active earlier and being expressed more strongly in XX embryos than in XY embryos. Sequences governing the early sexually dimorphic expression of *SxlPm* are included in the same 1.4-kb DNA segment that controls the on-or-off regulation of *SxlPe* (28). Importantly, the 1.4-kb region must work as an enhancer for *SxlPm* rather than exert an indirect effect in *cis* via activation of *SxlPe*, because deletion of the *SxlPe* core promoter has no effect on *SxlPm* activity. This, combined with the involvement of the XSEs *scute* and *runt* in *SxlPm* regulation, suggests that *SxlPm*, like *SxlPe*, responds directly to the number of X chromosomes present in the embryo. However, the fact that neither loss of the strong XSE *sisA* nor loss of the potent maternal co-repressor Groucho affects *SxlPm* suggests that the mechanism of X-counting at *SxlPm* differs from that at *SxlPe*, despite their shared common *cis*- and *trans*-acting components. We suspect that additional transcription factors contribute to both early *SxlPm* activation and the female/male differences in timing.

The existence of a shared regulatory region between *SxlPe* and *SxlPm* raises the question of how enhancer activity is directed to the correct promoter at the appropriate time. The 1.4-kb region regulates *SxlPe* from cycle 12 through early cycle 14, yet the enhancer does not lead to significant expression from *SxlPm* until cycle 14. Expression from the two promoters overlaps briefly before *SxlPe* is silenced and *SxlPm* fully controls *Sxl* transcription. We imagine two general mechanisms that might explain how the enhancer can chose between the two promoters (34, 35). First, an insulator situated between the enhancer and the upstream promoter might block the 1.4-kb region from interacting with *SxlPm* until the insulating protein is removed from the DNA or its activity is overcome by additional positive signals. Second, promoter choice could be dictated by differences in the transcription machinery at the two promoters (34) or by a temporally restricted transcription factor that recruits the enhancer to one of the two *Sxl* promoters. The developmentally regulated competition between the promoters of the chicken ε-globin and β-globin genes for their common enhancer provides a precedent for the latter mechanism (36). The rapid changeover from *SxlPe* to *SxlPm* coincides with the *Drosophila* maternal-to-zygotic transition, when expression of the zygotic genome begins in earnest and numerous early mRNAs and proteins are eliminated from the embryo (37). It would not be surprising if the rapid changes at *Sxl* were directly connected to more general regulatory events occurring during this dynamic period of development.

Methods

P-Element Vectors and Transformation. *Sxl* genomic fragments were made using the Expand Long-Template polymerase chain reaction system (Roche), cloned into pCRII-TOPO (Invitrogen), and ligated into P-element transformation vectors based on pCaSpeR-AUG-ßgal. Germline transformations were performed by Genetic Services, Inc, Cambridge, MA. Transgenes with internal deletions were cloned as upstream and downstream fragments and joined at primer-derived PacI sites. The -1.8-kb *SxlPm-lacZ* was made with primers 1.8Pm5′ and 1.8Pm3′; *SxlPm[+Pe]-lacZ*, with primers 1′ and 4′; *SxlPm[Δ-88Pe]-lacZ*, with primers 1′ and 2′/PacI and 3′PacI and 4′; and *SxlPm[Δ-1.4Pe]-lacZ*, with primers 1′ and 5′/PacI and 3′PacI and 4′. Control transgenes *SxlPe[L2]-lacZ* and *SxlPe[Δ-88,L2]-lacZ* were similar to *SxlPm[+Pe]-lacZ* and *SxlPm[Δ-88Pe]-lacZ*, except that sequences distal to 1.4 kb upstream of exon E1 were absent and the vector was pPelican. Two independent lines of *SxlP[L2]-lacZ* were expressed similarly to previous 1.4-kb *SxlPe-lacZ* lines (18, 28), but deletion of the core *SxlPe* promoter left both tested lines of *SxlPe[Δ-88,L2]-lacZ* inactive. Primer sequences were 1.8Pm5′-CTCACGCTAGAGAACACCGATCATTC; 1.8Pm3′-GACTTTCCTTCTTCGGCAAC; 1′-CCATCCGATCCGCGAGTCCA; 4′-GCACGCTCACTGTGCTTTCCTCTC; 2′/PacI-CC*attaattaa*GGAGGCAAGGTGCGCGT; 3′/PacI-CC*attaattaa*CGTAACTTTGTGATTATCCC; 5′/PacI-CC*ttaattaa*ATGCGAGCAGCGGAGAAGGG.

In Situ Hybridization. Nonfluorescent in situ hybridization used digoxygenin or fluorescein-labeled probes (38). *D. melanogaster* and *D. simulans SxlPm* intron probes (1.4 kb) were transcribed from templates made using the following primers: Pm5′-CCCTTCTCCGCTGCTCGCAT and T3Pm-aattaaccctcactaaagggCCAGGTAGAAGATCGAAGGA. Templates for corresponding *D. yakuba* and *D. virilis SxlPm* probes were made with yakPm5′-CACCACCCCATTCCACCCG and T3Pm, or virPm5′-CGAGCCTTTCCGTAACTGTTCG and virT3Pm-aattaaccctcac-

taaagggTGCGCTACCTGTTGACAGTG. Probes for *lacZ* and exon E1 (*SxlPe*) have been described previously (9, 18, 26). Fluorescent detection of *SxlPe* transcripts was as detailed previously (see http://superfly.ucsd.edu/~davek/). Nascent transcripts, visible as dots within stained nuclei, were seen with all probes but were more difficult to detect from *lacZ* transgenes. For X-linked genes, the number of nuclear dots indicates chromosomal sex. Times within cycle 14 were estimated by nuclear shape and length, as well as by the extent of membrane furrow invagination (20, 21). Specific developmental time estimates were based on the literature, but embryos grouped within specified time periods were staged as close as possible to one another.

Genetic Analysis. The alleles $sisA^1$, da^1, and sc^{sisB3} are near null for sex determination. gro^{E48} and run^3 are null alleles. Embryos homozygous or hemizygous for sc^{sisB3} and $sisA^1$ were generated using the constitutive Sxl^{M4} allele to bypass female-lethal effects (19). Null allele Sxl^{f1} suppressed the male lethality of the 2X *P(miniw*$^+$*, sisA*$^+$*)* and 2X *P(miniw*$^+$*,* sc^{sisB+}*)* chromosome (19). Nascent transcripts from Sxl^{f1} and Sxl^{M4} are not detectably different than those from wild type. Germline clones (39) were generated in larvae of genotype *P{hsFLP}1,* y^1 w^{1118}/w^{1118}*;* $P\{neoFRT\}82B$ ry^{506} $gro^{E48}/P\{neoFRT\}82B$ *P{ovoD1–18}3R*; and *P{hsFLP}1,* y^1. Females with recombinant germlines were crossed with w^{1118}/Y males. Embryos were collected at 25 °C. Other mutations and chromosomes are described at http://flybase.bio.indiana.edu. The sc^{sisB3} allele and transgenic XSE duplications were provided by T. Cline (University of California Berkeley). *FRT82B* gro^{E48} was provided by P. Simpson (University of Cambridge). *D. virilis* was provided by S. Johnson (Texas A&M University). *D. simulans*, *D. yakuba*, and *D. simulans* were provided by D. Barbash (Cornell University). Fly stocks for FLP/FRT recombination were obtained from the Bloomington *Drosophila* stock center.

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