The Drosophila Female-Specific Sex-Determination Gene, Sex-Lethal, Has Stage-, Tissue-, and Sex-Specific RNAs Suggesting Multiple Modes of Regulation

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The *Drosophila* female-specific sex-determination gene, *Sex-lethal*, has stage-, tissue-, and sex-specific RNAs suggesting multiple modes of regulation

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For proper sexual development of females, the *Sex-lethal* (*Sxl*) gene must be activated early in development and remain on during the rest of the life cycle. Conversely, in males, *Sxl* must remain functionally off through development. Here, we show that the *Sxl* transcription unit spans a DNA segment of >20 kb and encodes at least 10 distinct, but overlapping, RNA species. These RNAs range in size from 4.4 to 1.7 kb and exhibit sex, stage, and tissue specificity. Six RNAs, three female specific and three male specific, are first detected by mid-embryogenesis and persist through the adult stage: Their expression reflects the on/off regulation of *Sxl*’s activity at the level of sex-specific alternate splicing. Four *Sxl* RNAs are found in adult females. Two of these RNAs are dependent on the presence of a functional germ line and may be relevant to *Sxl*’s role in adult germ-line development. All four are present in unfertilized eggs. Finally, three *Sxl* RNAs are found only transiently during very early embryogenesis; we suggest that the expression of these RNAs may reflect an early regulation of *Sxl* at the level of transcription and that these transcripts are involved in the initial selection of the *Sxl* activity state in response to the primary sex-determination signal, the X/A ratio.

[Key Words: *Drosophila melanogaster*, *Sex-lethal*, sex determination; dosage compensation; sex-specific transcripts]

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later in development. One of these appears to be required for the stable activation of the Sxl gene [Cline 1984, 1988a,b, Maine et al. 1985b, Salz et al. 1987]. Another very early function appears to be involved in the down-regulation of the X chromosome dosage-compensation system [see Gergen 1987].

Once the initiation process is complete and Sxl is on in the female embryo, the maintenance of a commitment to the female pathway ('determination' in the strict developmental sense) requires that Sxl remain in an active state from embryogenesis through the adult stages. The stable commitment to the female pathway is thought to be achieved via a positive autoregulatory function of Sxl, which keeps it in an active mode [Cline 1984, 1988a,b, Maine et al. 1985b, Bell et al. 1988]. The other level is 'differentiation.' Sxl is required at later stages in development for the proper expression of the female developmental pathway [Cline 1979a, 1984, Sanchez and Nothiger 1982]. Genetic and molecular studies have shown that Sxl controls the activity state of genes downstream in the sex-determination pathway and dosage compensation system. The most immediate known target of Sxl in the sex-determination pathway is transformer (tra) [Cline 1979b, Baker and Ridge 1980, Nagoshi et al. 1988]. Like Sxl, tra must be on in females but off in males. The on/off regulation of tra is post-transcriptional and involves the sex-specific splicing of the tra RNA [Boggs et al. 1987]. Moreover, it has been suggested that Sxl may turn on tra by directing the female-specific splicing of the tra transcript [Boggs et al. 1987, Bell et al. 1988; Nagoshi et al. 1988]. Sxl is also required to keep the dosage-compensation system off in females [Lucchesi and Skripsiy 1981, Cline 1983, 1984, Gergen 1987]. Although a number of genes involved in regulating some aspects of dosage compensation have been characterized [for review, see Lucchesi and Manning 1987], the most immediate target(s) for Sxl's action is not known [Cline 1984, Gergen 1987]. In addition to these somatic functions, Sxl is also required for the normal development of the female germ line [Schüpbach 1985; Salz et al. 1987].

To understand more fully the role of Sxl in the development of sexual dimorphism, we have undertaken a molecular analysis of the gene. The Sxl gene was isolated initially using P-element tagging and chromosome walking [Maine et al. 1985a]. In the work reported here, we have extended the initial chromosomal walk and have examined the transcripts encoded by a ~44-kb DNA segment around the Sxl locus in both wild-type and mutant Sxl alleles. These studies have permitted the identification of the Sxl transcription unit and indicate that it covers a DNA segment of ~20 kb. [This analysis substantially revises the preliminary map of the transcription unit reported in Maine et al. 1985b]. We find that the functional complexity of Sxl evident in genetic studies is paralleled at the molecular level: Sxl encodes a complex set of transcripts that show sex, stage, and tissue specificity. The analysis of RNAs in female and male animals reported here provided the first evidence for the sex-specific processing of Sxl transcripts and suggests that it occurs throughout most of the life cycle. Studies on Sxl cDNAs from adult animals reported elsewhere [Bell et al. 1988] have confirmed the sex-specific processing of the Sxl transcripts and indicate that alternate splicing is a mechanism for the on/off regulation of the gene. The complex transcription pattern also affords the first hints as to the molecular basis for the sex-specific activation of Sxl very early in development and its possible relationship to the regulation and function of the gene later in development.

Results

Localization of the Sxl transcription unit

A physical map of the ~44-kb DNA segment from the cytogenetic region 6F, which contains the Sxl gene, is shown in Figure 1. In our initial characterization of this DNA segment [Maine et al. 1985a], we found that DNA rearrangements associated with Sxl mutations were clustered in an 11-kb DNA segment extending from coordinate 0 to -11. We further defined sequences required for the various Sxl functions by analyzing a series of deficiencies in the 6F region generated by the mobilization of a P element (SxlP) inserted at coordinate 0 [Salz et al. 1987]. The limits of some of these deficiencies within the chromosomal walk are indicated at the top of Figure 1.

To identify transcribed sequences in the 6F DNA segment, we used nick-translated restriction fragments to probe Northern blots of poly[A]+ RNA prepared from 0- to 5-hr embryos and adult females and males. The results of this analysis are summarized at the bottom of Figure 1. At least 16 different RNA species are detected with probes from the 44-kb region. Because Sxl is required in females but not in males and also must be active in very young embryos, a comparison of the transcripts hybridizing to the various probes in the two sexes and at the different stages should suggest which of these transcripts might be products of the Sxl gene. On the basis of these criteria, the transcripts encoded by sequences on the proximal and distal sides of our walk would not appear to be good candidates for Sxl RNAs. On the centromere proximal side [left], e.g., probes a–d extending from coordinates +15 to +0.5 [extend to +0.5] hybridize to four different RNA species that are present in adults of both sexes and are also found in early embryos. On the distal side [right] probe 1 labels two poly[A]+ RNAs. These are also found in adult males and females but are absent in early embryos.

A different result is obtained with probes derived from near the middle of the walk. Fragments from a region extending from coordinates 0 to -22 hybridize to a complex array of overlapping transcripts that show both sex and stage specificity. A blot probed with fragment h (see Fig. 1), which hybridizes to all of the major transcripts from this region, is shown in Figure 2. In adult females, three major size classes of RNA are observed: 4.2, 3.3, and 1.9 kb. A similar set of three RNA size classes is found in adult males; however, as can be seen
in Figure 2, the male-specific RNA species are ~100–300 bp larger than the corresponding female RNAs (4.4, 3.6, and 2.0 kb) in each case. The transcript pattern in 0- to 5-hr embryos is more complex. We observe three RNAs which, by mobility, appear to correspond to the three major size classes of RNA found in adult females. In addition, there are two bands of 3.1 and 1.7 kb, respectively, which are only observed in embryos. On higher percentage gels, a third embryo-specific transcript of ~4.0 kb can be resolved from the large 4.2-kb female-specific transcript [data not shown].

Several lines of evidence indicate that this complex group of overlapping RNAs corresponds to the SxI transcripts. First, the transcription unit that would encode these RNAs includes the 11-kb region [see Fig. 1] that contains the SxI DNA rearrangements used in the initial identification of the locus [Maine et al. 1985a]. Second, we have found that a number of these loss-of-function mutations alter the SxI transcript pattern. [Because these mutations are female lethal, the SxI transcripts were examined in adult males.] For example, all three male-specific transcripts are absent in adult males carrying the
loss-of-function mutation SxPb [which has a P-element transposon inserted at coordinate 0]. In contrast, the sex nonspecific RNA species encoded by sequences just proximal to coordinate 0 [e.g., the 2-kb RNA of fragment d or the 4-kb RNA of fragment b; see Fig. 1] are unaffected by the P-element insertion [data not shown].

This conclusion is supported by analysis of the transcripts produced by males carrying small deletions that affect SxI function. In the experiment presented in Figure 3, RNA was prepared from four SxI deficiency mutations and probed with either fragment h2 [which hybridizes strongly to the small and middle RNA species (2- and 3-kb transcripts) but only weakly to the large (4-kb) RNAs [see Fig. 4]] or with fragment d [which labels the proximal 2.0-kb sex-nonspecific transcript]. As indicated in Figure 1, two of these deficiencies, 7BO and 3G2, completely lack both fragment h and d DNA. As would be expected, the three male-specific SxI transcripts and the 2.0-kb RNA are missing in these two deletion mutants [see Fig. 3]. In other experiments [data not shown] we have found that the more proximal 4.2-, 1.9-, and 1.5-kb RNAs [see Fig. 1] are still present in males carrying the smaller deletion 3G2.

Deficiency ED1 has fragment d sequences but is deleted for fragment h2, as well as more distal sequences beyond the endpoint of our chromosomal walk [Fig. 1]. As can be seen in Figure 3, the 2.0-kb proximal RNA is expressed in males carrying this deficiency, whereas none of the male SxI RNAs is observed. The smallest deletion, SxTbA1, lacks sequences extending from coordinate 0 to -6.5 but retains fragment h DNA. Adult males carrying this small deficiency express the proximal sex nonspecific 2-kb RNA [data not shown]. In contrast, the male-specific transcripts are absent in RNA from this mutant [Fig. 3b], and instead we observe a weakly labeled smear of hybridization [which is more evident in longer exposures]. Because the proximal breakpoint of 7A1 is located downstream of the 5' end of the adult SxI transcription unit [see below], we presume that the SxI gene may still be transcribed at some level in the 7A1 mutant males and that some processing defect results in
Figure 4. Organization of the Sxl transcription unit. Probes used in this analysis were more sensitive and smaller than those used in Fig. 1 to localize the transcribed regions more precisely. Single-stranded probes were made from fragments d2–h2, as well as fragment j. These probes were used to determine the direction of transcription. Fragments i and k were labeled by nick-translation. As in Fig. 1, the transcripts detected by each fragment are indicated by the solid bars directly below the fragment in decreasing order of size. Those transcripts of the same size and temporal, tissue (see Fig. 7), and sex specificity are presumed to arise from a single RNA species and are joined by a dashed line to indicate the putative introns. Note that there are two different 3.3-kb female-specific RNAs, one of which is dependent on a functional germ line (see Fig. 7). Although not indicated here, other studies (Bell et al. 1988) show that the 2-kb male-specific transcript also hybridizes to fragment g2. Poly(A)* adult RNA (5 μg) or poly(A)* 0- to 5-hr embryo RNA (2.5 μg) was loaded per lane for this analysis.

the production of abnormal RNAs.

Finally, in other experiments [data not shown], we have examined the expression of the distal 2.0- and 1.9-kb RNAs labeled by fragment l. We found that both of these RNAs are expressed in males carrying deficiencies that delete large portions of the Sxl transcription unit [e.g., 7BO and 3G2] but retain fragment l DNA. Taken together, these findings indicate that the large transcription unit near the center of our chromosomal walk corresponds to the Sxl gene and that the RNAs encoded by the flanking proximal and distal regions must be encoded by transcription units distinct from Sxl.

Organization of the Sxl transcription unit

To define the Sxl transcription unit more precisely, smaller and more sensitive single-stranded probes were used to examine Sxl RNAs in embryos and adults. The results of this analysis are summarized in Figure 4. The salient findings are as follows: First, all of the Sxl RNAs are transcribed in the same direction, proximal to distal. Second, the Sxl transcription unit appears to extend over a DNA segment of at least 23 kb. Third, the three size classes of RNA observed in adults clearly differ in structure. Most of the differences between the size classes appear to arise toward the 3' end of the transcription unit in the region distal to fragment h1 (see Fig. 4). For instance, fragment i hybridizes to the RNAs in the 3-kb size class but does not hybridize to either the ~2- or ~4-kb size classes. Similarly, only the largest size class (~4 kb) hybridizes to fragment k. Fourth, the male and female RNAs in each size class hybridize [with one important exception, see below] to the same set of restriction fragment probes. Hence, at least at the level of resolution provided by Northern analysis, it appears that there is a close correspondence in structure between the male- and female-specific RNAs in a given size class. As indicated in Figure 4, the structure of the embryonic RNAs also appears to be closely related to that of the adult transcripts.
Two other results of the Northern analysis are of interest. The first is the probable location of the 5' end of the Sxl transcription unit. As indicated in Figure 4 and illustrated in the Northern blot shown in Figure 5A, the 5'-most probe that hybridizes to the adult Sxl RNAs is fragment d2. d2 is located proximal to coordinate 0 (see Fig. 4), and this would place the start site for the Sxl transcription unit upstream of both the P-element insertion of SxFvb and the proximal breakpoint of Df(1)SxlTM (see Figs. 1 and 4). Although d2 labels all three size classes of Sxl RNA in both adult males and females, it does not hybridize to any of the embryonic-specific RNAs (see Fig. 5A). This raises the possibility that the embryonic transcripts may have a different 5' start site from that of the adult RNAs. The existence of a distinct embryonic promoter was also suggested by genetic studies on Sxl deletion mutants [Salz et al. 1987].

The second result of interest is the difference between the adult male and female Sxl transcripts. Although the Northern experiments suggest that the male and female RNAs within a size class have similar structures, the male RNAs are ~200 bp larger than the corresponding female RNAs in each case (cf. Fig. 2). The difference between male and female RNAs appears to be due, at least in part, to the presence of an additional exon in the male RNAs. In the experiment shown in Figure 5B, RNA from adult males and females was probed with fragment g2, which is located near the middle of the Sxl transcription unit (see Fig. 4). This fragment does not hybridize to any of the female Sxl transcripts, but it does label the 4.4- and 3.6-kb Sxl male RNAs (see Fig. 5B). Though the 2.0-kb Sxl RNA is not observed in this Northern blot, it is much less abundant than the two larger male species (see Fig. 2). We suspect that the low level of the small transcript probably accounts for our inability to detect it with the genomic g2 probe. [In other experiments we have found that the Sxl exon in g2 labels all three male RNAs [Bell et al. 1988].]

**Developmental profile of the Sxl transcripts**

The Northern experiments described above indicate that the Sxl transcript pattern in 0- to 5-hr embryos differs from that in adults. It consists of two types of RNA: (1) a set of embryo-specific RNAs and (2) a set of RNAs which, by size and pattern of hybridization with different probes, appears to correspond to the Sxl transcripts found in adult females. We do not, however, detect transcripts corresponding to RNAs observed in adult males. These differences suggested that it would be of interest to learn more about the expression of the embryo RNAs and the transition to the transcript pattern found in adult females and males.

In the Northern blot shown in Figure 6, we have examined Sxl transcripts during the course of embryonic development. The transcript pattern in 0- to 2.5-hr and 2.5- to 5-hr embryos is roughly similar to that found in the longer 0- to 5-hr collections (cf. Fig. 6A with Fig. 2): It consists of a mixture of embryo-specific and adult female-specific transcripts. The 0- to 2.5-hr and 2.5- to 5-hr embryos do, however, show differences in the relative level of embryo-specific and female RNAs. The embryo-specific RNAs are more abundant in the 2.5- to 5-hr collection, whereas the female RNAs are more abundant in the earlier collection.

This observation suggested that it would be useful to determine the relative contributions of maternal and zygotic RNAs to the Sxl transcript pattern during the first
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observe RNAs corresponding to the three size classes found in adult females. In the 5- to 8-hr collection, the most abundant Sxl transcript is the 1.9-kb female-specific RNA, whereas the two larger species are only weakly labeled (cf. Fig. 6A and Fig. 2). In the 8- to 11-hr period, this pattern begins to change: The relative level of the two large female RNAs increases, and the level of the small RNA drops. In addition, transcripts corresponding in size to the adult male RNAs can first be detected during this time (e.g., the faint band just above the 3.3-kb female RNA in the 8- to 11-hr lane). In the later stages of embryogenesis, the two larger female

several hours of embryogenesis. As shown in Figure 7A, the female-specific RNAs in early embryos are found in unfertilized eggs and are likely to be of maternal origin. As can be seen in the detailed developmental profile shown in Figure 6B, these maternally derived female RNAs persist through the first 2 hr of embryogenesis. During the next hour (2-3 hr) the maternal female RNAs disappear and are replaced by the embryonic species. We presume that these embryonic transcripts are the first products of zygotic transcription of the Sxl gene. The level of the embryonic RNAs appears to peak in the 3- to 4-hr interval.

After this point, the early embryonic RNAs must turn over rather rapidly as these transcripts cannot be detected in 5- to 8-hr embryos (see Fig. 6A). Instead, we

Figure 6. Developmental profile of Sxl transcripts during the embryonic period. Fragment h was used as a probe because it detects the majority of the Sxl transcripts [Fig. 1]. All lanes contain 5 μg poly(A)+ RNA made from staged embryos at 25°C.

Figure 7. Sxl transcripts in unfertilized eggs and females without a functional germ line. Poly(A)+ unfertilized egg RNA (2.5 μg) or poly(A)+ female RNA (5 μg) were loaded per lane. The genotypes of the females without a functional germ line are as follows: (Lane 1) SxltsUSxl#1; (lane 2) ovoP1/+; (lane 3) females (tudor1/+) from females homozygous for the maternal effect mutation tudor1. (A) The blot was hybridized with fragment h; (B) the blot was hybridized with fragment j [Fig. 4].
transcripts become the predominant Sxl RNAs, whereas the small female RNA becomes a minor species. The relative abundance of the two larger male Sxl RNAs also increases during this period.

We have also examined Sxl RNAs in first instar larvae, in sexed second and third instar larvae, and in pupae (Maine 1984). The transcript pattern in the larval and pupal stages is quite similar to that found in 17- to 21-hr embryos: The major transcripts correspond to the larger (3 and 4 kb) female and male size classes, whereas the small (2 kb) size class is barely detectable. Moreover, in the sexed second and third instar animals, we find that these transcripts are sex specific. Male larvae have transcripts that appear to be identical to those observed in adult males, whereas female larvae have transcripts that correspond to RNAs found in adult females.

Germ-line dependence of Sxl transcripts

Sxl is known to be required in the female germ line (Schüpbach 1985), and mutations that differentially affect this aspect of its function have been identified (Salz et al. 1987). Hence, it was of interest to compare the Sxl transcript pattern in wild-type females with that of females lacking a functional germ line. Sterile females that differ in the severity of their germ-line defect were generated in three different ways: (1) Females lacking germ cells altogether were collected as progeny of mothers homozygous for tudor+ (Boswell and Mahowald 1985); (2) females with abnormal germ cells were collected as ovo'd1 heterozygotes (Busson et al. 1983; Oliver et al. 1987); (3) females with germ cell abnormalities because of defects in Sxl function were obtained as homozygotes for the viable but sterile partial loss-of-function Sxl allele, Sxl[61]. [The abnormal egg chambers in the ovaries of Sxl[61] females closely resemble those observed in chimeric females whose germ line is homozygous for a Sxl null mutation but whose soma is wild type for Sxl (Salz et al. 1987).]

Figure 7A shows the Sxl transcript pattern in wild-type and germ-line-deficient females detected with probe h. All of these sterile females exhibit abnormalities in their Sxl RNAs. First, the most abundant transcript in adult females, the 1.9-kb RNA, is barely detectable. In this respect, the transcript pattern in these mutant females closely resembles that found in the larval (and also pupal) stages when the female germ line is much less developed. Further analysis has revealed that the reduced level of labeling of the 3.3-kb band is due to the fact that this size class is composed of two different RNA species in wild-type females, one of which is absent in the sterile females. This is shown in the Northern blot probed with fragment j (see Fig. 7B). In wild-type females and also in unfertilized eggs, probe j hybridizes to a 4.2- and a 3.3-kb band; in contrast, the 3.3-kb band is missing in RNA from sterile females, whereas the 4.2-kb band remains. We therefore conclude that there are four rather than three transcripts in adult females. The level of two of these is dependent on the presence of a functional germ line; however, all four transcripts appear to be present in unfertilized eggs.

The sex-specific adult transcription pattern is not affected by tra or tra-2

Sxl is upstream of both tra and tra-2 in the regulatory hierarchy. Thus, we would predict that mutations in either tra or tra-2 would not affect the Sxl transcription pattern. To test this hypothesis, we examined the pattern of transcription in XX animals that were sexually transformed by mutations in either tra or tra-2. We found that the Sxl transcript pattern was essentially female, in agreement with the genetic model (Fig. 8). The relative level of the 3.3- and the 1.9-kb RNAs compared to the 4.2-kb RNAs is similar to that seen in females without a functional germ line. This is not unexpected because these sexually transformed animals do not have a functional germ line.

Discussion

The studies reported here provide some of the first insights into the underlying molecular basis for the genetic complexity of Sxl. We have identified the Sxl transcription unit and defined its probable limits. The transcription unit spans a DNA segment of at least 23 kb located in the 6F cytogenetic interval of the X chromosome. Many of the DNA lesions associated with altered Sxl function map entirely within this 23-kb DNA segment, and we have shown that such lesions alter the Sxl transcript pattern in a number of cases. The Sxl transcription unit is flanked on both the proximal and distal

Figure 8. Sxl transcripts in females sexually transformed by the 'downstream genes' tra and tra-2. All lanes contain 5 μg of poly(A)+ RNA. The blot was hybridized with a Sxl cDNA described in Bell et al. (1988). tra/tra sexually transformed females were isolated from the following crosses: y w f/y w f; mwh tra/TM5 × +/Y; Df(3L)stSlklZ(tra-)/TM6b. tra-2/tra-2 sexually transformed females were isolated from the following cross at 29°C: y/w; tra-2/SM1 × +/B'Y, tra-2/tra-2/CyO.
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sides by RNA-coding regions whose transcripts show no apparent sex and/or stage specificity. The RNAs produced by these flanking regions do not appear to be affected by mutations in Sxl that either eliminate or alter Sxl transcripts, and we conclude that they are encoded by genes that are distinct from Sxl. The functions of the genes in the flanking regions are unknown. Although extensive efforts have been made to identify vital genes in the vicinity of Sxl [Nicklas and Cline 1983], none of the genes discovered in mutant screens would be sufficiently close to Sxl to correspond to any of the flanking transcription units. In addition, some of the deletions extending from Sxl into the flanking regions have no apparent effect on male viability or fertility under laboratory conditions.

We have found that the Sxl transcription unit encodes a complex set of overlapping RNA species that differ in their stage and/or sex specificity. Using Northern blots to analyze the transcript pattern during the Drosophila life cycle, we have been able to detect 10 different Sxl RNA species. This is probably an underestimate of the number of distinct Sxl transcripts, as there may be RNAs of similar size with subtle structural differences that were not detected by our Northern analysis.

Given the complexity of Sxl function that has been revealed by genetic analysis, it is intriguing that the gene encodes multiple transcripts showing sex, stage, and tissue specificity. Although it unclear at this point how the specific transcripts relate to the different Sxl functions, the transcriptional complexity provides some valuable clues about the regulation and functioning of Sxl during development.

Male/female differences

Even though Sxl must be on in females and off in males, the gene is transcribed in both sexes. In adult females there are four major RNA species: a large 4.2-kb transcript, two different medium-sized transcripts of 3.3 kb, and a small 1.9-kb transcript. In adult males there are three slightly larger RNA species of 4.4, 3.6, and 2.0 kb transcribed off of the same strand as the female RNAs. At the level of resolution provided by Northern analysis, the large, medium, and small adult male transcripts appear very similar [but not identical] in structure to the large, to one of medium-sized, and to the small female transcript, respectively (a male transcript corresponding to the small female RNA is main-

fragment probe derived from near the middle of the Sxl transcription unit, whereas female RNAs do not. This would suggest that the male transcripts differ from the female RNAs by the presence of an additional internal exon. This suggestion has been confirmed in studies reported elsewhere [Bell et al. 1988]. All three of the male RNAs contain a short (~180 bp) exon that is located immediately downstream from the exon containing the presumptive translation initiation signal for the Sxl proteins. Within the male exon are in-frame translation stop signals that interrupt the large open reading frame. Thus, the male RNAs would encode a truncated, and presumably nonfunctional, protein product. In contrast, this exon is spliced out of the female transcripts. This would conserve the large open reading frame and would, in principle, permit the synthesis of fully functional protein products. Because adult forms of the Sxl transcripts appear to be present from mid-embryogenesis to the adult stage, the on/off regulation of Sxl during most of the Drosophila life cycle is apparently achieved by the alternate splicing of the male exon. Moreover, Sxl itself most probably participates in the splicing process. A feature common to the proteins encoded by the various female Sxl transcripts is two domains showing extensive sequence similarities to a family of RNA-binding proteins [Bell et al. 1988]. This observation would suggest that the positive autoregulatory function of Sxl may involve some type of interaction of the Sxl protein products with the splicing machinery to confer the female-specific splicing pattern on the late Sxl transcripts. In contrast, when the Sxl products are absent, the Sxl transcripts would be spliced in the male pattern [Bell et al. 1988].

Germ-line/soma differences in adult females

Two of the four Sxl RNAs found in adult females show a striking dependence on the presence of a functional germ line. In the absence of a germ line, the small 1.9-kb RNA is present at much lower levels and one of the medium sized 3.3-kb RNA species cannot be detected at all. The simplest interpretation of these observations is that these two RNAs are expressed primarily in the germ line or ovary of adult females, whereas they are expressed either at only very low levels or not at all in the rest of the animal. Consistent with this notion, we find that both of these RNAs are present in unfertilized eggs. The other two adult female transcripts [the 4.2-kb and other medium sized 3.3-kb RNA] are also present in unfertilized eggs. Hence, it seems likely that the latter two transcripts are expressed in both germ line and soma.

The germ-line dependence of two of the female transcripts is intriguing in light of the fact that Sxl is required for germ-line development and function. Genetic studies suggest that the role of Sxl in the germ line may be complex [Salz et al. 1987]. Several alleles of Sxl have been identified that appear to be specifically defective in germ-line function, but wild type with respect to somatic function. One of these Sxl alleles, Sxl\textsuperscript{Pat}, has been characterized in detail [Salz et al. 1987 and H. Salz, un-
Females homozygous for this viable but sterile allele produce abnormal egg chambers that are similar in phenotype to the cystic egg chambers observed in germ cells homozygous for a Sxl null allele in a wild-type somatic environment [Schüpbach 1985]. Surprisingly, the female sterility of Sxl Null is complemented by Sxl deletions that eliminate portions of the adult transcription unit. Three deletions that remove sequences proximal to coordinate 0 [see Fig. 1], and thus lack the probable 5’ end of the Sxl transcription unit [in fragment d2 of Fig. 4], complement the female sterility of this allele [Salz et al. 1987]. Because the female sterility of Sxl Null is also rescued by small internal deletions extending from coordinate 0 up to −6.5, it would appear that sequences sufficient for complementation are located 3’ to coordinate −6.5 [Salz et al. 1987]. One hypothesis suggested by these findings is that Sxl has an internal promoter and/or transcription start site that can be used in the germ line. Clearly, further studies are required to determine whether this is the molecular basis for the observed complementation.

Although the deletion mutants complement the female sterile Sxl Null allele, they lack some essential aspect of Sxl activity in the germ line, as they are unable to support normal germ-line development in homozygous clones [Salz et al. 1987]. We have found that these same deletions eliminate the three Sxl transcripts in adult males. Presumably, the corresponding female transcripts would be absent in homozygous mutant female germ-line clones. Hence, at least one of the adult female RNAs is likely to be involved in this aspect of Sxl’s germ-line function.

Maternal/zygotic transition

Although Sxl functions in both the maternal germ line and the early embryo, genetic experiments have not revealed any clear-cut Sxl maternal effect that would suggest a role for maternal Sxl products in zygotic sex determination [see Cline 1980, Oliver et al. 1988]. Thus, we had not anticipated finding the maternally derived adult female Sxl transcripts in unfertilized eggs and very early embryos. Given the potentially deleterious effects that functional Sxl products would have on chromosomally male embryos as a consequence of Sxl autoregulation [Cline 1984], we presume that these maternally derived Sxl transcripts [and/or their protein products] are either nonfunctional in the embryo or are inactivated prior to the time when the adult or late transcripts are first expressed in the embryo and the commitment to the male or female developmental pathway is firmly established. It is, however, conceivable that the maternally derived Sxl products might be required at a point earlier in embryogenesis for the initial response of the zygotic Sxl gene to the X/A signal [see below]. In this respect, it is of interest that the maternally derived Sxl RNAs persist only during the first 2 hr of development, disappearing at or just prior to the time when the X/A signal is first assessed and the activity state of Sxl is selected initially. It will be important to determine whether the translation products of these maternal Sxl RNAs are also found for a brief period during very early embryogenesis.

Early/late transcription differences

The functioning of Sxl very early in development when its activity state is first selected in response to the X/A signal is genetically separable from its functioning during the remainder of development. During the first few hours after fertilization in chromosomal females, Sxl interacts with da, sis-a, and sis-b, as well as with itself, and there are mutations in Sxl that differentially affect these early interactions [Cline 1976, 1984, 1986, 1988a, Maine et al. 1985b, Salz et al. 1987]. Moreover, there is an early phase of X chromosome dosage compensation controlled by Sxl that is distinct from later phases [Gergen 1987].

In view of these special requirements for Sxl activity during the early phase of embryogenesis, it is particularly intriguing that there is a distinct set of embryonic or early Sxl transcripts. Because these early RNAs appear to be the first products of zygotic Sxl transcription, we suppose that they provide the early Sxl functions that have been identified in genetic experiments. Our experiments show that the early RNAs can first be detected after ~2 hr postfertilization. They are present for approximately a 2-hr period corresponding to the interval in which the early Sxl functions are likely to be required. At ~4 hr after fertilization, there is a transition to the late phase of Sxl expression: The embryonic RNAs disappear, and we begin to detect the late, or adult, female-specific RNAs.

Although our results suggest that the early RNAs share the same core region as the late transcripts [see Fig. 4], we note two potentially important differences. First, the 5’-most exon of the late RNAs appears to be located in fragment d2 [+1 to +0.5]. This exon does not appear to be present in the early transcripts, suggesting that they initiate at a site different from that used by the late transcripts. This possibility is supported by the analysis of Sxl deletion mutants generated in a P-element mobilization scheme [Salz et al. 1987]. Deficiencies that remove sequences proximal to the P-element insertion site at coordinate 0, including the probable 5’ adult exon, are defective in late somatic Sxl functions but fully complement Sxl P, an allele specifically defective in early Sxl functions [Maine et al. 1985b; Cline 1986]. These results would argue that the transcription start site for the early RNAs is internal, somewhere downstream of the probable adult start site in d2. Consistent with this notion, when we selected for Sxl mutants that failed to complement Sxl P, we recovered several internal deficiencies that remove sequences extending from coordinate 0 to a DNA segment around coordinate −6. One plausible interpretation of this observation is that there is an essential promoter/enhancer element or the transcription start site for the early RNAs located in the DNA segment from coordinate 0 to −6.

A second difference is that none of the early RNAs appears to contain the male-specific exon [which is located in fragment g2 between coordinates −8 and −10; see Fig. 4]. A possible explanation for this finding is that the early transcripts might initiate at a site downstream of the male-specific exon. However, preliminary analysis of partial embryonic cDNAs [L. Keys, unpubl.] sug-
gests that is not the case and that the early transcripts start at a site located upstream of the male-specific exon, somewhere in the region between coordinates 0 and –6. If this is correct, the male exon must be spliced out of the early transcripts. This would raise important questions about the possible mechanism for such a splicing process: Is it a structural feature of the early transcripts that permits the splicing machinery to skip over the male exon in the absence of Sxl products, or are some residual maternally derived Sxl proteins involved in the splicing of the early transcripts? It may be possible to answer these questions once we have a better understanding of the structure of the early RNAs.

The findings reported here raise the possibility that Sxl regulation early in development may be quite different from the alternate splicing mechanism used during most of the life cycle. Thus, the initial response of Sxl to the X/A signal may be at the level of transcription, resulting in the expression of the early Sxl RNAs in female but not in male embryos. Although conclusive evidence in support of sex-specific early transcriptional regulation will require probes specific for the embryonic RNAs, it should be noted that in situ hybridization experiments with a probe that labels all Sxl RNAs indicate that there are two classes of blastoderm-stage embryos: those that show strong hybridization and those that show weak hybridization (D. Coulter, unpubl.). What would the function of the early Sxl products be, and what mechanisms would account for the establishment of a stable commitment to either the female or male developmental pathway? Presumably, the proteins produced from the early transcripts would be responsible for the early dosage-compensation functions of Sxl and would facilitate the stable activation of Sxl in females. For example, when Sxl transcripton switches from the early to the late phase, ~4 hr after fertilization, the early Sxl proteins might be involved in promoting the female-specific splicing pattern of the newly synthesized late transcripts. Once the late Sxl proteins are processed, the autoregulatory functions of Sxl would then maintain a stable commitment to the female developmental pathway. In contrast, in male embryos, no functional early Sxl proteins would be present during the transition to the late phase, and the late transcripts would be spliced in the male mode with the male-specific exon included. It remains to be determined whether the somewhat delayed appearance of the late male transcripts in embryos reflects some type of transient sex-specific transcriptional regulation of the late promoter or arises from a decreased stability of RNAs containing a truncated open reading frame. Further studies will be required to test our model for the regulation of Sxl and also to determine the functional role of the different Sxl transcripts.

Materials and methods

Fly stocks

All crosses were carried out at 25°C on a standard cornmeal, dextrose, yeast medium (Cline 1978). Genes not listed in Lindsley and Grell (1968) are referenced in the text.

Recombinant DNA library construction, screening, and subcloning

The screening of recombinant phage libraries (to extend the walk described in Maine et al. (1985a)) and restriction digests were done as described in Maine et al. (1985a). Subcloning of genomic fragments was done essentially as described in Maniatis et al. (1982).

Genomic DNA isolation and Southern blotting

DNA was isolated as described in Ish-Horowicz et al. (1979). Genomic blots were done as described by Maine et al. (1985a).

RNA isolation and Northern blotting

Poly(A)+ RNA was prepared by the method of O’Hare et al. (1983). In general, we used 5 μg of poly(A)+ RNA per lane. RNA was fractionated on denaturing 0.7% agarose/6.7% formaldehyde gels made with a MOPS buffer, as described by Maniatis et al. (1982). RNA was transferred to nitrocellulose using 10× SSPE and hybridized using the same conditions as genomic Southern blots. Probes labeled with 32P were made either by nick translation, random primer extensions of isolated fragments, or single-stranded DNA probes from M13 subclones. Single-stranded probes of 1 kb or less were made by extending from the M13 sequencing primer with Klenow. Single-stranded probes longer than 1 kb were extended using the modified T7 polymerase sequencing kit from USB (Cleveland, Ohio). The probe was cut with the appropriate restriction enzyme, denatured, isolated on a 1.0–1.2% low-melt agarose gel, melted, and added directly to the hybridization mix. The Sxl RNAs were sized [1] by reprobing with probes specific for the Sc actin gene (1.95 kb, Fryberg et al. 1983), dorsal gene (2.8 kb, Steward et al. 1984), and rudimentary gene (7.3 kb; Segraves et al. 1983), and [2] by running the RNA alongside denatured DNA size standards in the range of 1–4 kb.

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