Autoregulation of *transformer-2* Alternative Splicing Is Necessary for Normal Male Fertility in Drosophila

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Manuscript received October 20, 1997
Accepted for publication April 3, 1998

ABSTRACT

In the male germline of Drosophila the *transformer-2* protein is required for differential splicing of pre-mRNAs from the *exuperantia* and *att* genes and autoregulates alternative splicing of its own pre-mRNA. Autoregulation of TRA-2 splicing results in production of two mRNAs that differ by the splicing/retention of the M1 intron and encode functionally distinct protein isoforms. Splicing of the intron produces an mRNA encoding TRA-2^{226}, which is necessary and sufficient for both male fertility and regulation of downstream target RNAs. When the intron is retained, an mRNA is produced encoding TRA-2^{179}, a protein with no known function. We have previously shown that repression of M1 splicing is dependent on TRA-2^{226}, suggesting that this protein quantitatively limits its own expression through a negative feedback mechanism at the level of splicing. Here we examine this idea, by testing the effect that variations in the level of tra-2 expression have on the splicing of M1 and on male fertility. Consistent with our hypothesis, we observe that as *tra-2* gene dosage is increased, smaller proportions of TRA-2^{226} mRNA are produced, limiting expression of this isoform. Feedback regulation is critical for male fertility, since it is significantly decreased by a transgene in which repression of M1 splicing cannot occur and TRA-2^{226} mRNA is constitutively produced. The effect of this transgene becomes more severe as its dosage is increased, indicating that fertility is sensitive to an excess of TRA-2^{226}. Our results suggest that autoregulation of TRA-2^{226} expression in male germ cells is necessary for normal spermatogenesis.

THE RNA-binding protein *transformer-2* (*tra-2*) affects alternative splicing of RNAs from several genes with critical roles in Drosophila sexual differentiation. In somatic tissues TRA-2 is required, in combination with the TRA protein, for the sex-specific processing of pre-mRNAs from both the *doublesex* (*dsx*) and *fruitless* (*fru*) genes (Nagoshi *et al.* 1988; Ryner *et al.* 1996). The resulting mRNAs from these genes encode products that in turn regulate sexual identity in different subsets of somatic tissues. In chromosomally female (*XX*) individuals lacking either TRA-2 or TRA, processing of these pre-mRNAs occurs in the male-specific rather than female-specific pattern resulting in sexual transformation.

In the male germline TRA-2 functions independently of TRA and is required for normal spermatogenesis. In the absence of functional TRA-2, male germ cells appear to initiate spermatogenesis normally but ultimately form spermatids with unelongated nuclei that are not motile (Belote and Baker 1983). As a result, such mutant males are sterile. The sex-specific processing of pre-mRNAs from two genes expressed during spermatogenesis, *exuperantia* (*exu*) and *alternative-testes-transcript* (*att*), are dependent on TRA-2 (Hazelrigg and Tu 1994; Madigan *et al.* 1996). The role of alternative splicing in these targets is largely unknown, however in the case of *exu* mRNA it has been shown that mutations affecting the alternatively spliced 3′ UTR lead to a significant reduction in the level of *exu* RNA that accumulates in male germ cells (Crowley and Hazelrigg 1995). Null mutations in *exu* result in male sterility (Hazelrigg *et al.* 1990) and, like *tra-2* mutations, lead to formation of spermatids with defects in nuclear elongation.

Two distinct TRA-2 protein isoforms (TRA-2^{226} and TRA-2^{179}) are expressed in the male germline (Amrein *et al.* 1990; Mattox *et al.* 1990). The relative levels of RNAs encoding these isoforms appear to be controlled by an autoregulatory mechanism (see Figure 1) in which the TRA-2^{226} isoform represses the splicing of the *tra-2* M1 intron (also called intron three) (Mattox and Baker 1991; Mattox *et al.* 1996). This intron interrupts the translation initiation codon for TRA-2^{226} itself. Thus, by repressing a processing pathway used to form TRA-2^{226} mRNA, this isoform acts to negatively regulate its own expression. When M1 splicing is repressed, TRA-2 mRNAs accumulate, encoding a truncated isoform (TRA-2^{179}) lacking the amino terminal RS domain. Analysis of transgenic strains that express either TRA-2^{226} or TRA-2^{179} individually indicated that, while TRA-2^{226} is necessary and sufficient for both sex-specific *exu* splicing and male fertility, TRA-2^{179} has no effect on either function (Mattox *et al.* 1996). This indicates

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that TRA-2^{226} is the primary functional product and that autoregulation of M1 splicing is part of a negative feedback pathway that serves to quantitatively limit the amount of RNA encoding functional protein (TRA-2^{226}) in the male germline.

Although the presence or absence of TRA-2^{226} clearly affects alternative splicing of the M1 intron, it is not known what biological role negative feedback regulation plays in *tra-2*$^+$ males. Here we show that variations in tra-2^+ gene dosage result in compensatory changes in the ratio of M1 splicing/retention, so as to maintain a constant level of TRA-2^{226} mRNA. Further, we find that feedback regulation of this mRNA in the male germline is required for nomal male fertility.

MATERIALS AND METHODS

Drosophila RNA and low-cycle reverse transcriptase (RT)-PCR: Drosophila RNA was isolated and RT-PCR was carried out essentially as has been described (Dauwalder *et al.* 1996) except that cDNA was produced using an oligo $dT_{(12–18\ nt)}$ primer, and PCR amplifications were carried out in 1 mm $MgCl_2$ with an annealing step of 60°. Amplification primers used to analyze the M1 intron were derived from *tra-2* exon three (5′CTCAGCCGATTCAGCTGGTGCTCTTG3′) and the exon five/six junction (5′CGCTGTGT/TTGTGCGTCAAT CA3′). Amplified fragments were electrophoresed, blotted, and then detected by hybridization with a ^{32}P-labeled DNA fragment extending from exon three to four. Relative levels of amplified products were quantified using a Molecular Dynamics (Sunnyvale, CA) Phosphoimager SI and ImageQuant software.

Construction of *P[ΔM1]* and *P[tra-2$^+$]* transgenic strains: P[tra-2^+] was constructed by first generating a plasmid (pTZ3.9AΔSac) containing the entire *tra-2* transcribed region, as well as flanking regions of 720 nt upstream and 523 nt downstream to it. This entire region was then inserted into the pCaSpeR *P*-element vector (Pirrotta 1988) as a 3.6-kb *Eco*RI-*Bam*HI fragment. To generate *P[ΔM1]*, the *Apa*I-*Bsm*I fragment from pTZ3.9AΔSac that encompasses the M1 intron was substituted by the analogous *Apa*I-*Bsm*I fragment from a TRA-2^{226}-encoding cDNA clone. The 3.4-kb *Eco*RI-*Bam*HI insert from this plasmid was then inserted into pCaSpeR. These *P* elements were injected directly into Drosophila embryos of the genotype w^{1118}/B^sY; *tra-*2^B*/CyO*. Transformed lines were identified in the G_1 progeny of injectees by w^+ eye pigmentation.

Fly strains and crosses: The *tra-*2^B mutation results from a nonsense codon within the RRM region (Mattox and Baker 1991). The allele *tra-*2^{PM6} is a deficiency that removes all *tra-2* coding sequences. The allele *tra-*2^{PM7} deletes the entire region between exon two and exon five of the *tra-2* gene. To generate individuals with multiple doses of *P* transposon insertions, lines were first generated that carry a *P*-element insertion on both the second and third chromosome by crossing w^{1118}/B^sY; *P/CyO*; *CxD/TM3*, *Ser* males to w^{1118};*Sco/CyO*; *P/TM3*, *Ser* females. The resultant doubly balanced lines were then crossed to one another to generate males with three or four *P* insertions.

Male fertility was tested by culturing a single male with three w^{1118} virgin females. In the fertility tests presented in Table 1, these flies were maintained together for five days in a single vial, which was then cleared and all F_1 progeny counted. In later fertility tests (Figures 5 and 6), flies were twice transferred to fresh vials at three-day intervals, and all F_1 progeny were counted from all vials. Vials in which any parents died before the final transfer were not counted. *P* values given in results for the hypothesis that experimental samples were identical to controls were calculated using the two-tailed *t*-test. For such pairwise comparisons, the fertility rates of *P[ΔM1]* genotypes were always compared to the *P[tra-*2^+*]* control cross that gave mean value closest to the experimental.

Splicing reporter genes and X-gal staining of testes: *P[CZP-ORF3]* is a transgene which expresses a TRA-2/β-galactosidase fusion protein specifically from the TRA-2^{179} translation initiation codon present in M1-containing RNAs. The construction of this reporter has been described previously (Mattox and Baker 1991). *P[CZPORF1,2]*, which specifically expresses a TRA-2/β-galactosidase fusion protein from RNAs in which the M1 intron has been spliced, was generated by inserting an *Eco*RI-*Nae*I fragment of the *tra-2* gene into the transformation vector pCaZPA (Mattox *et al.* 1990) so that β-galactosidase sequences are fused downstream to *tra-2* coding sequences within *tra-2* exon four. Germline translation in this case is driven by the TRA-2^{226} initiation codon. X-gal staining was performed as described previously (Mattox *et al.* 1990). All genotypes were fixed and stained in sequence for identical amounts of time so that direct comparison of staining levels could be made.

RESULTS

The ratio of alternative male-specific *tra-2* mRNAs is affected by gene dosage: If autoregulation of splicing serves to place an upper limit on the amount of TRA-2^{226} encoding mRNA produced, we expected that the ratio of TRA-2^{179} to TRA-2^{226} RNAs should increase as the number of transcribed copies of the *tra-*2^+ gene is increased. To test this, we examined the relative levels of these RNAs in flies with one, two, and four doses of the wild-type *tra-2* gene using low-cycle RT-PCR and the primers shown in Figure 1. Increased gene dosage led to an increase in the fraction of TRA-2^{179} (M1 unspliced) RNA relative to TRA-2^{226} (M1 spliced). Flies with a single dose of the *tra-*2^+ gene had a unspliced/spliced (U/S) ratio of only 0.42 (Figure 2, lane 4), while flies with four doses of *tra-*2^+ had an unspliced (U)/spliced (s) ration of 2.03 (Figure 2, lane 8). Overall levels of *tra-2*-derived PCR products were observed to increase in rough proportion to gene dosage. To control for the possibility that changes in the U/S ratio are due to artifactual changes in PCR efficiencies when different initial amounts of *tra-2* RNA are amplified, we performed RT-PCR on RNA from two-dose flies in amounts that were 0.5× and 2× that used in the above experiment (mimicking the one-dose and four-dose samples). The U/S ratios of these reactions (Figure 2, lanes 9 and 10) were very similar to that of the experimental two-dose sample (1.21 and 1.15 *vs.* 1.05). We conclude that male germ cells respond to an increase in the level of *tra-2* pre-mRNA by increasing repression of M1 splicing.

Since additional gene doses lead to an increase in the total amount of *tra-2* RNA produced in the above experiment, the change in U/S ratios observed may be due to a limiting factor other than TRA-2^{226} protein that

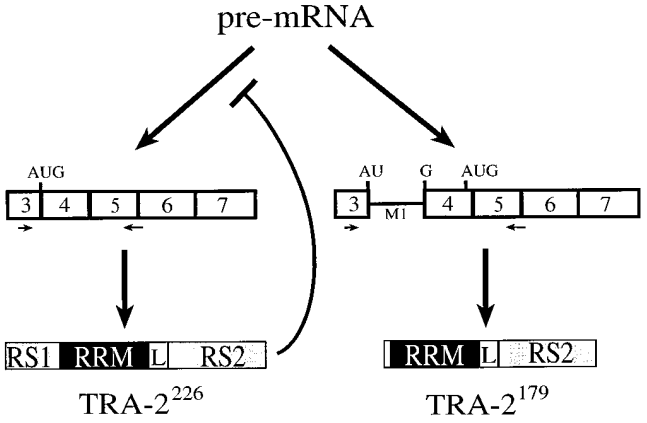

Figure 1.—Model for negative feedback regulation of TRA-2^{226} expression. A model for how repression of M1 splicing by TRA-2^{226} affects expression of different TRA-2 protein isoforms in the male germline. The protein isoforms produced from alternatively spliced *tra-2* mRNAs are shown. Numbered boxes represent exons and domains within proteins are indicated. RS1 and RS2 indicate arg-ser rich regions, RRM the RNA recognition motif, and L the conserved linker region of the protein. The M1 intron is represented by a line. As indicated, transcription initiates in exon three of the *tra-2* gene in this tissue and mRNA both with and without the M1 intron accumulate. The first in-frame translation initiation codon of each RNA is indicated. The initiation codon for TRA-2^{226} is split by the M1 intron. If the intron is retained, this codon is interrupted and the first in-frame initiation codon is that for TRA-2^{179}. The TRA-2^{226} protein represses removal of the M1 intron favoring formation of TRA-2^{179} encoding mRNA. Horizontal arrows below these RNAs indicate PCR primers used to distinguish M1-containing and spliced mRNAs in dosage experiments presented in Figure 2.

affects M1 splicing. Therefore, we tested how variations in the number of functional *tra-2* gene doses affected M1 splicing when the total number of RNA-producing *tra-2* genes was kept constant. In Figure 2, lanes 12 and 14, RT-PCR products from males of the genotype *tra-*2^{+}*/tra-*2^{+}*;P[tra-*2^{+}*]/P[tra-*2^{+}] are compared to males of the genotype *tra-*2^{B}*/tra-*2^{+}*;P[CSP-ORF3]/P[CSP-ORF3]* where *P[tra-*2^{+}*]* is a *P* transposon containing an unaltered *tra-2* gene (Figure 3), *P[CSP-ORF3]* a transposon containing a mutation that blocks translation of TRA-2^{226} (Mattox and Baker 1991) and *tra-*2^{B} is a nonsense mutation in the endogenous *tra-2* gene (see materials and methods for more details). Importantly, both *P[CSP-ORF3]* and *tra-*2^{B} are known to produce normal levels of *tra-2* RNA (Mattox and Baker 1991). Consistent with the idea that TRA-2 itself is the limiting factor affecting M1 splicing, males with four wild-type doses of the gene had considerably higher proportions of M1-containing RNAs than did the four-dose males with only one *tra-2* allele producing functional protein. These results suggest that in response to increased levels of expression, TRA-2^{226} protein functions to reduce the level of M1 splicing to limit the production of its own mRNA.

The above dosage experiments were carried out on RNA derived from whole male flies. To examine whether gene dosage affects the ratio of alternatively spliced TRA-2 RNAs produced in testes, we examined the effect of zero, one, and two doses of *tra-*2^{+} on the amount of β-galactosidase activity produced from either of two different reporter genes that are responsive to splicing of the M1 intron (Figure 3). *P[CZP-ORF3]* produces RNAs that are translated to produce TRA-2/β-galactosidase fusion proteins only when the M1 intron is retained (Mattox and Baker 1991), while *P[CZP-ORF1,2]* produces TRA-2/β-galactosidase fusion protein only from mRNAs in which the M1 intron has been precisely spliced out (see materials and methods). As expected, we observed that the intensity of X-gal staining depended on the number of *tra-*2^{+} genes present. Individuals carrying *P[CZP-ORF3]* and two doses of functional *tra-*2^{+} had stronger staining than those with one (*tra-*2^{B}*/tra-*2^{+}) or zero (*tra-*2^{B}*/tra-*2^{B}) functional *tra-*2^{+} alleles indicating that a larger fraction of M1-containing RNAs accumulate as the number of functional gene doses is increased (see Figure 4, A–C). Conversely, testes from males carrying *P[CZP-ORF1,2]* stained weakly when two doses of functional *tra-*2^{+} were present and progressively stronger in individuals with one and zero doses (Figure 4, D–F), indicating that more M1 splicing occurs in males with fewer doses of *tra-*2^{+}. These results are consistent with our RT-PCR analysis and support the idea that an increase in the level of transcripts potentially encoding functional *tra-2* products is accompanied by a compensating increase in the ratio of unspliced to spliced M1 intron.

A *tra-2* gene lacking the M1 intron is functional in both the female soma and male germline: Since autoregulation of M1 splicing appears to limit the production of RNAs encoding TRA-2^{226} in the male germline, we hypothesized that unregulated expression of this protein would lead to deleterious effects on male fertility. To test this idea, we constructed a *P*-element transformation vector, *P[ΔM1]*, containing an altered version of the *tra-2* gene in which the M1 intron has been precisely deleted (Figure 3). Autoregulation of TRA-2^{226} expression by repression of M1 splicing is not possible in transcripts made from this construct. All other *tra-2* sequences needed for expression were left unchanged in the inserted fragment, including the gene's promoter, protein coding regions, and introns and untranslated flanking regions.

Transgenic individuals carrying *P[ΔM1]* were initially recovered in the background genotype $w^{1118}/B^{s}Y$; *tra-*2^{B}*/CyO* (males) or w^{1118}/w^{1118}; *tra-*2^{B}*/CyO* (females). In the presence of *P[ΔM1]*, the endogenous *tra-*2^{+} allele is expected to down regulate expression of TRA-2^{226}. In flies with only a single dose of *P[ΔM1]*, such down regulation may be sufficient to maintain a near-normal level of TRA-2^{226} mRNA. Thus we found it was possible

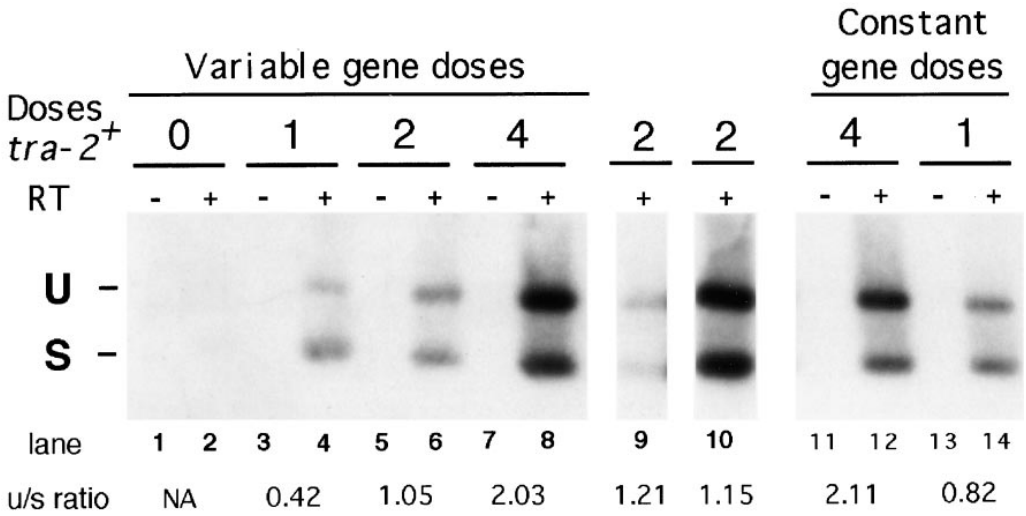

Figure 2.—The effect of changes in *tra-2*$^+$ gene dosage on the ratio of M1-containing and fully spliced RNAs in males. The ratio of RNAs in which the M1 intron is unspliced and spliced is compared for males with different numbers of *tra-2*$^+$ alleles per diploid genome. Each sample was amplified from cDNA synthesis reactions performed with (+) and without (−) reverse transcriptase. Primers used are shown in Figure 1. U/S ratios are shown below each pair of lanes. Genotypes of the males used are *tra-2*^{PM6}/*tra-2*^{PM7} (lanes 1 and 2); *tra-2*^{PM6}/*CyO, tra-2*$^+$ (lanes 3 and 4), *tra-2*$^+$/*CyO, tra-2*$^+$ (lanes 5 and 6); and *tra-2*$^+$/*CyO, tra-2*$^+$; *P[tra-2*$^+$*]*/*P[tra-2*$^+$*]* (lanes 7 and 8). In lanes 9 and 10 reactions were controls done on the same RNA used in lanes 5 and 6. In these cases cDNA synthesis was initiated with either 0.5× (lane 9) or 2.0× (lane 10) amounts of RNA. RNAs produced from four-dose individuals of the genotypes *tra-2*$^+$/*CyO, tra-2*$^+$; *P[tra-2*$^+$*]*/*P[tra-2*$^+$*]* (lanes 11 and 12) and *tra-2*^{B}/CyO, *tra-2*$^+$; *P[CSP-ORF3]*/*P[CSP-ORF3]* (lanes 13 and 14) are also compared.

to maintain vigorously fertile strains that carry a single dose of the transgene (see below).

Given this, we tested the ability of *P[ΔM1]* to provide *tra-2*$^+$ function sufficient to rescue *tra-2* mutant phenotypes (Table 1). Each of the eight independent insertions tested restored fertility to homozygous *tra-2*^{B} males

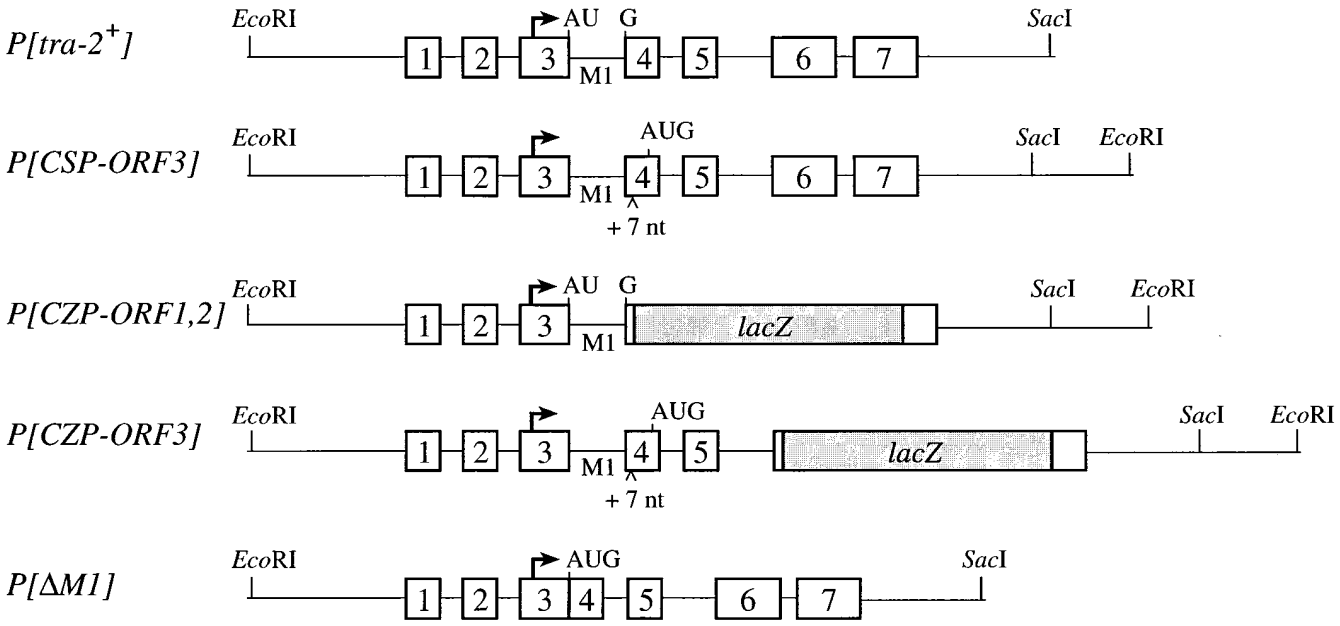

Figure 3.—P transposon inserts. Shown are the *tra-2* inserts contained in the various pCaSpeR transposon constructs that were used to generate transgenic strains. All exons indicated by unfilled boxes as well as the introns and flanking regions are from *tra-2*. Each insert contains over 500 nt both upstream and downstream of *tra-2* flanking sequences. Filled box indicates coding sequences from the *Escherichia coli lacZ* gene that are fused in frame with *tra-2* protein coding sequences. Arrows indicate the RNA 5′ end in the male germline. The major initiation codon(s) expected to produce male germline proteins in each construct is (are) indicated. A 7 nt sequence was inserted in exon four of both *P[CSP-ORF3]* and *P[CZP-ORF3]* to frameshift translation products that begin at the TRA-2^{226} initiation codon.

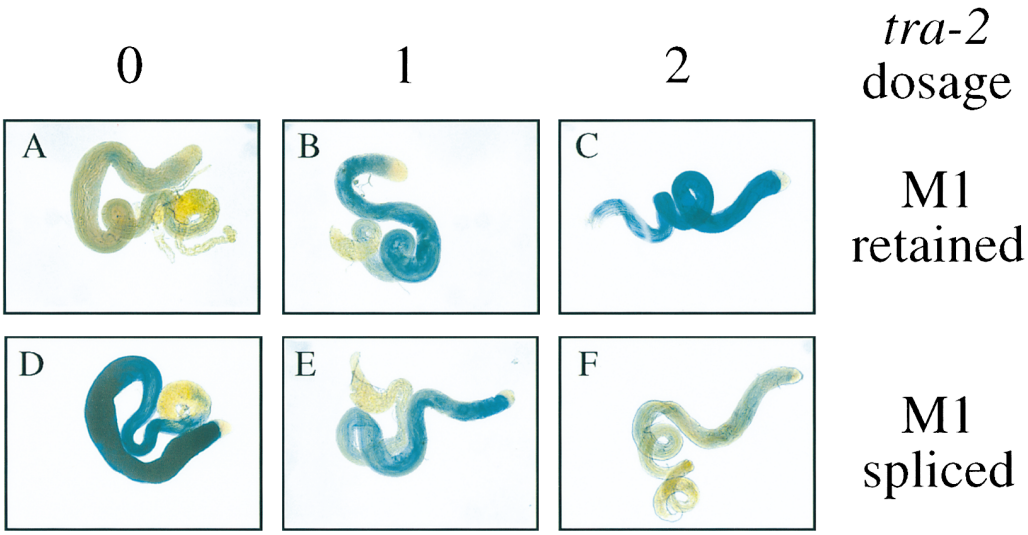

Figure 4.—Effects of changes in *tra-2*$^+$ gene dosage on expression of splicing reporter genes in the male germline. Testes from males carrying a single copy of either *P[CZP-ORF3]* (A–C) or *P[CZP-ORF1,2]* (D–F) that have been stained with X-gal under identical conditions are compared. The *tra-2* genotypes of the males were *tra-2*^{B}/*tra-2*^{B} (A and D), *tra-2*^{B}/*CyO*, *tra-2*^{B} (B and E), and *tra-2*$^+$/*CyO*, *tra-2*$^+$.

in varying degrees. Several of these lines produced numbers of progeny similar to that of *tra-2*^{B}/+ individuals. Seven out of eight of the insertions tested also provided *tra-2*$^+$ function in the soma sufficient to restore varying degrees of female somatic sexual differentiation to diplo-*X tra-2*^{B} homozygotes. Female differentiation was observed in a variety of cuticular structures including genitalia, sex combs, and the abdomen. Moreover, somatic rescue in four lines was sufficient to restore female fertility. The variation in the degree of rescue observed from line to line is probably due to differences in the position of *P* insertions and is similar to that obtained previously in experiments using *P* elements containing *tra-2*$^+$ (M. E. McGuffin and W. Mattox, unpublished data; Goralski *et al.* 1989; Mattox *et al.* 1996). We conclude from these observations that *P[ΔM1]* expresses functional products in both the male germline and the soma.

A single dose of *P[ΔM1]* increases the proportion of M1-containing RNAs produced by TRA-2 reporter genes in *trans*: If the *P[ΔM1]* transgene constitutively produces TRA-2^{226}, then it should have a greater effect on the proportion of M1-containing RNAs produced by a reporter than does *tra-2*$^+$. To test this, we introduced a single dose of *P[ΔM1]* into *tra-2*^{B}/*tra-2*^{B} males that simultaneously carry *P[CZP-ORF3]*. As expected, we ob-

TABLE 1

Rescue of *tra*-2 mutant phenotype by a single dose of *P[ΔM1]* transposon

P[ΔM1] insertion line	Chromosome carrying transgene	Sexual phenotype of XX; *tra-2*^{B}/*tra-2*^{B} individuals carrying a single copy of transgene	Single dose rescue of fertility in XY; *tra-2*^{B}/*tra-2*^{B} males[a]	
			No. fertile/No. tested	No. of progeny per fertile male[b]
3-1	*X*	Male	9/10	117 ± 10.0
4-2	*X*	Female	10/10	113 ± 6.3
6-1	*2*(*CyO*)	ND	ND	ND
6-3	*X*	Female	9/10	88 ± 7.5
20-1	*X*	Female-like intersex	10/10	121 ± 6.3
24-1	*2*	Female	7/10	122 ± 5.7
34-2	*3*	Female-like intersex	10/10	115 ± 7.9
34-3	*2*	Female	10/10	106 ± 5.7
41-7	*2*	Female-like intersex	8/10	46 ± 7.7
Controls				
tra-2^B/+		Female	8/8	102 ± 12.7
tra-2^B/*tra-2*^{B}		Male	0/10	0

ND, not determined.

[a] In these tests, progeny produced by individual males kept with 3 females in a single vial for 5 days were counted. In tests shown in Figures 6 and 7, males and females were transferred to fresh vials two times at 3-day intervals (3 vials total), and all progeny were counted.

[b] Values are mean ± standard error of the mean (SEM).

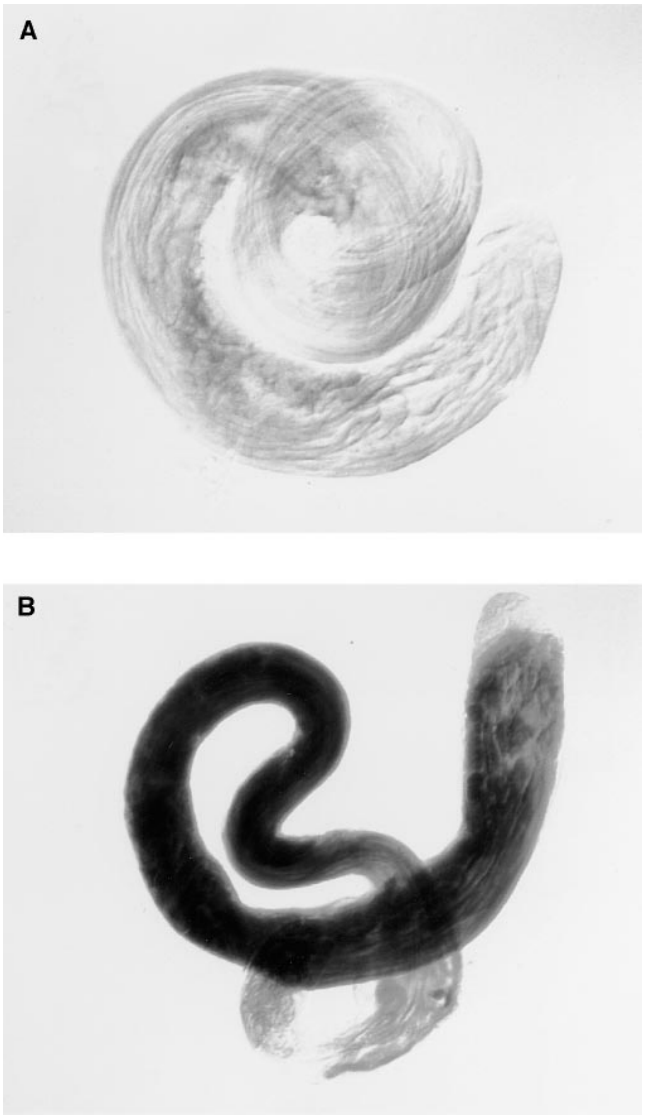

Figure 5.—Male germline expression of a TRA-2^{179} reporter gene is enhanced by the presence of a single dose of *P[ΔM1]*. DIC microscopy of testes from males carrying the reporter *P[CZP-ORF3]*, and a single dose of either *tra-2*$^{+}$ or *P[ΔM1]* are compared under identical X-gal staining conditions. The testes shown in A derive from a *tra-2*$^{+}$/*tra-2*^{B}; *P[CZP-ORF3]*/+ male and that in B from a *tra-2*^{B}, *P[ΔM1, 41-7]*/*tra-2*^{B}; *P[CZP-ORF3]*/+ male. It should be noted that these samples were stained for a shorter period of time than those shown in Figure 4.

served that X-gal staining of *P[CZP-ORF3]* testes was greater in these males than when a single dose of *tra-2*$^{+}$ was present, indicating that a larger proportion of reporter RNA retains the M1 intron (see Figure 5). These results provide functional evidence that excess TRA-2^{226} is produced by *P[ΔM1]*.

Males with multiple doses of *P[ΔM1]* have reduced fertility: To test whether *P[ΔM1]* interfered with male fertility, we examined *tra-2*^{B}/+ individuals carrying one or more doses of *P[ΔM1]*. As mentioned above, preliminary analysis indicated that males carrying a single dose of any of the transgene insertions are fertile. To determine if fertility is subtly effected in such males, the number of progeny produced by transgene-bearing *tra-2*^{B}/+ males from several independent lines was determined (see Figure 6). Males bearing a single dose of *P[ΔM1]* produced progeny in numbers that did not differ significantly from control males carrying a single copy of the *P[tra-2*$^{+}$*]* transgene. These data indicate that a single dose of *P[ΔM1]* does not substantially affect male fertility.

In contrast, *tra-2*^{B}/+ males carrying *two* doses of *P[ΔM1]* showed a quantitative reduction in male fertility. In Figure 6, males carrying any of four different combinations of two *P[ΔM1]* insertions are compared to control males carrying two copies of *P[tra-2*$^{+}$*]*. It is worth noting that in these controls the number of progeny produced per male was *elevated* relative to that observed in the above experiment with flies carrying a single dose of the *P[tra-2*$^{+}$*]*. Since these experiments were done in parallel, this indicates that fertility is improved by additional doses of *tra-2*$^{+}$.

Relative to these controls, males with any of four different combinations of *P[ΔM1]* insertions produced fewer progeny. Although the reductions in progeny observed were in each genotype statistically significant ($P < 0.001$ for *each* of the four *P[ΔM1]* combinations examined relative to either *P[tra-2*$^{+}$*]* combination), the magnitude of this reduction varied considerably depending on the particular insertions present. In one case (*P[ΔM1, 34-3]; P[ΔM1, 34-2]*) males produced progeny in numbers that were only slightly less than that of males with a single dose of either transgene alone. On the other hand, in two of the combinations of insertions tested, males produced <30% as many progeny as did controls. In one of these genotypes three of the twelve males tested were completely sterile. Taken together these results demonstrate a significant reduction in the level of male fertility when two doses of *P[ΔM1]* are present.

If the effects on fertility observed with two doses of *P[ΔM1]* are due to excess tra-2^{226}, then we would expect further increases in the dosage of *P[ΔM1]* to result in still lower levels of fertility. Therefore, we generated lines of flies carrying combinations of two insertions and crossed these to produce individuals with three or four doses of *P[ΔM1]* or *P[tra-2*$^{+}$*]*. As shown in Figure 7A, males carrying three doses of *P[ΔM1]* produced reduced numbers of progeny. Parallel comparisons of individuals carrying either two or three doses of similar insertions revealed that males with three doses of *P[ΔM1]* were slightly less fertile than those with two doses (data not shown).

Dramatic effects were observed when dosage of *P[ΔM1]* was increased to four copies. Males of this type were found to be uniformly sterile in each of several combinations examined (Figure 7B and data not shown). In contrast, males carrying four doses of *P[tra-*

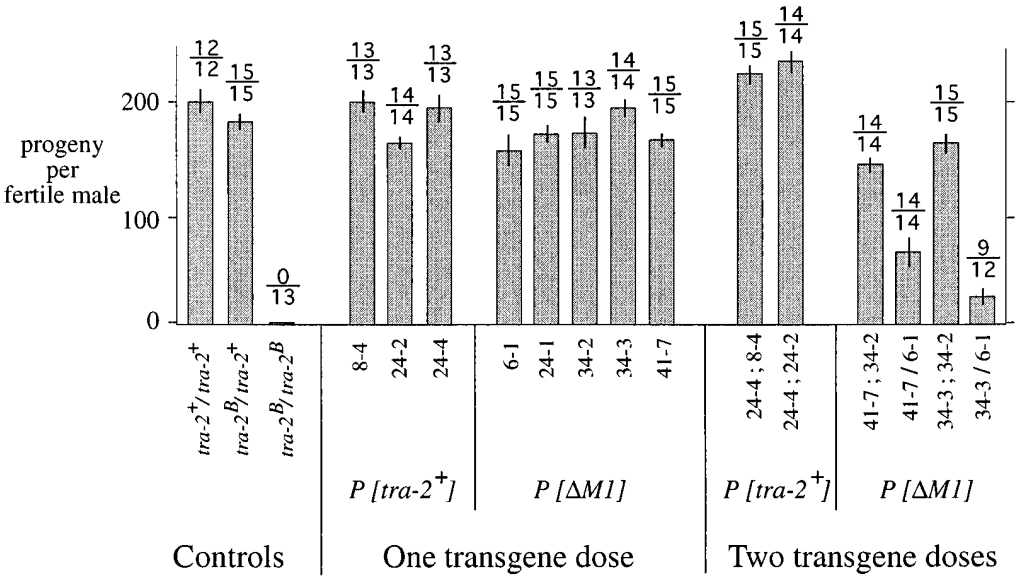

Figure 6.—Effect of *P[ΔM1]* on fertility of *tra-2*^{B}/*tra-2*$^{+}$ males. One experiment is shown testing the effects of one or two doses of *P[tra-2*$^{+}$*]* and of *P[ΔM1]* transgenes on male fertility. Bars represent the numbers of progeny produced per fertile male for controls and for *w*^{1118}/*B*^{s}*Y*; *tra-2*^{B}/*tra-2*$^{+}$, *CyO* adults with one or two transgene insertions. Below each bar is indicated the genotypes or numerical designation of each transgene insertion corresponding to those in Table 1. Standard error of the mean is indicated by the line on each bar. Numbers above each bar indicate the number of fertile males/total number of males tested for each genotype.

2^{+}*]* had nearly wild-type levels of fertility. These results strongly indicate that unregulated overexpression of *P[ΔM1]* leads to reduced male fertility.

Expression of *tra-2* products from multiple doses of *P[ΔM1]* causes a defect in spermatogenesis: Male sterility in *P[ΔM1]* males could result from either abnormal somatic sexual differentiation or a defect in spermatogenesis itself. To address the possibility that somatic structures in *XY* flies carrying four doses of this transgene are sexually transformed, we examined these flies for signs of female transformation. These individuals are clearly male in appearance. Close examination of a variety of sexually differentiated tissues (*i.e.*, gonads, sex combs, genitalia, tergite pigmentation) revealed that they were morphologically indistinguishable from those of wild-type males (data not shown). We therefore examined differentiating germ cells within the testes of these males to determine if *P[ΔM1]* affected spermatogenesis. Examination of live testes preparations revealed no morphological abnormalities in gonial cells, spermatocytes, meiotic stages or mature spermatids, all of which were observed in roughly normal numbers. Unlike *tra-2* loss-

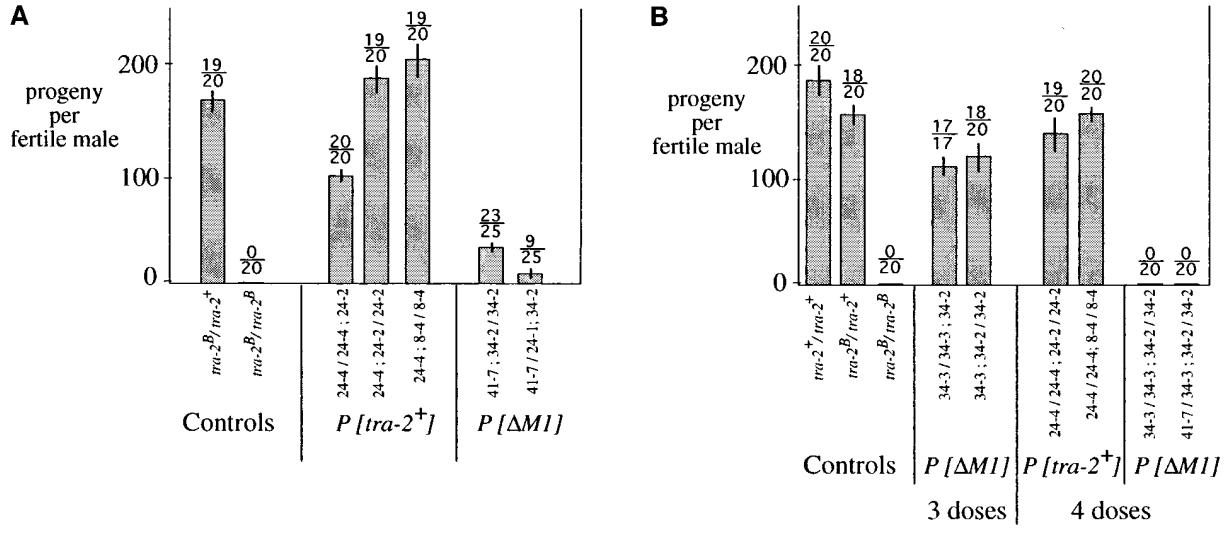

Figure 7.—Effect of multiple doses of *P[ΔM1]* on male fertility. Effects of three and four doses of *P[tra-2*$^{+}$*]* and *P[ΔM1]* on male fertility are shown. Bars indicate number of progeny per fertile male and lines the standard error. Number of fertile males/total number tested are given above each bar. Trangene insertions used to produce three- and four-dose males are indicated below each bar. Two experiments are shown. In the first, (A) males with three doses of each transgene are compared. In the the second, (B) males with three and four doses of *P[ΔM1]* are tested.

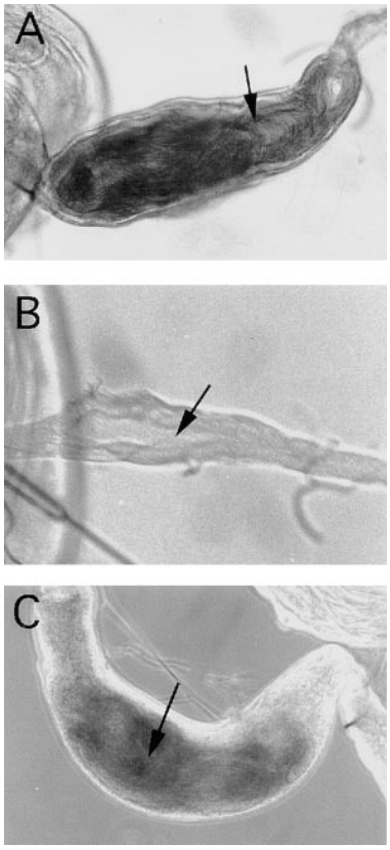

Figure 8.—Mature sperm capable of migrating to the seminal vesicle fail to form in four-dose *P[ΔM1]* males. Motile sperm move into the lumen of the seminal vesicle (arrow) of w^{1118} males (A) but not into that of $w^{1118}/B^{s}Y$; *P[ΔM1, 41-7], tra-2*^{B}*/P[ΔM1,34-3], tra-2*^{B}P[ΔM1, 34-2]/P[ΔM1, 34-2] males (B). The failure of the latter sperm to migrate into the seminal vesicle is not simply due to homozygosity for the *tra-2*^{B} mutation since *P[ΔM1,34-3], tra-2*^{B}*/P[ΔM1, 34-3], tra-2*^{B} males accumulate many sperm in their seminal vesicles (C).

of-function mutants, which form spermatids with unelongated nuclei, the nuclear morphology of mature spermatids present in four-dose *P[ΔM1]* males was normal. However, it was observed that the bundles of mature spermatids, which are normally tightly aligned, were sometimes disarrayed in four-dose *P[ΔM1]* males. More dramatically, the coiled mature sperm that formed at the base of the testis in these males failed to migrate to the seminal vesicle, indicating that they were amotile (Figure 8, A–C). Moreover, sperm were never observed in the spermatheca or seminal receptacles of females that had copulated with four-dose *P[ΔM1]* males (data not shown). These results suggest that sterility in these males results from the failure to form normal motile sperm.

DISCUSSION

Negative feedback mechanisms are important for a wide variety of biological processes, but only a few cases where feedback regulation occurs at the level of alternative pre-mRNA splicing have yet been described. Here we have examined the functional importance of a negative feedback mechanism affecting the ratio of alternative *tra-2* mRNAs produced in the male germline of Drosophila. Our data support a model in which feedback control quantitatively limits the amount of mRNA expressed that encodes a functional TRA-2 isoform, TRA-2^{226}. Increases or decreases in the overall level of *tra-2* transcription caused compensatory changes in the ratio of the alternative mRNAs. For example, increased *tra-2* gene dosage produced proportionally higher levels of mRNA encoding the nonfunctional TRA-2^{179} isoform and proportionally less mRNA encoding the functional isoform TRA-2^{226}. Analysis of the response of TRA-2/β-galactosidase fusion genes to increased dosage confirms that increased gene dosage affects the relative amounts of translated product generated by TRA-2^{179} and TRA-2^{226} mRNA in accord with the shift in the relative levels of these transcripts. These results support the model proposed in Figure 1 wherein expression of TRA-2^{226} above a certain basal level leads to a proportional increase in repression of M1 splicing and the accumulation of mRNAs encoding TRA-2^{179}.

Although some of the above dosage studies employed flies with up to four doses of *tra-2*$^{+}$, it should be noted that negative feedback regulation is not a mere oddity of flies with unnaturally high levels of *tra-2* expression. Rather, this phenomenon is also observed in wild-type male flies where approximately 60% of germline-specific *tra-2* mRNAs contain the M1 intron (Amrein *et al.* 1990; Mattox and Baker 1991; Mattox *et al.* 1990). The level of TRA-2^{226} mRNA expressed normally must therefore be limited by M1 repression. M1 repression observed in such males is entirely dependent on the presence of the TRA-2^{226} protein since males homozygous for point mutations affecting this isoform accumulate only fully spliced *tra-2* mRNAs (Mattox and Baker 1991).

We previously hypothesized that this feedback mechanism functions to prevent expression of TRA-2^{226} at levels that are deleterious to male fertility. We have tested this idea in this study by generating transgenic males carrying *P[ΔM1]*, a transgene from which the M1 intron had been deleted. Because the intron is missing, production of TRA-2^{226} mRNA in this case cannot be limited by M1 repression. Our results show that the unregulated transgene was clearly deleterious to male fertility in a dose-dependent manner. Males carrying four doses of the *P[ΔM1]* transgene were most severely affected, being uniformly sterile. Significant effects on fertility were also observed in males carrying only two or three doses of the transgene. Such males produced progeny, but in numbers that were significantly reduced relative to control males carrying a wild-type transgene. The observation that the level of fertility observed is dependent on the dose of *P[ΔM1]* is consistent with the idea that

overexpression of TRA-2^{226} is deleterious to spermatogenesis.

Since TRA-2^{226} mRNA normally accounts for only about 40% of the total amount of poly A$^+$ *tra-2* mRNA that accumulates in the male germline we anticipated that down regulation of the endogenous *tra-2*$^+$ genes would compensate for most if not all overexpression observed in flies with a single dose of the transgene. Consistent with this idea we found that such males had near normal fertility and produced significantly increased levels of M1-containing mRNA expressed from a reporter gene. These observations agree well with our model and further support the notion that the level of TRA-2^{226} mRNA produced is limited by negative feedback.

While it is apparent from these observations that unregulated expression of TRA-2^{226} reduces male fertility, the primary defect in spermatogenesis remains to be elucidated. Examination of the distribution of epitope-tagged TRA-2^{226} protein expressed within primary spermatocytes of flies carrying two doses of *P[ΔM1]* revealed that it is localized, as usual, to discrete sites in the nucleus (B. Dauwalder and W. Mattox, unpublished results). Thus, reduced fertility in such flies is probably not attributable to mislocalized TRA-2^{226} protein. Morphological examination of the testes from sterile males carrying four doses of the *P[ΔM1]* transgene revealed that although they produce mature spermatids without gross abnormalities, motile sperm are not produced in significant number. While this phenotype suggests that spermatogenesis is disrupted at a late stage, it may also result from secondary consequences of an earlier primary defect. For instance, it is known that null mutations in *tra-2* manifest late defects in spermatid nuclear elongation although experimental data using temperature-sensitive *tra-2* alleles indicate that the critical period for *tra-2* function is earlier during a short, well-defined interval near the late primary spermatocyte/meiosis/early spermatid stages (Belote and Baker 1983). Consistent with this, we have observed that epitope-tagged TRA-2^{226} protein is expressed in late primary spermatocytes but rapidly disappears as cells enter the meiotic G2/M transition and does not reappear at later stages (B. Dauwalder and W. Mattox, unpublished results). Given that the TRA-2 protein normally functions during stages preceding meiosis in a process that manifests itself phenotypically at later stages in spermatogenesis it seems possible that the phenotype observed here also results from a subtle primary defect at an earlier stage.

In addition to the data presented here, the functional significance of feedback regulation of TRA-2^{226} expression is suggested by the observation that it is conserved in distantly-related Drosophilids. Recently, a homologue of *tra-2* from *Drosophila virilis*, a species that diverged from *Drosophila melanogaster* over 60 million years ago, was described (Chandler *et al.* 1997). The *D. virilis tra-2* gene also produces multiple isoforms through alternative splicing. Interestingly, alternative mRNAs encoding isoforms analogous to TRA-2^{226} and TRA-2^{179} are produced in the male germline of this species as well. Introduction of the the *D. virilis* TRA-2 gene into the *D. melanogaster* genome resulted in appropriate, interspecific feedback regulation of *D. virilis tra-2* RNA by the *D. melanogaster* TRA-2 protein indicating that a similar mechanism is utilized in the *D. virilis* germline.

A number of other genes encoding RNA-binding proteins are thought to autoregulate their expression by affecting the processing of their own pre-mRNAs (Boelens *et al.* 1993; Chabot *et al.* 1997; Jumaa and Nielsen 1997). One important example of this, also from Drosophila, is the *Sex-lethal* protein that functions in a positive feedback mechanism that affects the sex-specific skipping/inclusion of an exon containing a stop codon that interrupts the *Sex-lethal* open reading frame (Bell *et al.* 1991). Feedback in this case plays a significant role in development, since it is needed to maintain the female-specific expression of *Sex-lethal* protein that is essential for normal dosage compensation and sex determination.

Other functionally significant feedback mechanisms have been reported for both the yeast ribosomal protein L32 (Li *et al.* 1996) and the Drosophila *suppressor-of-white-apricot* protein (SWAP) genes (Zachar *et al.* 1987, 1994). Interestingly, it has recently been shown that elements of the negative feedback mechanism used in SWAP are conserved in the human and *Caenorhabditis elegans* homologues of this gene (Spikes *et al.* 1994; Sarkissian *et al.* 1996). Given that mammalian homologues of *tra-2* have now been identified (Matsuo *et al.* 1995; Dauwalder *et al.* 1996; Segade *et al.* 1996), it will be interesting to determine if the expression of functional proteins from these genes is subject to tissue-specific negative feedback regulation using a strategy similar to that of Drosophila.

We thank Steve Marcus and Mitzi Kuroda for their helpful comments on this manuscript. D.C. was supported by a National Institutes of Health (NIH) training grant (CA09299). This work was supported by an NIH First Award (GM50825) to W.M. W.M. is a Pew Biomedical Scholar.

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Communicating editor: R. S. Hawley