

The Control of Alternative Splicing at Genes Regulating Sexual Differentiation in *D. melanogaster*

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Summary

The transformer (*tra*) and doublesex (*dsx*) genes produce sex-specific transcripts that are generated by differential RNA processing. We have examined the effects of mutants in other regulatory genes controlling sexual differentiation on the patterns of processing of the *tra* and *dsx* RNA transcripts. Our results demonstrate that the genes suggested by genetic studies to act upstream of *tra* or *dsx* in the sex determination hierarchy regulate these two loci at the level of RNA processing. Our data suggest that the order of interaction of the factors controlling sex is X:A>*Sxl*>*tra*>*tra-2*>*dsx*>*ix*>terminal differentiation. While these results cannot preclude regulatory interactions at other levels, the regulation of RNA splicing revealed by these experiments is sufficient to account for all of the known functional interactions between the regulatory genes in this hierarchy.

Introduction

The control of sexual differentiation in *Drosophila melanogaster* is brought about by one of the best understood regulatory hierarchies in higher eukaryotes. The primary determinant of sex is the X:A ratio, the number of X chromosomes relative to the number of sets of autosomes. The evaluation of the X:A ratio initiates a regulatory cascade that controls both somatic sexual differentiation and dosage compensation. This binary decision between male and female development is mediated by at least 12 regulatory genes whose positions in this regulatory cascade have been deduced from genetic studies (for reviews see Baker and Belote, 1983; Cline, 1985; Baker et al., 1987). Included among these loci are genes that regulate both somatic sexual development and dosage compensation, as well as genes that regulate either just sexual development or dosage compensation. Here we are concerned with just the part of the regulatory cascade that controls somatic sexual differentiation; this includes the *Sex lethal* (*Sxl*), *transformer* (*tra*), *transformer-2* (*tra-2*), *doublesex*

(*dsx*), and *intersex* (*ix*) loci. Genetic experiments suggest a model for the order in which these genes interact (Figure 1; for reviews see Baker and Belote, 1983; Cline, 1985).

Female development is elicited by an X:A ratio of 2:2 that results in the active expression of the *Sxl* gene (Cline, 1985, 1986). The *tra* and *tra-2* genes are believed to act after (downstream of) the *Sxl* locus, although the genetic studies that suggest this (Cline, 1979) do not preclude the possibility that *tra* and *Sxl* act in parallel. Both the *tra* and *tra-2* loci are necessary in females for all aspects of somatic sexual differentiation; apparent null mutations in either gene transform chromosomally female individuals into phenotypic males. Both the *tra* and *tra-2* genes function to control the activity of the *dsx* locus (Baker and Ridge, 1980). The *dsx* gene is unique in the hierarchy in that it actively controls both male and female sexual differentiation. This regulation is negative; in females the *dsx* gene functions to repress male differentiation, whereas in males, the *dsx* gene functions to repress female differentiation. The *dsx* locus therefore acts as a bifunctional switch whose state of expression is determined by the activity of the *tra* and *tra-2* genes. Female differentiation also depends on the expression of the *ix* locus that has been suggested to act downstream of the *dsx* gene (Baker and Ridge, 1980).

The control of sexual differentiation in males differs from that in females in that many of the genes in the regulatory hierarchy are inactive (Figure 1). In XY flies only one sex determination regulatory gene, *dsx*, is known to function. The male-specific *dsx* function acts to repress the genes involved in female sexual differentiation. The genes specifying male-specific terminal differentiation are not repressed, thereby leading to male development. No regulatory functions are known that are necessary only in males for *dsx* to be expressed in this manner. This suggests that the expression of the *dsx* male function is the default state of expression of this gene, a condition that occurs in the absence of regulation by the rest of the sex determination hierarchy (Baker et al., 1987).

Genetic studies have also delimited when and how the various components in this hierarchy function in the establishment and maintenance of sexual differentiation during development. The reading of the X:A ratio that initially determines sex occurs once early in development, and that determination is remembered by each cell thereafter (Sanchez and Nöthiger, 1982; Baker and Belote, 1983). One proposed mechanism for this cellular "memory" is the positive autoregulation of the *Sxl* gene (Cline, 1984). In contrast to the early and irreversible reading of the X:A ratio, somatic sexual differentiation requires the active and continual functioning of the sex determination regulatory genes throughout much of development (Baker and Ridge, 1980; Sanchez and Nöthiger, 1982; Cline, 1985; Wieschaus and Nöthiger, 1982; Belote and Baker, 1987). For example, studies done with temperature-sensitive *tra-2* alleles show that wild-type *tra-2* function is needed from at least second instar until the late pupal

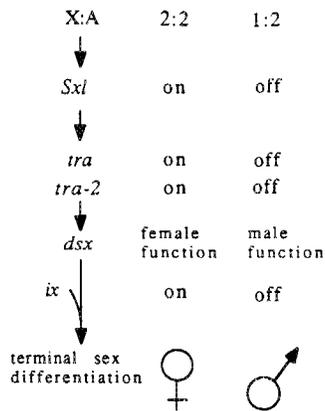


Figure 1. Model for the Sex Determination Regulatory Hierarchy
Genetic experiments have defined a regulatory hierarchy for sexual differentiation (Baker and Belote, 1983; Cline, 1985). Those parts of the hierarchy relevant to this study are shown. The initial signal for this process is the X:A ratio that is proposed to regulate the activity of the *Sxl* locus in females via the *da* and *sis-a* genes (Cline, 1985, 1986). *Sxl* is believed to activate the *tra* and *tra-2* genes, which in turn regulate the bifunctional *dsx* locus. The *ix* locus acts with the *dsx* female function to allow female differentiation. An X:A ratio of 1:2 results in male development by keeping the *Sxl* locus inactive. This in turn does not allow the expression of the *tra* and *tra-2* female-specific functions. In the absence of upstream regulation, the *dsx* male function is expressed.

period for normal sexual differentiation of adult cuticular structures (Belote and Baker, 1987). The *tra-2* product is also necessary in adults to maintain the sexually differentiated state of at least some sexually dimorphic tissues. Thus, in adult females both the occurrence of yolk protein synthesis and absence of male courtship behavior require the continued presence of the wild-type *tra-2* product (Belote et al., 1985; Belote and Baker, 1987). Taken together, these results suggest that this regulatory hierarchy is functioning throughout much of development to both bring about and maintain sexual differentiation.

Some insights into the molecular basis of the sex-specific functions of these regulatory genes have come from the cloning and characterization of the *Sxl* (Maine et al., 1985), *tra* (Butler et al., 1986; McKeown et al., 1987), and *dsx* (Baker and Wolfner, 1988) loci. In each case, a complex transcription pattern is found that includes one or more sex-specific RNA species, as well as nonspecific RNAs. In the case of the *tra* gene, there is both a female-specific and a sex-nonspecific transcript. These two RNAs arise via differential splicing that generates a long open reading frame in only the female-specific product (Boggs et al., 1987). The *dsx* locus produces both male-specific and female-specific RNAs, consistent with the need for *dsx* expression in both sexes, which we believe represent the functional *dsx* products on the basis of the locations of *dsx* mutations and the time of expression of these RNAs (Baker and Wolfner, 1988). These sex-specific *dsx* transcripts contain long open reading frames that would encode different proteins; they share common 5' exons but differ in the pattern of splicing and polyadenylation at their 3' ends (K. B. and B. B., unpublished data).

This suggests that a major aspect of the control of *tra* and *dsx* expression occurs at the level of RNA processing.

We examined at the molecular level how the *tra* and *dsx* genes are controlled by the other regulatory loci in the sex determination hierarchy. Our results demonstrate that mutations at some of these other loci alter the processing of the *tra*⁺ and *dsx*⁺ transcripts in a manner sufficient to account for all the functional interactions between these genes that are seen in genetic studies. In addition, we show that in adult females, the female-specific pattern of the *dsx* transcripts is dependent on the continual presence of functional *tra-2* product. This suggests that the maintenance of the sexually differentiated state in the adult is mediated by the *dsx* locus that requires continual regulation by the genes upstream of it in the sexual hierarchy.

Results

We are interested in determining the manner in which the expression of the *tra* and *dsx* genes is controlled by other regulatory genes in the sex determination hierarchy. The *tra* and *dsx* loci produce different RNAs in males and females that result, at least in part, from differential RNA processing. Presumably, these sex-specific patterns of RNA processing are mediated by one or more of the sex determination regulatory genes that act prior to *tra* and *dsx*. However, not all regulation of *tra* and *dsx* need occur through RNA processing as there could also be translational and/or posttranslational control. Genes that regulate at levels other than RNA transcription or processing would not be expected to affect the sex-specific patterns of the *tra* and *dsx* RNAs. Additional regulation of *tra* or *dsx* could occur via feedback or cross-regulation by genes that are either downstream of or parallel to them in the hierarchy. To distinguish among these possibilities, we have examined the patterns of transcripts produced by wild-type copies of both the *tra* and *dsx* genes in flies carrying mutations in other sex determination regulatory loci.

Regulation of the transformer Gene

The *tra* locus gives rise to a 1.1 kb transcript that is found in both sexes, and a 0.9 kb RNA that is present only in females (Figure 2; McKeown et al., 1987; Boggs et al., 1987). These two RNAs are produced by alternative splicing of a common precursor RNA (Boggs et al., 1987; Boggs and McKeown, unpublished data). To determine whether the occurrence of the female-specific splice is controlled by other sex determination regulatory genes, we have examined the pattern of transcripts produced by wild-type *tra* genes in chromosomally female flies mutant at either the *Sxl*, *tra-2*, or *dsx* loci. As a probe for *tra* transcripts in these experiments, we used a cDNA that hybridizes to both the female-specific and sex-nonspecific *tra* transcripts (Figure 2A).

Since null *Sxl* mutations are lethal in females, we examined *tra* expression in flies carrying a heteroallelic combination of leaky *Sxl* alleles that allows viability of some diplo-X progeny. These flies undergo complete male somatic differentiation (Cline, 1984). Blots prepared from

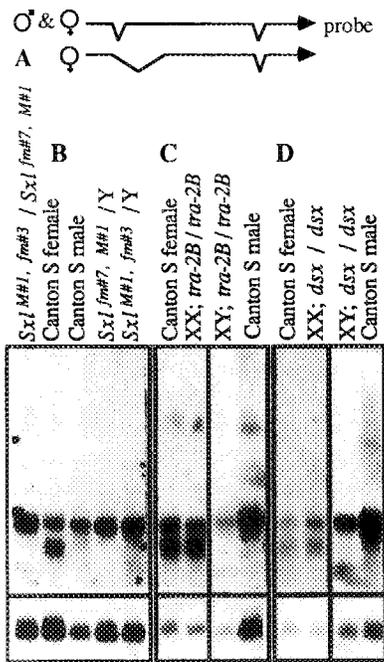


Figure 2. The Expression of *tra* RNAs in Different Mutant Backgrounds

The sex-specific *tra* RNA patterns in different mutant backgrounds are shown.

(A) Diagram of the female-specific and sex-nonspecific transcripts from the *tra* locus. The probe was a nick-translated cDNA of the sex-nonspecific *tra* RNA. (B–D) Two to five micrograms of poly(A)⁺ RNA from wild-type and mutant adult flies were electrophoretically separated and blotted to nitrocellulose. Filters were hybridized first with a probe derived from a *tra* cDNA (top panels). As a control for loading variations, the filters were reprobbed with a nick-translated sequence from the ribosomal protein gene *rp49* (bottom panels; O'Connell and Rosbash, 1984).

(B) *Canton S* represents the wild-type *tra* RNA pattern. *Sxl^{tm⁷M#1}* *Sxl^{M#1, f^{m#3}}*, *Sxl^{f^{m#7}M#1}/Y*, and *Sxl^{M#1, f^{m#3}/Y}* are phenotypic males.

(C) Both XY and XX; *tra-2^B/tra-2^B* flies are sterile phenotypic males.

(D) Both XY and XX; *dsx/dsx* flies are intersexual. Smudges below the *tra* transcripts are nonspecific background. These flies were probed by the following crosses: (B) *cm Sxl^{tm⁷M#1} ct⁶ v*; *Dp(1,3)sn^{13a1} × y w Sxl^{M#1, f^{m#3} ct⁶ v/Y}*; (C) *B^SY/+*; *cn tra-2^B bw/CyO × +/+*; *cn tra-2^B bw/CyO*; (D) *B^SY/+*; *p⁰dsx/TM6b × +/+*; *p⁰ dsx/TM6b*.

RNA from such flies were probed with a *tra* cDNA and the results are shown in Figure 2B. A reprobing of the same blot with a ribosomal protein mRNA (*rp49*) specific probe (O'Connell and Rosbash, 1984) is included as a control for variations in both the mobility between lanes and in the amount of RNA loaded. This figure demonstrates that XX, *Sxl*⁻ flies display the male-specific pattern of *tra* transcripts rather than the female-specific pattern, indicating that the wild-type function of the *Sxl* locus is necessary for the production of the female-specific *tra* transcript. In chromosomally male (XY) flies carrying either of these *Sxl* alleles, only the sex-nonspecific transcript is produced and there is no discernable change in the amount of *tra* RNA present. Therefore, while the transcription of the *tra* precursor RNA is independent of the *Sxl* gene, the female-specific processing of the *tra* precursor RNA depends on *Sxl*⁺ expression. This demonstrates that the *Sxl* product

Alternative regulatory pathways

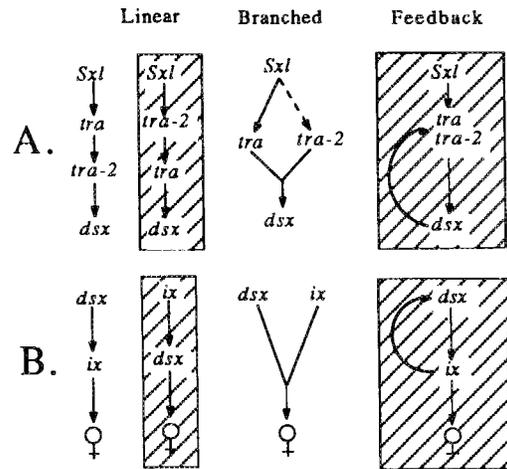


Figure 3. Models for the Interactions of *tra*, *tra-2*, *dsx*, and *ix*

The possible sex determination regulatory pathways that are consistent with the genetically derived hierarchy described in Figure 1 are diagrammed. (A) Possible interactions between *tra* and *tra-2*. (B) Possible interactions between *dsx* and *ix*. The molecular data in this paper disprove the pathways placed in cross-hatched boxes. The remaining two pathways describe the order of gene interactions at the RNA level, but they do not take into account possible translational or posttranslational controls. The dotted arrow between *Sxl* and *tra-2* indicate the possibility that *tra-2* may be directly or indirectly regulated by *Sxl* or may act independently of *Sxl* control. The existing data do not preclude either possibility.

acts upstream of the *tra* gene in the regulatory hierarchy as suggested by genetic studies (Cline, 1979) and precludes the possibility that the two genes act in parallel pathways.

In order to determine if *tra-2* controls *tra* at the RNA level, we examined *tra*⁺ expression in flies mutant for *tra-2*. Figure 2C shows that diplo-X, *tra-2*⁻ flies, which develop as morphological males, express the female pattern of *tra*⁺ RNAs that is consistent with their chromosomal sex and *Sxl*⁺ genotype. There is also no effect of the *tra-2* genotype on *tra*⁺ expression in XY flies. This indicates that, at the RNA level, *tra-2* neither regulates *tra* nor is involved in the *Sxl* dependent female-specific processing of the *tra*⁺ RNA. These data are consistent with the hypothesis that the *Sxl* product acts directly on the *tra* RNA.

Genetic studies suggest that *dsx* acts after *tra* and *tra-2* in the regulatory hierarchy that controls sex. In accord with this expectation, we find that the pattern of RNAs produced by the *tra* locus is independent of the functioning of the *dsx* locus (Figure 2D). Thus, both XX, *dsx*⁻ and XY, *dsx*⁻ flies express the *tra* RNA pattern consistent with their chromosomal sex. This indicates that the control of the sex-specific *tra* RNA patterns by *Sxl* is neither mediated by, nor requires, the presence of any *dsx* product.

The finding that *dsx* mutations do not affect the pattern of RNAs produced by the *tra* locus precludes the possibility of a feedback loop in which the *dsx* products, either directly or indirectly, regulate the expression of the *tra* RNAs (Figure 3). Moreover, we show below that in diplo-X

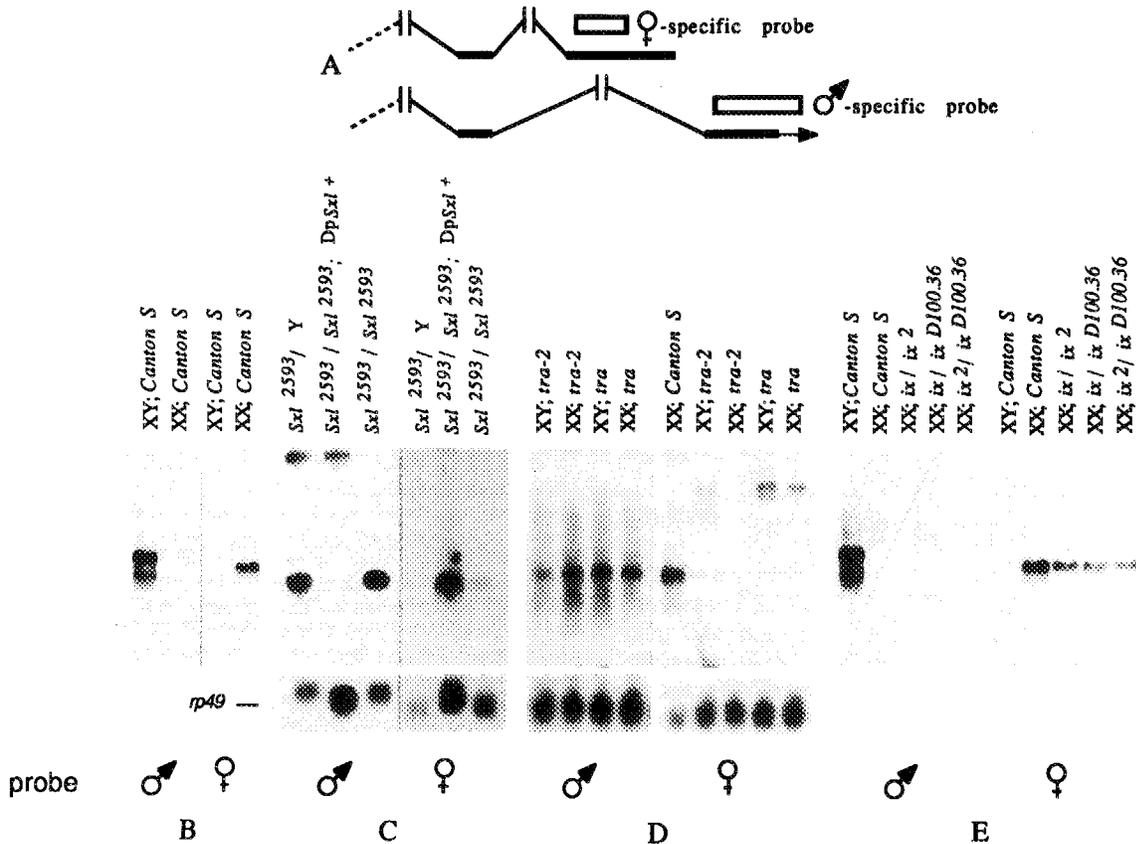


Figure 4. The Expression of the Male and Female-Specific *dsx* RNAs in Different Mutant Backgrounds

The sex-specific *dsx* RNA patterns in different mutant backgrounds are shown. The two autoradiograms in each set represent separate blots derived from the same RNA preparations and prepared in parallel.

(A) Diagram of the sex-specific portion of the *dsx* transcripts and the location of the *dsx* sequences used as probes. (B-E) Ten to twenty micrograms of total RNA from wild-type and mutant adult flies was electrophoretically separated and blotted to nylon filters. Filters were hybridized first with a single-stranded *dsx* probe, then, with the exception of Figure 4E, rehybridized with a nick-translated sequence from *rp49*. We believe that any differences in band intensities in Figure 4E are due to variations in the amount of RNA loaded per lane, as subsequent studies show no detectable difference in the level of *dsx* RNA expression in *ix* mutants versus wild-type (data not shown).

(B) Wild-type *dsx* RNA patterns.

(C) *Sxl*²⁵⁹³/Y flies are male while *Sxl*²⁵⁹³/*Sxl*²⁵⁹³; *Dp*(1,3)*Sxl*⁺ flies are female (*Dp*(1,3)*Sxl*⁺ connotes *Dp*(1,3)*sn*^{13a1}). *Sxl*²⁵⁹³/*Sxl*²⁵⁹³ flies are intersexual in phenotype. In the filter probed with the male-specific sequence, a high molecular weight band is seen in the *Sxl*²⁵⁹³/Y and the *Sxl*²⁵⁹³/*Sxl*²⁵⁹³; *Dp*(1,3)*Sxl*⁺ lanes but not in the *Sxl*²⁵⁹³ mutant RNA. Bands of this size are infrequently seen in other RNA preparations, including wild-type, and could represent a rare or unstable precursor RNA species.

(D) All *tra-2* and *tra* mutant flies are phenotypic males. Higher molecular weight bands present in some of the lanes are only occasionally seen in these genotypes and could represent relatively rare or unstable precursor RNA species.

(E) XX; *ix*¹/*ix*¹ flies are intersexual in phenotype. Flies of the above genotypes were produced by the following crosses: (C) *y Sxl*²⁵⁹³ *ct*⁶ *snly Sxl*²⁵⁹³ *ct*⁶ *sn*; *Dp*(1,3)*sn*^{13a1} × *y Sxl*²⁵⁹³ *ct*⁶ *snly*⁺ Y. (D) *FM6/y w cin*; *tra-2*/*CyO* × *y w cinly*⁺ Y; *tra-2*/*CyO* and *FM6/y w cin*; *tra*/*TM6b* × *y w cinly*⁺ Y; *tra*/*TM6b*. (E) *B⁵Yl*⁺; *ix*⁻¹/*CyO* × *1*/*+*; *ix*⁻¹/*CyO* (*ix*⁻¹ = *pr cn ix*, *ix*², or *ix*^{D100.36}).

flies, *tra-2* mutations alter the expression of the *dsx* locus such that only the male-specific *dsx* transcript is produced without affecting *tra* expression (Figure 2C). This result demonstrates that the presence of the *dsx* male product does not prevent the expression of the female-specific *tra* RNA and that the presence of the *dsx* female product is not necessary for the female-specific pattern of *tra* RNAs.

Regulation of the *doublesex* Gene

The *dsx* locus express both male-specific and female-specific RNAs that are first detectable at the end of the larval period (Baker and Wolfner, 1988; K. B. and B. B., unpublished data). Sequence analysis of cDNAs corre-

sponding to these transcripts indicate that they differ from each other as a consequence of differential RNA splicing and poly-A addition sites. The presence of sex-specific exons in these *dsx* RNAs has enabled us to make probes specific for the male and female *dsx* transcripts (Figure 4A). The wild-type *dsx* RNA patterns detected by these probes are shown in Figure 4B. The male-specific *dsx* probe detects two sex-specific transcripts. Both Northern and cDNA analyses indicate that the larger, more abundant transcript, and probably the smaller one as well, undergo the male-specific splice at *dsx* (K. B. and B. B., unpublished data). In Figures 4C and 4D, the smaller band is visible only after long exposure. We do not believe this

difference in the relative abundance of the two transcripts is due to the *Sxl*, *tra*, or *tra-2* mutations tested since RNA from phenotypically wild-type males in these autoradiographs also shows reduced levels of this transcript (i.e. *Sxl*²⁵⁹³/Y, XY; *tra-2*, and XY; *tra*) and we have found similar variability in the relative abundance of these two transcripts in wild-type males. This may reflect a differing sensitivity in the expression of the two transcripts to the age of the adults tested and/or the genetic background. Because of these considerations, we will refer only to the larger male-specific *dsx* transcript in the following analyses.

Our current understanding of the hierarchy controlling sex determination in flies (Figure 1) suggests that the *Sxl*, *tra*, and *tra-2* loci are all necessary in females to switch the *dsx* locus from expressing its male-specific function to expressing its female-specific function. To determine if this regulation occurs at the RNA level, we examined the pattern of *dsx* transcripts in flies homozygous for mutants in these other regulatory genes.

Because null mutations at *Sxl* are lethal when homozygous in females, we examined the effects of a hypomorphic allele of *Sxl* (*Sxl*²⁵⁹³) on the pattern of RNAs produced by a wild-type *dsx* gene. When homozygous, *Sxl*²⁵⁹³ allows some diplo-X flies to survive and they display an intersexual phenotype (Marshall and Whittle, 1978; Cline, 1984). Diplo-X flies homozygous for *Sxl*²⁵⁹³ produce both the male and female *dsx* transcripts, consistent with the hypomorphic nature of *Sxl*²⁵⁹³ and the observed intersexual phenotype (Figure 4C). In contrast, phenotypically normal females that carry one copy of *Sxl*⁺ express only the female *dsx* RNA (Figure 4C). These results indicate that the wild-type *Sxl* function is necessary to prevent the formation of the male-specific *dsx* RNA. We interpret these results to mean that in the leaky *Sxl*²⁵⁹³ mutants there is insufficient functional *Sxl*⁺ product to convert all of the *dsx* transcript into the female-specific RNA, instead, some *dsx* transcript is processed into the male-specific form. The fact that the male-specific *dsx* transcript is produced in this genotype is consistent with the view that the expression of the *dsx* male function is the default form of *dsx* expression. This default state will occur in either chromosomally male or female individuals whenever the upstream female-specific controls are absent or inadequately expressed (Baker et al., 1987). The pattern of *dsx* transcripts in XY flies is not affected by the *Sxl*²⁵⁹³ mutation, a result expected since neither *Sxl* null alleles nor *Sxl*²⁵⁹³ cause a detectable mutant phenotype in XY flies.

Genetic studies suggest that the *tra* and *tra-2* loci are necessary for the expression of the female-specific function of the *dsx* locus; in the absence of either wild-type *tra* or *tra-2* activity, the *dsx* male-specific function is active (for review see Baker and Belote, 1983). The results in Figure 4D show that the wild-type products of both genes are necessary in females for the generation of the female-specific *dsx* transcript. In both XY and XX flies homozygous for *tra* or *tra-2* mutations, only the male *dsx* RNA is produced. These results indicate that the wild-type functions of the *tra* and *tra-2* loci are required for the female-specific processing of the *dsx* transcript, whereas the ab-

sence of wild-type activity of either of these genes results in the male-specific RNA splicing pattern.

Epistasis experiments indicate that the *ix* gene acts downstream of the *tra* and *tra-2* loci. However, the similar phenotypes produced by *ix* and *dsx* mutants make it difficult to order these two genes in the hierarchy (Baker and Ridge, 1980). Examination of three different *ix* mutant combinations show that despite the substantial amounts of male differentiation occurring in these diplo-X, *ix*⁻ flies, only the female-specific *dsx* transcript is produced (Figure 4E). Thus, the *ix* gene does not regulate the expression of the *dsx* gene at the RNA level.

In Figure 2 and Figure 4, occasional bands can be seen that do not correspond with known *tra* or *dsx* transcripts. These do not correlate with any mutant genotype, are of relatively low abundance, and are not consistently reproducible. We suggest that they could be rare or unstable precursor RNAs or cross-homologies with other *Drosophila* transcripts. The latter possibility is particularly relevant to *dsx* since cognate sequences have been identified by low-stringency hybridizations with *dsx* probes to genomic DNA libraries (Andrew, 1987). We do not preclude the possibility of novel transcripts arising as a consequence of these mutations, however, we see no evidence for them in our experiments.

The Role of the *doublesex* Gene in Maintaining the Sexually Differentiated State of the Fly

The regulatory hierarchy described in Figure 1 controls the differentiation of sexually dimorphic adult cuticular structures, a process completed by the end of the pupal period (Baker and Belote, 1983). That these regulatory genes interact in the same hierarchical manner to establish at least some of the sexually dimorphic internal characteristics of the adult has been shown by studies on the control of expression of the female-specific yolk protein genes (Ota et al., 1981). Furthermore, even in the adult stage after sexual differentiation has occurred, yolk protein synthesis and female mating behavior require the continuous expression of the *tra-2* gene (Belote et al., 1985; Belote and Baker, 1987). However, it is not known if the *tra-2* gene maintains the sexually differentiated state in the adult via the same cascade of regulatory interactions that establishes the pattern of sexual differentiation during development. Alternatively, it is possible that the action of *tra-2* in the maintenance of sexual differentiation in the adult occurs independently of the rest of the hierarchy.

To distinguish between these possibilities, we examined the pattern of *dsx* transcripts produced in response to temperature shifts of adult flies carrying a temperature-sensitive *tra-2* allele. Diplo-X individuals homozygous for the *tra-2*^{ts2} allele were raised at the semipermissive temperature of 18°C until eclosion. These flies were phenotypically female, although occasionally a female-like intersex was found. The adults were maintained either at 18°C or placed at 29°C for 2 days, at which time their RNA was isolated and analyzed (Figure 5). Flies maintained at 18°C showed significant amounts of the female *dsx* transcript and low, though noticeable, levels of the *dsx* male tran-

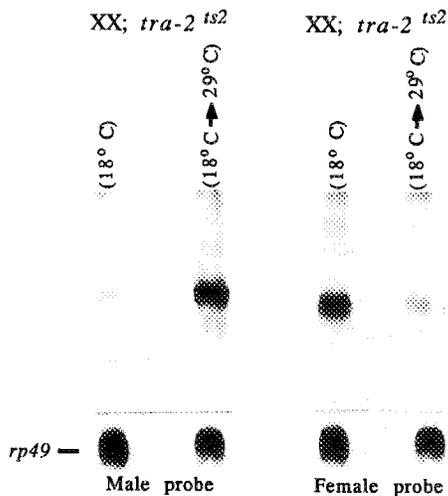


Figure 5. Changes in *dsx* Expression in XX; *tra-2^{ts2}* Females Following Temperature Shift to 29°C

The sex-specific *dsx* RNA patterns in XX; *tra-2^{ts2}* flies are shown. XX; *tra-2^{ts2}* *bw* females were raised and kept at 18°C until 24–48 hr after eclosion, then either kept at 18°C for 2 days or shifted to 29°C for 2 days before analysis by Northern blotting. Total RNA (10–20 µg/lane) from adult flies were electrophoretically separated and blotted to a nylon filter. The two autoradiograms represent separate blots derived from the same RNA preparations and prepared in parallel. Blots were hybridized first with single-stranded probes prepared from sequences specific for either the male or female *dsx* transcripts (see Figure 4A). Blots were subsequently reprobbed with nick-translated sequences from *rp49*.

script. After a shift to the restrictive temperature of 29°C, the opposite result was seen; these flies had reduced levels of the female *dsx* transcript relative to the amount of male-specific transcript. These results indicate that even after sexual differentiation is established, *dsx* expression is still under the control of the *tra-2* gene product. Thus, the expression of the *dsx* gene is not irreversibly fixed at the time of sexual differentiation. Instead, the continuous regulation by the *tra-2* gene, and by inference the rest of the sex determination loci, is needed to maintain the sex-specific pattern of *dsx* expression. This result is consistent with *tra-2* acting together with the rest of the sex regulatory hierarchy to maintain the sexually differentiated state of the adult.

Discussion

The cloning of the *tra* and *dsx* loci has allowed the analysis of the regulation of sexual differentiation at the molecular level. Transcriptional studies at *tra* (Butler et al., 1986; McKeown et al., 1987) and *dsx* (Baker and Wolfner, 1988) have identified RNAs for both genes that are specific to the sex in which these genes are known to be active. The molecular data presented here demonstrate that the formation of these sex-specific RNAs is controlled by the sex determination regulatory hierarchy through the alternative splicing of sex-specific exons.

We have used our molecular data to order the sex

regulatory genes in an epistatic hierarchy. By assuming that loci affecting the *tra* or *dsx* RNA patterns act prior to these genes, while loci that fail to do so function either in parallel with or downstream of *tra* and *dsx*, an epistasis pattern of *Sxl*>*tra*>*tra-2*>*dsx*>*ix* is derived (Figure 3) that is consistent with the hierarchy deduced from genetic studies. It is particularly striking that in every case where genetic studies infer that a gene acts prior to *tra* (or *dsx*), mutations in that gene alter the *tra* (or *dsx*) RNA processing pattern. While these results obviously cannot preclude regulatory interactions at other levels, the regulation of RNA splicing revealed by these experiments is sufficient to account for all of the known functional interactions between *tra* or *dsx*, and the other regulatory genes in this hierarchy.

We find no evidence for feedback regulation of the *tra* or *dsx* genes by downstream functions. Specifically, our data show that neither ectopic expression of the *dsx* male product (occurring in XX; *tra-2⁻* flies) nor the absence of wild-type *dsx* activity affects *tra* expression. Similarly, the production of the *dsx* transcripts is independent of *ix* mutations; in diplo-X, *ix⁻* flies only the female-specific *dsx* transcript is produced. Therefore, the *ix* product is not needed to maintain the expression of the *dsx* female RNA nor to prevent the formation of the *dsx* male transcript.

These data demonstrate that everything necessary for the male-specific processing of the *dsx* RNA is normally present in diplo-X individuals. The failure to produce the male-specific *dsx* RNA in females is due to the sequential functioning of the wild-type products of the *Sxl*, *tra*, and *tra-2* genes, which impose the alternative (female-specific) pattern of RNA processing. This is in keeping with our suggestion (Baker et al., 1987) that the expression of the *dsx* male function is a default state of gene expression that depends solely on the basic housekeeping machinery of the cell and not on any sex-specific controls.

Our experiments have also provided insights into some previously ambiguous aspects of the sex determination regulatory hierarchy. The similarity in the phenotypes produced by *tra* and *tra-2* mutations, as well as by *dsx* and *ix* lesions, precludes a determination based on genetic experiments of the order in which these genes function (Baker and Ridge, 1980). We found that the regulation by *Sxl* of *tra* RNA processing is not mediated by the *tra-2* locus, indicating that either the *tra-2* gene functions downstream of, or parallel to, the *tra* locus (Figure 3A), or that it regulates *tra* at some level other than transcription or RNA processing. Similarly, the *ix* gene must act downstream of, or in parallel with, the *dsx* locus in females (Figure 3B).

These results provide experimental support for a recently proposed molecular model that attempts to consolidate several important features of this regulatory hierarchy. Boggs et al. (1987) proposed that *Sxl* regulates the *tra* gene by controlling sex-specific alternative splicing. In addition, they suggested that the positive autoregulation of the *Sxl* locus (Cline, 1984) could be mediated via the same RNA splicing activity. The female-specific product of the *Sxl* locus could both result from as well as cause a female-specific pattern of splicing on the primary *Sxl* transcript.

This would potentially provide a molecular explanation for the cellular "memory" of the X:A ratio that Cline (1984) suggested was mediated by the autoregulation of the *Sxl* gene. An intriguing possibility raised by this model is that the other proposed function of *Sxl*, the regulation of dosage compensation through the control of the male-specific lethal genes (*msl*s; Uenoyama et al., 1982; Lucchesi and Skripsy, 1983), might also be regulated by alternative splicing. For example, the wild-type action of the *Sxl* product in preventing the expression of the *msl* genes in females could occur by its mediating the splicing of the *msl* transcripts into nonfunctional RNAs. This model would therefore consolidate the regulation of such genetically and functionally distinct processes as the establishment of the sexually determined state, the "memory" of the X:A ratio in individual cells, and the establishment of dosage compensation, to a single RNA splicing activity that is encoded at or regulated by *Sxl*. The data in this paper directly demonstrate the validity of one crucial aspect of this model: that the *Sxl* locus functions in females to direct the formation of the female-specific *tra* RNA. The molecular analysis of the *Sxl* and the *msl* genes should allow similar testing of the rest of this hypothesis.

Experimental Procedures

Fly Strains

The *Sxl* alleles used in these studies are partially functional mutations that allow the survival of diplo-X flies but result in sexual transformation. *Sxl*^{M¹,M²,M³} and *Sxl*^{fm¹,M¹} are described in Cline (1984), and *Sxl*^{P²937} is described in Marshall and Whittle (1978) and Cline (1984). *Dp(1,3)sn^{13a1}* is a *cm⁺*, *Sxl⁺*, *ct⁺* duplication (Cline, 1978). Three *tra-2* alleles were used in these studies: *tra-2* (Watanabe, 1975), *tra-2^B* (Belote and Lucchesi, 1980), and *tra-2⁵²* (Belote and Baker, 1983). The *ix^{2102,35}* allele is an EMS-induced recessive mutation obtained from Ian Duncan. Descriptions of mutants and rearrangements not referred to in the text are found in Lindsley and Grell (1968). Flies were raised on a standard cornmeal, molasses, yeast, agar media containing propionic acid as a mold inhibitor and supplemented with live yeast.

RNA Preparation

RNAs for experiments on the *tra* locus were made as described in McKeown et al. (1987) with the exception that flies were neither frozen nor ground prior to homogenization. All RNAs are poly(A)⁺ and were prepared by batch purification in microfuge tubes. RNAs for the *dsx* experiments were obtained as follows: 10–50 adult flies were homogenized in a Dounce homogenizer in 10 ml of 1:1 homogenization buffer: phenol (homogenization buffer: 0.15 M NaCl, 1.5 mM MgCl₂, 10 mM Tris-HCl [pH 8.0], 0.5% sodium dodecyl sulfate). The supernatant was extracted twice with 1:1 phenol:chloroform then ethanol precipitated. The nucleic acid precipitate was resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and the concentration was determined by OD 260.

RNA Blotting and Hybridization

For the Northern analysis of the *tra* locus, blots and probes were as described in McKeown et al. (1987) and Boggs et al. (1987). For the analysis of the *dsx* gene, 10–20 µg of total RNA were loaded per lane in a formaldehyde gel and blotted to Nytran (Schleicher and Schuell) as described in Maniatis et al. (1982). Single-stranded DNA probes were derived from *dsx* genomic and cDNA sequences subcloned into the M13 vector utilizing primed synthesis from a 17mer M13 primer. Hybridizations were done in 5× SSPE (or 5× SSC), 5× Denhardt solution, 50 µg/ml salmon sperm DNA, 0.2% SDS at 42°C. To control for variations in loading, the filters were reprobbed with the ribosomal protein gene *rp49* (O'Connell and Rosbash, 1984), which appears to be uniformly expressed in both sexes.

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References

- Andrew, D. J. (1987). A search for new genes regulating sex determination in *Drosophila melanogaster*. Ph.D. thesis, University of California, San Diego, California.
- Baker, B. S., and Belote, J. M. (1983). Sex determination and dosage compensation in *Drosophila melanogaster*. *Annu. Rev. Genet.* 17, 345–397.
- Baker, B. S., and Ridge, K. (1980). Sex and the single cell: on the action of major loci affecting sex determination in *Drosophila melanogaster*. *Genetics* 94, 383–423.
- Baker, B. S., and Wolfner, M. F. (1988). A molecular analysis of *doublesex*, a bifunctional gene that controls both male and female sexual differentiation in *Drosophila melanogaster*. *Genes Dev.*, in press.
- Baker, B. S., Nagoshi, R. N., and Burtis, K. C. (1987). Molecular genetic aspects of sex determination in *Drosophila*. *Bioessays* 6, 66–70.
- Belote, J. M., and Baker, B. S. (1983). The dual functions of a sex determination gene in *Drosophila melanogaster*. *Dev. Biol.* 95, 512–517.
- Belote, J. M., and Baker, B. S. (1987). Sexual behavior: its genetic control during development and adulthood in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 84, 8026–8030.
- Belote, J. M., and Lucchesi, J. C. (1980). Male-specific lethal mutations of *Drosophila melanogaster*. *Genetics* 96, 165–186.
- Belote, J. M., Handler, A. M., Wolfner, M. F., Livak, K. J., and Baker, B. S. (1985). Sex-specific regulation of yolk protein gene expression in *Drosophila*. *Cell* 40, 339–348.
- Boggs, R. T., Gregor, P., Idriss, S., Belote, J. M., and McKeown, M. (1987). Regulation of sexual differentiation in *Drosophila melanogaster* via alternative splicing of RNA from the *transformer* gene. *Cell* 50, 739–747.
- Butler, B., Pirotta, V., Irminger-Finger, I., and Nöthiger, R. (1986). The sex-determining gene *tra* of *Drosophila*: molecular cloning and transformation studies. *EMBO J.* 5, 3607–3613.
- Cline, T. W. (1978). Two closely linked mutations in *Drosophila melanogaster* that are lethal to opposite sexes and interact with *daughterless*. *Genetics* 90, 683–698.
- Cline, T. W. (1979). A male-specific lethal mutation in *Drosophila* that transforms sex. *Dev. Biol.* 72, 266–275.
- Cline, T. W. (1984). Autoregulatory functioning of a *Drosophila* gene product that establishes and maintains the sexually determined state. *Genetics* 107, 231–277.
- Cline, T. W. (1985). Primary events in the determination of sex in *Drosophila melanogaster*. In *Origin and Evolution of Sex*, H. O. Halvorson and A. Monroy, eds. (New York: Alan R. Liss), pp. 301–327.
- Cline, T. W. (1986). A female-specific lethal lesion in an X-linked positive regulator of the *Drosophila* sex determination gene. *Sex-lethal*. *Genetics* 113, 641–663.
- Lindsley, D. L., and Grell, E. H. (1968). Genetic variations of *Drosophila melanogaster*. Carnegie Inst. Wash. Pub. 627.
- Lucchesi, J. C., and Skripsky, T. (1981). The link between dosage compensation and sex differentiation in *Drosophila melanogaster*. *Chromosoma* 82, 217–227.

- Lucchesi, J. C., and Skripsky, T. (1983). Intersexuality resulting from the interaction of sex-specific lethal mutations in *Drosophila melanogaster*. *Dev. Biol.* 94, 153–162.
- Maine, E. M., Salz, H. K., Schedl, P., and Cline, T. W. (1985). *Sex-lethal*, a link between sex determination and sexual differentiation in *Drosophila melanogaster*. *Cold Spring Harbor Symp. Quant. Biol.* 50, 595–604.
- Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). *Molecular Cloning*. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).
- Marshall, T., and Whittle, J. R. S. (1978). Genetic analysis of the mutation *female-lethal* in *Drosophila melanogaster*. *Genet. Res.* 32, 103–111.
- McKeown, M., Belote, J. M., and Baker, B. S. (1987). A molecular analysis of *transformer*, a gene in *Drosophila melanogaster* that controls female sexual differentiation. *Cell* 48, 489–499.
- O'Connell, P., and Rosbash, M. (1984). Sequence, structure and codon preference of the *Drosophila* ribosomal protein 49 gene. *Nucl. Acids Res.* 12, 5495–5514.
- Ota, T., Fujunaga, A., Kawabe, M., and Oishi, K. (1981). Interactions between sex transformation mutants of *Drosophila melanogaster*. I. Hemolymph vitellogenins and gonad morphology. *Genetics* 99, 424–441.
- Sanchez, L., and Nöthiger, R. (1982). Clonal analysis of *Sex-lethal*, a gene needed for females sexual development in *Drosophila melanogaster*. *Roux's Arch. Dev. Biol.* 191, 211–214.
- Uenoyama, T., Fukunaga, A., and Oishi, K. (1982). Studies on the sex-specific lethals of *Drosophila melanogaster*. V. Sex transformation causes by interactions between a female-specific lethal, *Sxl^{tr1}*, and the male-specific lethals *mle(3)32*, *msl-2^{tr}*, and *mle*. *Genetics* 102, 233–243.
- Watanabe, T. K. (1975). A new sex-transforming gene on the second chromosome of *Drosophila melanogaster*. *Jap. J. Genet.* 50, 269–271.
- Wieschaus, E., and Nöthiger, R. (1982). The role of the *transformer* genes in the development of the genitalia and analia of *Drosophila melanogaster*. *Dev. Biol.* 90, 320–334.