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Genetics

Sex-specific processing of the *Drosophila* exuperantia transcript is regulated in male germ cells by the *tra-2* gene

TULLE HAZELRIGG[†‡] AND CHI TU[§¶]

[†]Department of Biological Sciences, 602 Fairchild, Columbia University, New York, NY 10027; and [§]Biology Department and Howard Hughes Medical Institute, University of Utah, Salt Lake City, UT 84112

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ABSTRACT **The *Drosophila* exuperantia (*exu*) gene encodes overlapping sex-specific, germline-dependent mRNAs. In this work, the structural differences between these sex-specific *exu* mRNAs were determined by sequence analysis of 9 ovary and 10 testis cDNAs. The transformer 2 (*tra-2*) gene functions in sex determination of female somatic cells through its role in regulating female-specific splicing of doublesex (*dsx*) RNA. We report here that *tra-2* is required in male germ cells for efficient male-specific processing of *exu* RNA; in the absence of *tra-2*, *X/Y* males produce a new mRNA which is processed at its 3′ end so that it contains sequences normally specific to the female 3′ untranslated region. Although the processing event that requires *tra-2* occurs in an untranslated region of the *exu* transcript, the isolation and characterization of a male-specific *exu* allele which deletes male 3′ untranslated sequence indicate that this processing is biologically significant.**

The exuperantia (*exu*) gene, which is required for correct localization of bicoid (*bcd*) mRNA during Drosophila oogenesis (1–3), was first identified and characterized by maternal-effect mutations (4–7). Most mutations of the gene, however, also confer male sterility; mutant defects in spermatogenesis include the presence of micronuclei and abnormally large nebenkerns in early spermatids, a failure of spermatid nuclei to completely elongate, and dispersal of these nuclei along spermatid bundles in later stages (7). The *exu* gene encodes overlapping, sex-specific mRNAs whose expression is limited to germ cells: a 2.9-kb male mRNA and a 2.1-kb female mRNA (7). A rare 2.5-kb mRNA common to both sexes is present in somatic cells (7). The protein product of the female germline mRNA has been described before (8, 9). To determine how different mRNAs are generated in the male and female germlines, we isolated and characterized a set of 9 additional female cDNAs from ovaries, and a set of 10 male cDNAs from testes. Analysis of these cDNAs shows that the male and female mRNAs differ at both their 5′ and 3′ ends due to sex-specific RNA processing and also exhibit apparent sex-specific promoter utilization.

Alternative processing of transcripts is a frequently occurring phenomenon whereby cell-specific products of single genes are produced during development and differentiation (10–13). While much is known about cis-acting signals in pre-mRNA that mediate splicing- and polyadenylylation-site choices (11), relatively few trans-acting factors for alternative RNA processing have been identified (12, 13). Some of the genes controlling sex-determination in *Drosophila* have been shown to regulate alternative splicing in a sex-specific manner (reviewed in ref. 14). One of these, transformer 2 (*tra-2*), is required for female somatic sex determination. In the absence of *tra-2* function in *X/X* flies, somatic cells are sexually transformed into male cells (15–19). The primary transcript of the doublesex (*dsx*) gene is sex-specifically spliced, producing different polypeptides in males and females (20, 21). Molecular and genetic studies have shown that in female somatic cells, *tra-2* is required for splicing of the *dsx* mRNA in its female form (22). Both genetic and molecular studies indicate that the mode of action of *tra-2* in female somatic cells is by activation of the *dsx* female splice-site choice (21, 23–25). Direct binding of *tra-2* protein to the *dsx* transcript has been demonstrated *in vitro* (26).

Whereas *tra-2* function is not required in male somatic cells, it is required in male germ cells for normal spermatogenesis (15, 27, 28). *tra-2* autoregulates processing of its own primary transcript in male germ cells (29). However, an autoregulatory role of *tra-2* in male germ cells does not directly address the question of the biological role of the *tra-2* gene product in the male germline. An understanding of the function of *tra-2* in male germ cells has awaited identification of target RNAs encoded by genes required for normal male germ-cell determination and/or differentiation. The existence of differentially processed *exu* transcripts in the germlines of the two sexes provided an opportunity for testing the hypothesis that *tra-2* regulates RNA processing in male germ cells. We show that the *tra-2* gene is required for male-specific processing of the *exu* transcript in spermatogenesis and that the RNA processing choice (cleavage and polyadenylylation within an intron versus splicing of this intron) is different from the choices previously attributed to *tra-2* function. In addition, the isolation and characterization of a male-specific *exu* allele with a deletion of male-specific 3′ untranslated sequences provide strong evidence for the biological significance of *tra-2*-mediated processing of *exu* RNA in male germ cells.

MATERIALS AND METHODS

Molecular Characterization of Transcripts. The testis cDNA library was constructed by Stratagene's Custom Library Service in λZAPII, from 10 μg of poly(A)$^+$ RNA isolated from hand-dissected wild-type *Drosophila melanogaster* (Canton-S strain) testes. An ovary cDNA library was similarly constructed by Stratagene from 10 μg of poly(A)$^+$ RNA prepared from hand-dissected Canton-S ovaries. We also screened a λgt11 ovary cDNA library generously provided by L. Kalfayan. *exu* cDNAs were identified by hybridization with labeled ovary cDNA pC8 insert (9).

Clones from the λgt11 library were subcloned into pBluescript KS(−), and phagemids were isolated from the λZAPII libraries according to Stratagene's instructions. DNA sequencing (9) used a set of synthetic oligonucleotides as primers. The Intelligenetics PC/GENE program was used for comparisons between cDNA and genomic sequences (9).

Molecular Analysis of Transcripts and Genomic DNAs. Southern blot analysis of DNA from *exu*^{DP3} DNA was done

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[‡]To whom reprint requests should be addressed.
[¶]Present address: Nexagen, Inc., 2860 Wildneress Place, Suite 200, Boulder, CO 80301.

as described (7). Nick-translated probes included the entire cDNA insert of testis cDNA TC17, a 5′ fragment from TC17 (a 740-bp *Eco*RI–*Hin*dIII restriction fragment extending from the *Eco*RI λZAPII polylinker cloning site at the 5′ end of TC17 to the *Hin*dIII site at genomic map position 2.3), and a 3′ TC17 fragment (a 561-bp nick-translated *Hin*dIII–*Eco*RI restriction fragment from genomic map position 3.8 to the *Eco*RI polylinker λZAPII site at the 3′ end of TC17).

Isolation of poly(A)$^+$ RNA and Northern blot analysis were done as described (7). For Fig. 2, the probe was a ^{32}P-labeled nick-translated ovary pC8 insert (7, 9), and ≈6 μg of poly(A)$^+$ RNA was loaded in each lane. The ^{32}P-labeled female antisense probe (Fig. 3*a*) was transcribed from *Hpa* I/*Pst* I-digested pC8 DNA. The male-specific probe (Fig 3*b*) was a nick-translated 0.7-kb *Bgl* II/*Xba* I fragment from the 3′ end of pMC1 insert DNA. [pMC1 is a partial testis cDNA which includes the 3′ end of the transcript (W. S. Watkins and T.H., unpublished work)]. For the blots shown in Fig. 3, it was estimated that 2 μg of poly(A)$^+$ RNA was loaded in each lane, but reprobing the Fig. 3*a* blot with an *rp49* transcript probe revealed that more RNA was inadvertently loaded in the Canton-S (CS) ovary lane.

Genetic Screen to Isolate Male-Specific *exu* Alleles. The crossing scheme was identical to the mutagenesis G_2 screen described in ref. 7, figure 1*C*, except that x-rays were used instead of ethyl methanesulfonate, and male G_2 males, not females, were tested for fertility, by mating with wild-type Canton-S virgin females. G_0 males were irradiated with 4000 rads (1 rad = 0.01 Gy) at 434.4 rads/min with an Astrophysics Torrex 120D x-ray machine. In the G_2 test crosses, vials lacking progeny after 3–5 days were identified, and the putative male-sterile mutation was isolated by crossing the sibling *cn bw*/SM1* males to *Tft/CyO* virgin females to establish a stock, and recrossing also to *cn Df(2R) exu^1 bw sp/SM1* virgin females to obtain males to retest and confirm the male sterility.

***Drosophila* Strains.** The *tra-2^B* allele is a point mutation believed to be a loss-of-function allele (28, 30). *X/X;tra-2^B/tra-2^B* flies are transformed into males. *X/Y;tra-2^B/tra-2^B* flies were distinguished from their *X/X;tra-2^B/tra-2^B* siblings by the presence of the dominant *Bar^{Stone}* (*B^S*) marker on the Y chromosome in the *cn tra-2^B bw/CyO* stock. Marker mutations and balancer chromosomes are described in ref. 31.

RESULTS

***exu* Male and Female Germline mRNAs Differ in Untranslated Sequences at Both Their 5′ and 3′ Ends.** To determine the differences between the *exu* gene's germline female and male mRNAs, several cDNAs were isolated and sequenced from ovary and testis cDNA libraries. The differences between the male and female *exu* mRNAs are diagrammed in Fig. 1. Based on sequences of cDNAs extending the furthest either 5′ or 3′, the male and female mRNAs have the following unique 5′ and 3′ regions. At the 5′ ends, the data are consistent with the existence of sex-specific *exu* promoters in the male and female germlines, with the female promoter lying within a 5′ male-specific intron. At the 3′ end, a male-specific splice yields an 868-nt untranslated exon unique to the male mRNA. The female transcript is polyadenylylated within this 3′ male intron, producing a female mRNA with 229 nt of unique 3′ untranslated sequence. Because the differences in the male and female mRNAs are in untranslated regions, the polypeptides predicted from both mRNAs are the same. The predicted protein of 532 aa has been described (8, 9).

Processing of the Male-Specific *exu* mRNA in Germ Cells Is Regulated by the *tra-2* Gene. Given the role of the *tra-2* gene in *dsx* mRNA processing in female somatic cells and its known, but not understood, function in spermatogenesis (15, 27, 28), we considered that *tra-2* might regulate the processing of male germline transcripts required for spermatogenesis, and *exu* might be one of its targets. To test this, we

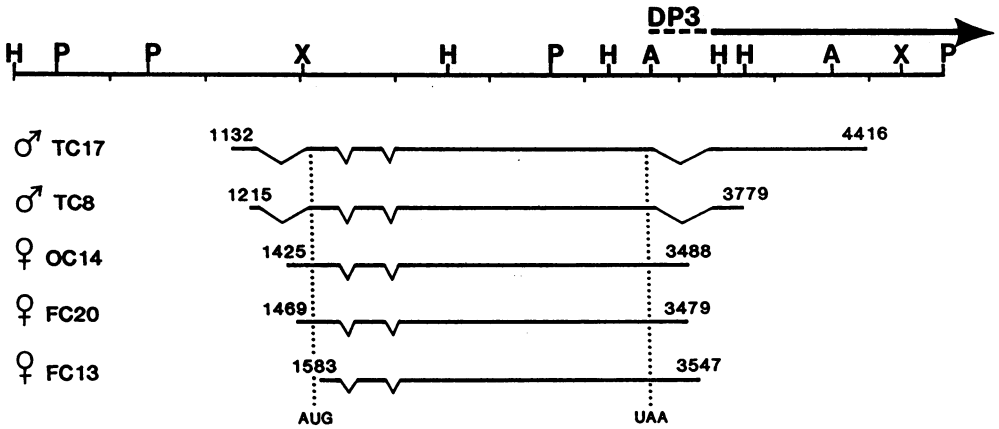

FIG. 1. Map of the *exu* male and female transcripts showing their structural differences. Shown here are the structures of two completely sequenced testis cDNAs and three fully sequenced ovary cDNAs. The top line is a restriction map of the genomic DNA, based on sequence. The position of the left breakpoint of the *exu^{DP3}* deletion, a male-specific *exu* allele, is shown above the map. The numbers at the beginning and end of each cDNA are the nucleotide positions in the genomic sequence. TC17, FC20, and FC13 cDNAs have poly(A) tails and thus represent true 3′ ends, but TC8 and OC14 cDNAs do not contain poly(A) tails and probably were initiated from random priming in the construction of the λZAPII libraries. Of 10 testis cDNAs, 4 were sequenced in full. Partial sequences, including the 5′ and 3′ ends, were obtained for the other 6. Four testes cDNAs had poly(A) tails; three polyadenylylation sites are suggested by the positions of these 3′ ends. There is a consensus AAUAAA polyadenylylation signal positioned 18 nt upstream of two cDNAs that share the same 3′ end. The 3′ end of a third male cDNA lies 12 nt downstream. There is no good candidate for a consensus polyadenylylation signal upstream of the 3′ end of TC17. Two of the 10 testis cDNAs have 5′ ends within the 5′ male intron; these cDNAs probably represent partially processed primary transcripts. Of 9 ovary cDNAs analyzed, 3 were sequenced in full and 6 were sequenced partially, including their ends. At their 5′ ends, 2 female cDNAs have fusions of DNA not encoded by *exu*, apparently as the result of artifacts in construction of the cDNA library. Six of the remaining 7 ovary cDNAs have 5′ ends within the first male intron. None of the ovary cDNAs have 5′ ends within the first male exon. All 5 ovary cDNAs with poly(A) tails terminate within the male-specific intron 4; of these, 4 were clustered within a 15-nt region. No consensus polyadenylylation signal exists upstream of these, but the sequence AAUAUA is present upstream of this cluster of 3′ ends (10–24 nt upstream, depending on the cDNA). There is also an AAUAUA 19 nt upstream of the 3′ end of a fifth female cDNA. Some nucleotide differences were found in a few of the cDNAs, probably representing polymorphisms and alterations brought about during cDNA synthesis. A, *Hpa* I; H, *Hin*dIII; P, *Pst* I; X, *Xba* I.

examined exu expression in *tra-2 X/Y* and *X/X* flies. Northern blots show that both the male-specific 2.9-kb mRNA and a new mRNA of about 2.1 kb are present in *X/Y;tra-2^B* males (Fig. 2). The new mRNA is slightly larger than the wild-type female-specific mRNA. Northern blots hybridized with probes specific to either the male or the female 3′ untranslated region show that this new mRNA hybridizes to the female-specific probe (Fig. 3). [The Northern blots in Fig. 3 were done with poly(A)+ RNA isolated from hand-dissected ovaries and testes, whereas the Northern blot in Fig. 2 was done with poly(A)$^+$ RNA from whole flies. More RNA was inadvertently loaded in the Canton-S (CS) ovary lane in the Fig. 3*a* blot than in the other lanes.] This novel mRNA also hybridizes with a probe unique to the 5′ male-specific untranslated exon (data not shown), which normally hybridizes to the male 2.9-kb transcript, but not the female 2.1-kb transcript. These results indicate that the unusual mRNA in *tra-2* mutant males is transcribed from the male germline promoter but is processed at its 3′ end so that it contains sequences which are normally unique to the female mRNA. That it is slightly larger than the female transcript may reflect a difference in length of the poly(A) tail in male germ cells.

The presence in *X/Y;tra-2* mutant males of an RNA with female-specific 3′ UTR sequences is probably not the result of sexual transformation of *X/Y* germ cells, but rather a result of *tra-2*-mediated processing of the *exu* primary transcript in the male germline. There is no evidence that somatic or germ cells in the testes of *X/Y;tra-2* mutant males are transformed sexually (28, 32).

Homozygous *X/X;tra-2^B* flies, which are chromosomal females phenotypically transformed into males, have low levels of both transcripts seen in *X/Y;tra-2^B* males (Fig. 2). (The gonads of these flies are very underdeveloped, containing few germ cells, so that it is not surprising that the level of these transcripts is correspondingly low.) That these sexually transformed flies express *exu* in the male germ cell mode provides strong molecular evidence for the conclusions of Nöthiger *et al.* (32), based on morphological data, that germ cells in mutant *X/X;tra-2* flies can enter the spermatogenic pathway. That the normal 2.1-kb female mRNA is not detected in *X/X;tra-2^B* flies may reflect the presence of a higher number of germ cells entering spermatogenesis rather than oogenesis. Nöthiger *et al.* (32) report that about 56–58% of the differentiating germ cells in *X/X;tra-2* mutant flies cannot be identified by morphology as oogenic or spermatogenic. Our results suggest that these cells may be entering the spermatogenic pathway.

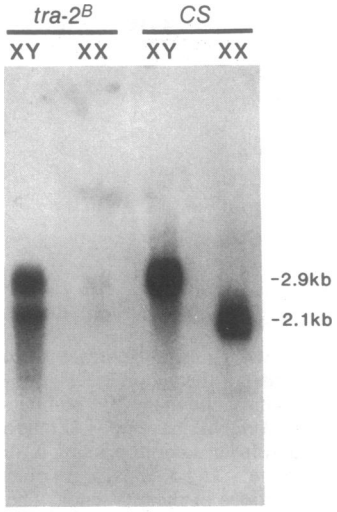

FIG. 2. *tra-2* is required for processing of the *exu* transcript in the male mode. Poly(A)$^+$ RNA isolated from wild-type Canton-S (CS) and homozygous *tra-2^B* flies was probed with labeled pC8 insert DNA, which detects both overlapping sex-specific mRNAs (7). The wild-type CS male and female mRNAs are 2.9 and 2.1 kb, respectively. The 2.9-kb mRNA and a new mRNA that is slightly larger than 2.1 kb are present in homozygous *tra-2^B;X/Y* males. Low levels of both mRNAs are also seen in the *tra-2^B;X/X* transformed males. The chromosomal sex is indicated above each lane.

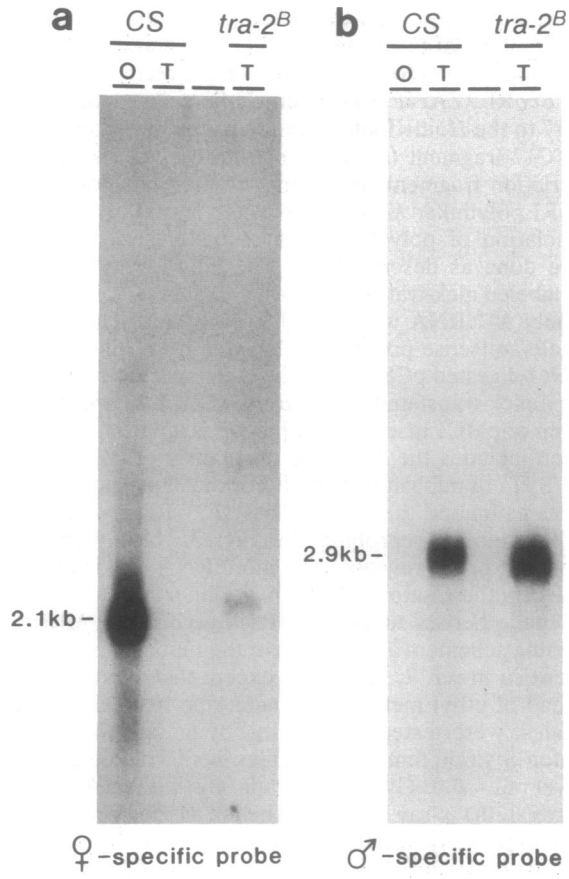

FIG. 3. The new mRNA in *tra-2* mutant males contains female-specific 3′ untranslated sequence. (*a*) Northern blot of wild-type Canton-S (CS) ovary (O) and testes (T) poly(A)$^+$ RNA and *X/Y;tra-2^B* testes (T) poly(A)$^+$ RNA, probed with a labeled antisense RNA specific for unique sequence in the 3′ end of the female mRNA, showing that the new mRNA appearing in homozygous *tra-2^B* males is processed to contain female sequence at its 3′ end. (*b*) Northern blot identical to the blot in *a*, probed with a ^{32}P-labeled DNA fragment unique to the male 3′ exon. This probe detects only the 2.9-kb male mRNA and does not detect the new mRNA present in *tra-2* mutant males.

Isolation of a Male-Specific *exu* Allele. The differences in the *exu* male and female germline mRNAs are in untranslated regions such that the mRNAs encode the same predicted protein in both sexes. This left open the question whether there was biological significance to *tra-2*-mediated *exu* RNA processing in male germ cells. Genetic experiments were undertaken to identify regions of the male transcript required for male-specific expression of *exu* in spermatogenesis.

Male-sterile *exu* mutations were sought in the mutagenesis screen described in *Materials and Methods*. One male-sterile allele, *exu^{DP3}* (hereafter designated *DP3*) was isolated in a G_2 screen of 3185 x-irradiated second chromosomes. It was retested for female fertility in *DP3/Df*(2R)*exu^1* flies and was found to be female-fertile. Complementation tests with several existing *exu* alleles showed that DP3 is a male-specific *exu* allele. Testes squashes of *DP3/DF*(2R)*exu^1* males revealed that these males have defects in spermatogenesis similar to those described previously for other *exu* mutants.

Molecular Characterization of *DP3* Shows That It Is a Deletion and That DP3 Males Make a Truncated *exu* mRNA. Southern blot analysis of *DP3* DNA shows that it contains a deletion that removes most, if not all, of the male-specific 3′ untranslated region (data not shown). Using probes from the 5′ or 3′ end of male cDNA TC17 (see *Materials and Methods*), the left breakpoint of the deletion was mapped between a *Hpa* I site in the fourth male intron and a *Hin*dIII site within the last male untranslated exon (Fig. 1); this deletion extends for about 1 kb downstream of the 3′ end of the male transcript. A Northern blot of RNA from mutant *DP3* males shows they produce a truncated mRNA which is smaller than the normal male mRNA but larger than the female mRNA by about 300 nt (data not shown).

DISCUSSION

The *exu* gene functions in both oogenesis and spermatogenesis and encodes overlapping, sex-specific, germline-dependent mRNAs. In this work, the structural differences between the male and female transcripts have been determined by characterization of a set of cDNAs isolated from testis and ovary cDNA libraries. These differences are found in both the 5′ and 3′ untranslated regions of the transcripts. The predicted polypeptides in both sexes are the same.

We have shown that sex-specific processing at the 3′ end of the *exu* primary transcript is dependent on the function of the *tra-2* gene in male germ cells. In the absence of *tra-2*, some of the *exu* primary transcript in male germ cells is processed so that it contains sequences unique to the female 3′ untranslated region. Since some of the RNA remains processed in the male mode, *tra-2* is not absolutely required for male-specific processing. Rather, *tra-2* affects the efficiency of processing in the male mode. We have isolated a male-specific *exu* allele (male-sterile and female-fertile) that deletes most or all of the male-specific 3′ UTR and expresses a truncated *exu* mRNA in males. That these mutant males are sterile indicates that at least part of the male-specific 3′ untranslated exon is necessary for normal functioning of *exu* in male germ cells and suggests that *tra-2*-regulated processing of *exu* RNA is biologically important for *exu* function in the male germline.

The *exu* gene is a candidate downstream target RNA for *tra-2*-regulated processing in male germ cells. The present data do not demonstrate whether this effect is direct or indirect; further research is needed to determine the exact mechanisms whereby *tra-2* regulates *exu* transcript processing. Since current evidence about the role of *tra-2* in processing of the *dsx* primary transcript indicates that there is a direct interaction between the *tra-2*-encoded protein and *dsx* pre-mRNA (23, 25, 26), it is likely that in the case of *exu* transcript processing, *tra-2* acts directly in the alternative processing reactions.

Consideration of the phenotypic effects of both *tra-2* and *exu* mutants (for both genes the phenotypic effects of null alleles have been described) suggests that additional targets for *tra-2* may exist in male germ cells. *exu* mutant males first show defects shortly after meiosis, when micronuclei appear, and nebenkern formation is not normal (7). Subsequent steps of spermiogenesis are also abnormal. The first defects described by Belote and Baker (28) for *tra-2* mutant males occur later than those first seen in *exu* mutants; in some *tra-2* mutant sperm bundles no sperm head elongation occurs. In *exu* mutant males, sperm heads partially elongate but do not complete elongation. Thus, the *tra-2* defects are both less and more severe than the *exu* defects: although the first defects in *tra-2* mutant males occur later (i.e., at the stage of sperm head elongation), the sperm head elongation defect can be more severe than that seen in *exu* mutants. Later, *exu* and *tra-2* mutants share a similar phenotype: sperm heads appear disconnected from tails and are dispersed throughout the sperm bundles (7, 28). Since *tra-2* males still make some *exu* male mRNA (see Fig. 2), some defects may not be as severe as an *exu*-null phenotype simply because these males still have some wild-type *exu* function. That later defects are more severe in *tra-2* mutant males (i.e., sperm head elongation is more severely disturbed) suggests that *tra-2* has additional RNA targets in the male germline.

Most cases of alternative RNA splicing are regulated by negative control of splice-site selection (12). The results presented here and previously reported data indicate that the *tra-2* gene participates in various types of RNA processing events. In the case of *dsx* transcript processing, the *tra-2* gene acts positively to promote female-specific splicing (23–26). Mattox and Baker (29) have shown that *tra-2* autoregulates processing of its own transcripts in the male germline. In this case a *tra-2* product is required to repress splicing of an intron, leading to the accumulation of a *tra-2* mRNA that retains this intron. In contrast, the evidence presented here shows that in male germ cells a *tra-2* gene product acts to promote the accumulation of a spliced *exu* male mRNA, either through activation of male-specific splicing or through repression of female polyadenylylation-site choice.

It has been proposed that the various types of RNA processing events seemingly mediated by the *tra-2* gene are likely to be a result of a combination of different *tra-2* polypeptides interacting with other tissue-specific factors, such as the female somatic transformer (*tra*) gene product (29, 30, 33–36). However, our results and those of Mattox and Baker (29) indicate that not all of the different effects of *tra-2* are due to interactions with different tissue-specific factors since in one tissue, the male germline, two different sorts of RNA processing events are mediated by the *tra-2* gene: the *exu* transcript processing and the autoregulated *tra-2* transcript processing appear to be different types of events. It is formally possible that these different processing events are temporally regulated in the male germline, such that different cofactors are present at different times of spermatogenesis when these two processing events occur. However, since most transcription occurs during the primary spermatocyte stage (37), this sort of temporal regulation seems unlikely.

The different RNA processing events regulated by *tra-2* in the male germline may be mediated by different *tra-2* products. Three major variant *tra-2* polypeptide products containing ribonucleoprotein CS-type RNA-binding domains are predicted from mRNAs expressed in different cell types (29, 34–36). In the male germline, two different polypeptides are predicted from identified *tra-2* transcripts, one of which, designated type C by Mattox and Baker (29) and called msT_{maj} by Amrein *et al.* (36), is encoded by the unspliced *tra-2* RNA. The other predicted *tra-2* protein in male germ cells, type E (msT_{min}) is the same polypeptide predicted to be translated from a minor form of *tra-2* mRNA also present in male and female somatic cells. Recent evidence shows that the type E protein is responsible for repression of the *tra-2* splice (W. Mattox and B. Baker, personal communication). It is possible that *exu* transcript processing is regulated by the male germline product encoded by the type C (unspliced) transcript. The results of Mattox and Baker (29) and Amrein *et al.* (36) indicate that the type C polypeptide alone cannot rescue male sterility. Thus, in the male germline both of these products may be required to perform different RNA processing reactions, all of which are required for male fertility. It is also possible that the different regulatory events governed by *tra-2* are context dependent and that different cis-acting RNA signals are involved in directing the choices governed by *tra-2*. The *exu* and *tra-2* RNAs provide an excellent system to determine the mechanisms whereby *tra-2* regulates different types of RNA processing events, since both are expressed in the same cell type and are processed differently.

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