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Evolutionary Conservation of Regulatory Strategies for the Sex Determination Factor *transformer-2*

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Sex determination in *Drosophila melanogaster* is regulated by a cascade of splicing factors which direct the sex-specific expression of gene products needed for male and female differentiation. The splicing factor TRA-2 affects sex-specific splicing of multiple pre-mRNAs involved in sexual differentiation. The *tra-2* gene itself expresses a complex set of mRNAs generated through alternative processing that collectively encode three distinct protein isoforms. The expression of these isoforms differs in the soma and germ line. In the male germ line the ratio of two isoforms present is governed by autoregulation of splicing. However, the functional significance of multiple TRA-2 isoforms has remained uncertain. Here we have examined whether the structure, function, and regulation of *tra-2* are conserved in *Drosophila virilis*, a species diverged from *D. melanogaster* by over 60 million years. We find that the *D. virilis* homolog of *tra-2* produces alternatively spliced RNAs encoding a set of protein isoforms analogous to those found in *D. melanogaster*. When introduced into the genome of *D. melanogaster*, this homolog can functionally replace the endogenous *tra-2* gene for both normal female sexual differentiation and spermatogenesis. Examination of alternative mRNAs produced in *D. virilis* testes suggests that germ line-specific autoregulation of *tra-2* function is accomplished by a strategy similar to that used in *D. melanogaster*. The similarity in structure and function of the *tra-2* genes in these divergent *Drosophila* species supports the idea that sexual differentiation in *D. melanogaster* and *D. virilis* is accomplished under the control of similar regulatory pathways.

Metazoan sexual differentiation is controlled by complex regulatory pathways that govern the developmentally coordinated expression of large groups of male- and female-specific gene products (26, 47). A central component of the regulatory hierarchy controlling somatic sexual differentiation in *Drosophila melanogaster* is a cascade of splicing factors which each function to regulate sex-specific alternative processing of RNAs from genes immediately downstream to them in the hierarchy (6, 39). This cascade of splicing factors ultimately controls the selection of alternative 3′ splice sites in pre-mRNA from the *doublesex* (*dsx*) gene (5, 10). The alternative *dsx* mRNAs produced encode transcription factors with opposite activities regulating the expression of male- and female-specific sexual differentiation genes (4, 11, 14).

The *transformer-2* (*tra-2*) gene plays a key role in this regulatory cascade. TRA-2 proteins function in combination with the female-specific *transformer* protein (TRA) to direct female-specific *dsx* splicing (37, 39). In the absence of either of these factors, *dsx* pre-mRNA is spliced by using the male-specific 3′ splice site, which appears to be the default choice of the general splicing machinery. To facilitate female-specific splicing, TRA-2 binds directly to *dsx* RNA by recognizing both a series of 13-nucleotide (nt) repeated elements and a single purine-rich element located in the *dsx* female-specific exon (24, 30). TRA facilitates binding of TRA-2 to these sequences, which further enables the *dsx* pre-mRNA to interact with other SR family splicing factors (31, 52, 53). This regulatory complex facilitates assembly of spliceosomal complexes at the branch-point-polypyrimidine tract region adjoining the female-specific 3′ splice site (13).

In addition to its effect on *dsx* splicing, TRA-2 also affects processing of mRNAs from other genes involved in sexual differentiation. In the nervous system of the fly, TRA and TRA-2 alter splicing of RNA from *fruitless* (46), a gene affecting sex-specific aspects of both mating behavior and muscle differentiation (19, 20, 51). In the germ line, TRA-2 is required for normal spermatogenesis and affects sex-specific processing of pre-mRNA from *exuperantia* (*exu*), *alternative testis transcripts* (*att*), and *tra-2* itself (7, 15, 23, 34). Thus, TRA-2 plays multiple roles in sexual differentiation, promoting both the specification of female sexual identity in the soma and the completion of spermatogenesis in the male germ line.

RNAs encoding three distinct isoforms of TRA-2 (TRA-2^{264}, TRA-2^{226}, and TRA-2^{179}) are expressed during development (2, 36). Functional studies on these isoforms (1, 35) indicate that TRA-2^{264} and TRA-2^{226} redundantly regulate alternative splicing of *dsx* pre-mRNA in the soma. In the male germ line both TRA-2^{226} and TRA-2^{179} are expressed, but only the former is necessary and sufficient for male fertility. TRA-2^{179}, on the other hand, appears to be nonfunctional. Autoregulation of *tra-2* pre-mRNA processing in male germ cells governs the relative expression of these two isoforms. Splicing of an intron that splits the initiation codon for TRA-2^{226} is repressed by TRA-2^{226} itself. Retention of the intron results in the formation of an mRNA encoding the nonfunctional TRA-2^{179} isoform. It has been hypothesized that autoregulation serves as a negative feedback mechanism that limits the amount of functional TRA-2 isoform (TRA-2^{226}) expressed in the male germ line (35), but it is not known whether feedback regulation is itself required for male fertility.

Given that TRA-2 has multiple regulatory functions, it

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seems logical that these isoforms exist to perform specialized roles. However, genetic analysis of transgenic strains expressing individual isoforms suggests that the production of multiple isoforms is unnecessary. For example, strains expressing only TRA-2^{226} under normal developmental control grow vigorously and show no apparent defects in somatic sex or male fertility (35). Moreover splicing of *dsx*, *exu*, and *tra-2* mRNAs is normal in these strains.

To determine what features of the *tra-2* gene are conserved during evolution, we have examined whether a *tra-2* homolog with similar functional capabilities exists in *Drosophila virilis*, a species separated from *D. melanogaster* by over 60 million years. We find that as with other members of the sex determination splicing cascade from *D. melanogaster* (9, 25, 40), the *tra-2* gene is also conserved in *D. virilis*. Moreover, the homolog of TRA-2 present in this species has a gene architecture similar to that observed in *D. melanogaster* and produces a similar set of protein isoforms. Our data suggest that both somatic and germ line TRA-2 functions, including autoregulatory strategies, have been conserved between these distantly related *Drosophila* species.

MATERIALS AND METHODS

Isolation of *D. virilis* phage clones. Approximately 10^5 phage from a *D. virilis* λEMBL3A genomic library (kindly provided by P. Macdonald) were screened by using a ^{32}P-labeled *tra-2* cDNA clone that includes the complete protein-coding region (36). For low-stringency hybridizations, filters were prehybridized in 30% formamide–1 M NaCl–0.1 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.8)–0.2% sodium dodecyl sulfate–0.2% Ficoll–0.2% bovine serum albumin–0.2% polyvinylpyrrolidone–100 μg of denatured salmon sperm DNA per ml for 3 h at 42°C. Denatured probe DNA was then added to the mixture at a concentration of 5×10^5 dpm/ml, and the filters were allowed to incubate for 24 h. The filters were then washed four times in 0.15 M sodium chloride–0.015 M sodium citrate at 42°C. For high-stringency hybridizations similar conditions were used, except that prehybridization and hybridization were in 50% formamide and washes were performed in 0.015 M sodium chloride–0.0015 M sodium citrate at 50°C.

In situ hybridization to *D. virilis* polytene chromosomes. *D. virilis* polytene chromosome squashes were prepared from the salivary glands of wild-type larvae (Pasadena-lethal free strain) and hybridized with biotinylated λV4 DNA (3).

Northern blot hybridization of *Drosophila* RNAs. Two micrograms of oligo(dT)-cellulose-selected RNA per lane was resolved on agarose-formaldehyde gels and transferred to nylon membranes for hybridization (45) under the high-stringency conditions given above.

Isolation and sequence analysis of RT-PCR products. The structures of *D. virilis* mRNAs were ascertained by using several combinations of oligonucleotide primers that were designed based on the *D. virilis* genomic sequence and alignments with sequences from *D. melanogaster*. The organization of 5′ untranslated region sequences in *D. virilis* TRA-2 was initially determined by using products obtained with a 5′ RACE (rapid amplification of cDNA ends) kit (Gibco-BRL) for the amplification of RNA 5′ ends. This identified the positions of exons 1 and 2, allowing the subsequent design of primers for reverse transcription-PCR (RT-PCR) experiments and of probes for RNase protection assays.

For RT-PCR, first-strand cDNA synthesis was performed with male and female *D. virilis* poly(A)$^+$ RNA by using the SuperScript Preamplification System (catalog no. 18089-011; Gibco-BRL). Poly(A)$^+$ RNA (0.8 μg) and oligo(dT)$_{12–18}$ (New England Biolabs) (0.5 μg) were denatured at 70°C for 10 min in a volume of 12 μl and incubated on ice for 1 min. The subsequent annealing, cDNA synthesis, reaction termination, and removal of RNA were done according to the protocol provided with the kit. Seven microliters of the first-strand cDNA was amplified directly by using *Taq* polymerase and the primers L (5′CATTTACA TCGCATTGATAAGC3′), S (5′GTTTTTCAAGCGAGCAGGTTCT3′), and VX7 (5′GGTGATACTGACGACGATTC3′). PCRs were carried out in 100 μl by incubating the cDNA and primers in a buffer containing 1.5 mM $MgCl_2$ at 94°C for 5 min and then cycling 20 times at 94°C for 1 min, 58°C for 30 s, and 72°C for 30 s, followed by a final incubation at 72°C for 5 min. PCR products were separated and purified on a 1.5% agarose gel and then ligated directly into the plasmid PCR II (Invitrogen) for DNA sequencing.

Quantitative male fertility test. To determine the relative number of progeny generated by transgenic and control flies, males were placed individually into vials along with three virgin females of the genotype *y* w^{67c23}. To maintain a low culture density, all flies were transferred to fresh vials at 3-day intervals. All adult progeny obtained from three vials corresponding to each male were counted. Flies were maintained at 25°C on cornmeal-molasses-containing medium.

Preparation and analysis of *D. virilis* gonad RNA. The testes of 50 male flies and ovaries of 50 female flies were dissected in 0.7% NaCl. The testes, ovaries, and respective fly carcasses were homogenized with a Tekmar Tissuemizer in 2 ml of phenol combined with 2 ml of 2× NETS (200 mM NaCl, 20 mM EDTA, 20 mM Tris, pH 8.0). Phases were separated by centrifugation, and the aqueous phase was collected. After an additional phenol extraction, the RNA was precipitated with 2 volumes of ethanol. The RNA was resuspended in RNase-free water and quantified by absorbance. After treatment with DNase at 37°C for 1 h, samples were heat inactivated for 10 min at 70°C, phenol extracted, and precipitated with 2 volumes of ethanol. RT-PCR was performed with 10 μg of each RNA under the conditions described above. *D. virilis tra-2* transcripts were detected after blotting by using a random hexamer-labeled 3.4-kb genomic DNA fragment encompassing all *D. virilis tra-2* coding sequences.

RNase protection assays. *Drosophila* poly(A)$^+$ RNA (3 to 4 μg) was coprecipitated with 80,000 cpm of a gel-isolated antisense, ^{32}P-labeled RNA probe. The subsequent assay was carried out as outlined for RPA II (Ambion). Protected RNA fragments were electrophoresed on 5% polyacrylamide–8 M urea gels.

Nucleotide sequence accession number. The genomic sequence for the *D. virilis tra-2* gene is available from GenBank under accession no. U72682.

RESULTS

Isolation of a homolog of *transformer-2* from *D. virilis*. To determine if a gene similar to *tra-2* is present in *D. virilis*, we screened a λEMBL3A phage genomic library at both high and low stringencies by using a single-stranded DNA probe derived from a nearly full-length *tra-2* cDNA clone (see Materials and Methods for details). Of 10^5 phages tested, four (λV3, -4, -5, and -7) hybridized at low stringency, while no phage hybridized under high-stringency conditions. Restriction mapping of the phage inserts revealed that λV4, λV5, and λV7 form an overlapping set of clones (Fig. 1C) and that the insert of λV3 did not fall into this set (not shown). To confirm that these clones contain DNA that cross-hybridizes with *tra-2*, *Eco*RI digests of each clone were compared to a similar digest of *D. virilis* genomic DNA on a Southern blot that was hybridized with a *tra-2* cDNA at low stringency (Fig. 1A). The most prominent *D. virilis* genomic fragment detected was 3.4 kb in length and comigrated exactly with the cross-hybridizing fragments in both λV4 and λV7. This suggests that DNA in these clones corresponds to the region of the *D. virilis* genome most strongly cross-hybridizing with *tra-2*. The hybridizing region corresponds to a terminal *Eco*RI fragment in the λV5 restriction map, which is considerably larger and also hybridized strongly.

DNA from λV3 failed to hybridize detectably in the experiment described above. However, a 1.2-kb *Eco*RI fragment from this clone specifically hybridized with *tra-2* cDNA when larger amounts of λV3 DNA were blotted (not shown). Further blot hybridization experiments indicated that the weakly cross-hybridizing region of λV3 DNA could be confined to a sequence of less than 600 nt. Complete sequencing revealed some short regions of similarity to *tra-2* at the DNA level that when conceptually translated did not produce protein sequences with significant similarity to any part of the *D. melanogaster* TRA-2 protein (data not shown). Therefore, this clone was not analyzed further.

To test whether the remaining clones arose from a region of the *D. virilis* genome that is syntenic with the 51B region of chromosome 2R, where *tra-2* resides in the *D. melanogaster* genome, we performed in situ hybridization to *D. virilis* polytene chromosomes by using DNA from λV7. Hybridization was localized to region 56D on chromosome 5 (Fig. 1B). This chromosome is partially homologous to *D. melanogaster* chromosome arm 2R (22). Notably, another gene deriving from the 51B region of *D. melanogaster* has been shown to hybridize to *D. virilis* chromosome 5 in the adjacent region 56C (55). Thus, the λV4, λV5, and λV7 phage clones are derived from a region of the *D. virilis* genome syntenic with the 51B region of *D. melanogaster*. These results suggest that these phages contain a

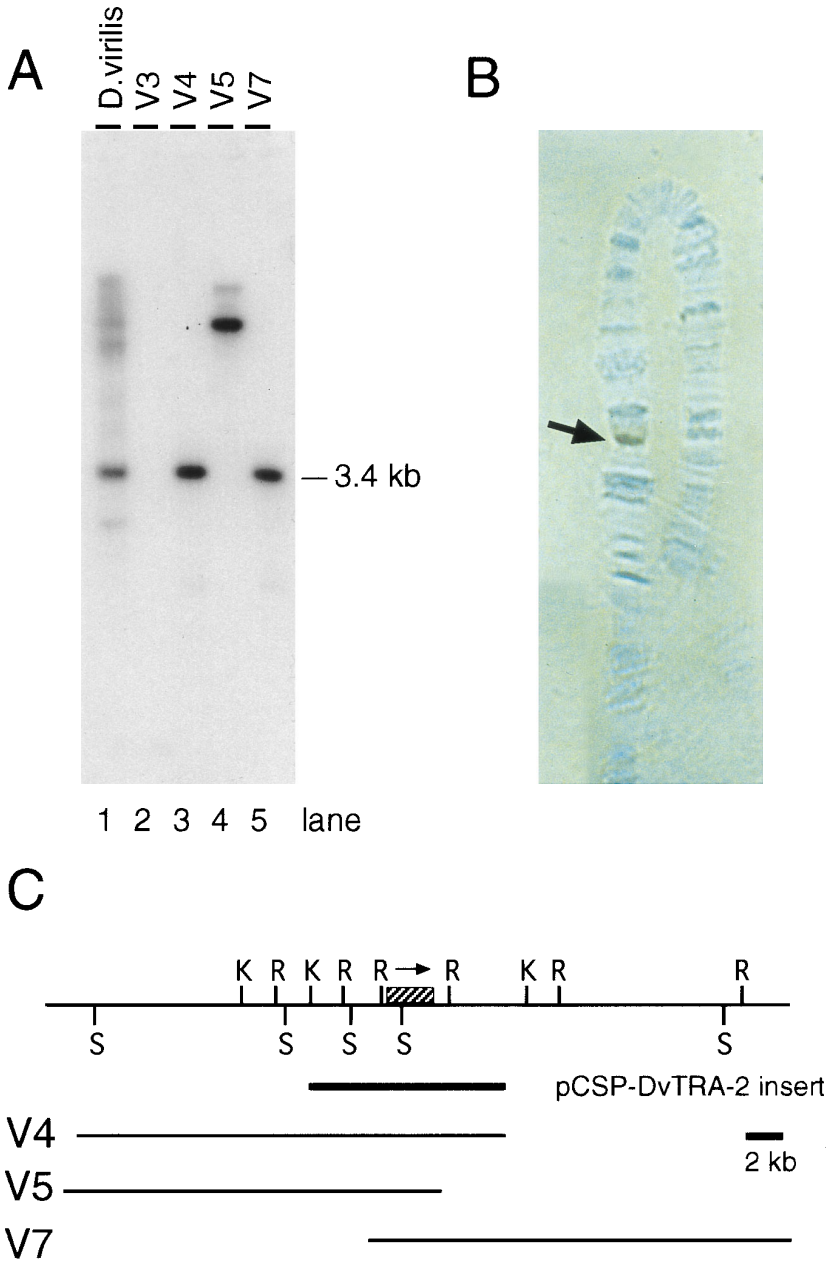


FIG. 1. Isolation and analysis of phage clones containing Dv*tra-2*. (A) Southern blot of 2 μg of *D. virilis* genomic DNA (lane 1) and 0.5 ng of DNA from each of four phage clones (lanes 2 to 5) cut with *Eco*RI. The blot was hybridized with a ^{32}P-labeled *tra-2* cDNA clone isolated from *D. melanogaster*. (B) In situ hybridization of *D. virilis* polytene chromosomes with λV7 DNA. The signal is localized to the 56D region of chromosome 5. The contrast in this image was adjusted by using Adobe Photoshop. (C) Map of the genomic region including the Dv*tra-2* gene (hatched box). The extents of DNA sequences in each of three overlapping phage clone inserts and in pCSPDvTRA2 are shown. Restriction sites for *Sal*I (S), *Eco*RI (R), and *Kpn*I (K) are shown. The arrow indicates the orientation of transcription for Dv*tra-2*.

homolog of the *tra-2* gene. For clarity we hereafter refer to this gene as Dv*tra-2* and the *D. melanogaster* gene as Dm*tra-2*.

Dv*tra-2* produces alternatively spliced mRNAs encoding protein isoforms like those found in *D. melanogaster*. We next examined whether Dv*tra-2* encodes proteins similar to those encoded by Dm*tra-2* by sequencing DNA from the cross-hybridizing restriction fragments. Sequencing of a 2.7-kb region from the V7 genomic clone revealed extensive similarities to Dm*tra-2*. When conceptually translated, several open reading frames similar to segments of the TRA-2 protein were detected. Interruptions in these reading frames frequently corresponded to positions where introns disrupt coding sequences in Dm*tra-2*, suggesting that the two genes are organized similarly (Fig. 2A).

To determine the splicing patterns of Dv*tra-2* mRNAs, we performed low-cycle-number RT-PCR with sense primers from putative exons 2 and 3 in combination with an antisense primer from exon 7. With each primer combination several different products were obtained, suggesting that Dv*tra-2* pre-mRNAs are alternatively processed. A Southern blot of these products, hybridized with the entire 3.4-kb *Eco*RI fragment encompassing the Dv*tra-2* gene, is shown in Fig. 3B. The major

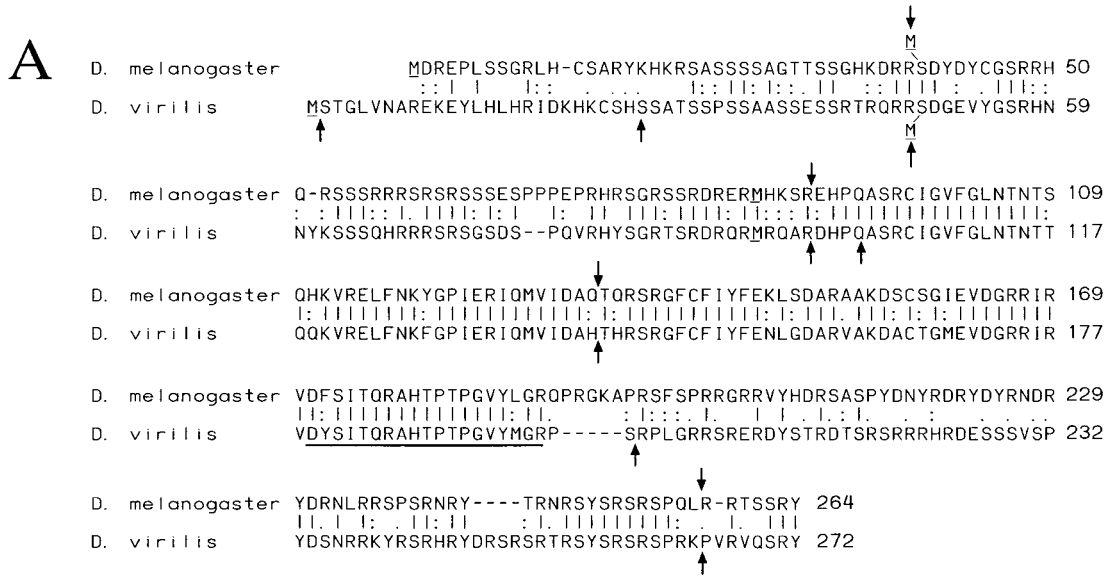
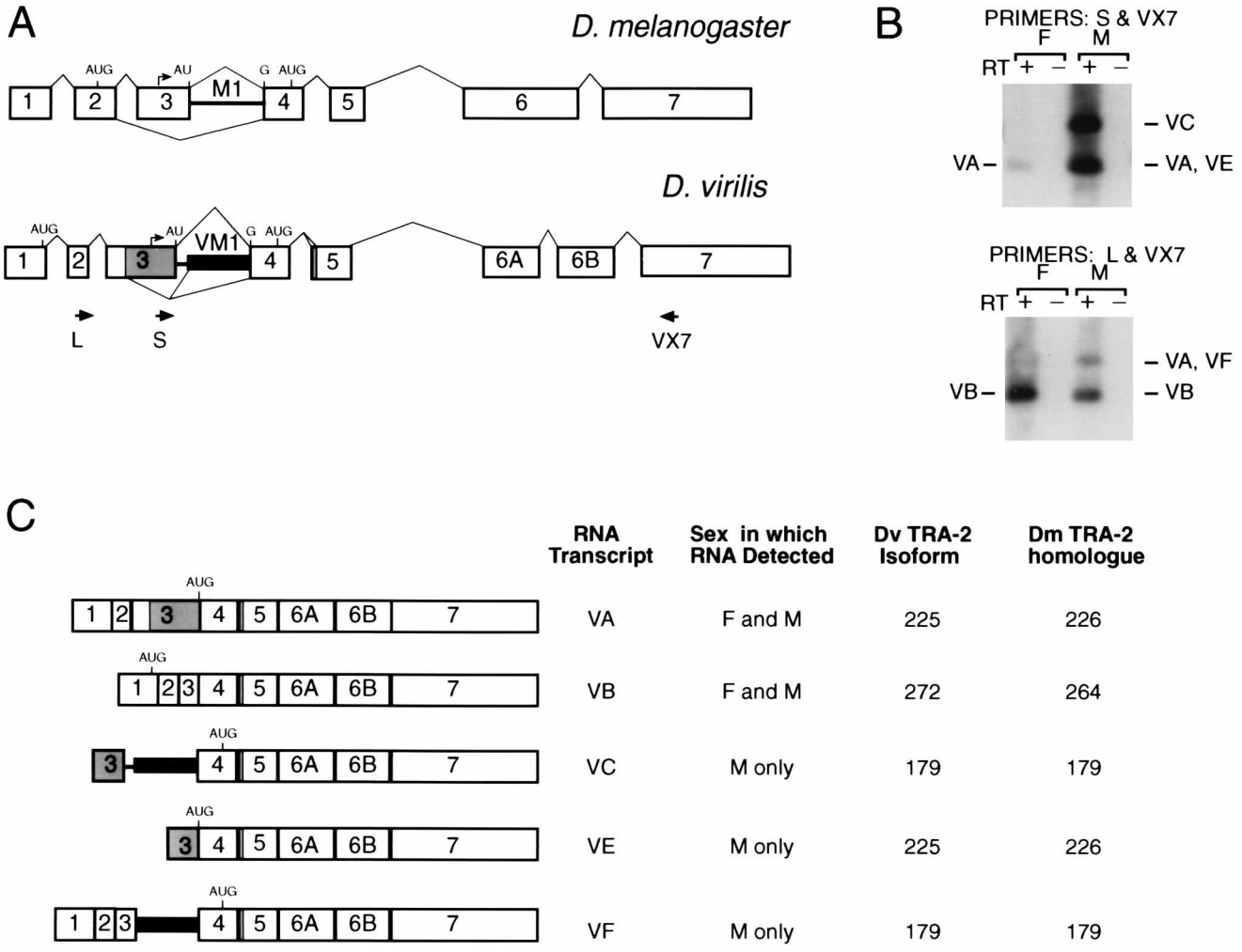


FIG. 2. Comparison of protein sequences from the Dv*tra-2* and Dm*tra-2* genes. (A) Alignment of the DmTRA-2^{264} and DvTRA-2^{272} proteins. Other isoforms differ only by amino-terminal truncation. The initiation codons used by each isoform are underlined, as is the 19-amino-acid conserved linker region. Arrows indicate the positions of splice junctions and show the alignment of several such junctions in the DvTRA-2 and DmTRA-2 protein sequences. For assignment of sequence similarities and compositions, the RS1 domains are defined as amino acids 21 to 85 (DmTRA-2^{264}) and 25 to 93 (DvTRA-2^{272}), and the RS2 domains are defined as amino acids 197 to 263 and 199 to 271. Sequences matching the RRM consensus extend from amino acids 99 to 170 in DmTRA-2^{264} and 107 to 178 in DvTRA-2^{272}. (B) Comparison of the arginine and serine compositions (by frequency) of RS1 and RS2 in DvTRA-2 and DmTRA-2.

products from these reactions were isolated and sequenced, and their splicing patterns were deduced by comparison with the genomic sequence (Fig. 3A and C). This analysis combined with RNase protection experiments with *D. virilis* RNA (see below) identified five alternatively spliced mRNAs (designated VA, VB, VC, VE, and VF) from adult males. Two of these mRNAs (VA and VB) are also found in females. As in *D. melanogaster*, the alternatively spliced mRNAs are predicted to encode distinct protein isoforms that differ at their amino termini. RNA form VB encodes a protein of 272 amino acids (DvTRA-2^{272}) initiating in exon 1, which most closely resembles DmTRA-2^{264}. RNA forms VA and VE are predicted to encode a 225-amino-acid protein (DvTRA-2^{225}) that resembles DmTRA-2^{226}. Like the larger DmTRA-2 isoforms, both DvTRA-2^{225} and DvTRA-2^{272} have a single RNA recognition motif (RRM) (44) flanked on both sides by RS-rich domains. VC and VF RNAs each potentially encode a truncated protein of 179 amino acids (DvTRA-2^{179}) initiating in exon 4 and lacking the amino-terminal RS domain. This protein is similar to DmTRA-2^{179}. Thus, *D. virilis* produces multiple *tra-2* mRNAs encoding three TRA-2 isoforms that are like those observed in DmTRA-2 (36).

An additional variation in *D. virilis* splicing patterns at the exon 3-exon 4 junction was observed in combination with the variants described above (Fig. 2 and 3). Here, two alternative 3′ splice sites, separated by 12 nt, are used. The upstream site is analogous to that used in *D. melanogaster*. Use of the downstream splice site deletes four internal amino acids from each of the above-described proteins. The amino acids affected are of unknown functional significance but are only a short distance upstream of the RRM. These amino acids are well conserved between *D. virilis* and *D. melanogaster*, but no similar alternative splice site is present in Dm*tra-2* exon 4.

Alignment of the DvTRA-2 protein sequences with the DmTRA-2 sequences revealed that, surprisingly, the most conserved region of the protein is the short linker segment immediately downstream of the RRM consensus, which has unknown function but is identical at 17 of 19 positions. Notably, other well-characterized RNA binding proteins have conserved regions carboxy terminal to their RRMs that are needed for RNA binding (41, 43). The RRM itself is also well conserved (85% identical), but both the RS1 and RS2 domains were more diverged, with identities of only 52 and 33%, respectively. It should be noted that the statistical significance of the primary sequence similarity in RS domains is further diminished by the low sequence complexity of these regions. Most of the identities observed consist of consecutive Arg or Ser residues or Arg-Ser dipeptides, suggesting that such simple repeats and/or the high overall Arg-Ser content of these domains are their only significant conserved features. Consistent with the idea that the RS composition rather than the primary sequence is conserved during evolution, a comparison of the amino acid compositions of RS1 and RS2 shows that they have well-conserved levels of arginine and serine (Fig. 2B).



C

RNA Transcript	Sex in which RNA Detected	Dv TRA-2 Isoform	Dm TRA-2 homologue
VA	F and M	225	226
VB	F and M	272	264
VC	M only	179	179
VE	M only	225	226
VF	M only	179	179

FIG. 3. Architecture of the *tra-2* genes from *D. melanogaster* and *D. virilis*. (A) Exon structures and splicing patterns of the Dm*tra-2* and Dv*tra-2* genes. Open boxes indicate exons, gray portions of these boxes indicate alternatively spliced portions of exons, and horizontal black lines indicate the alternatively spliced M1 or VM1 introns of the Dm*tra-2* and Dv*tra-2* genes, respectively. Splicing patterns are shown by angled lines above and below the gene structures. L, S, and VX7 represent the primers used to produce RT-PCR products. (B) RT-PCR products were resolved, blotted, and hybridized with the genomic 3.4-kb *Eco*RI fragment containing Dv*tra-2*. RT-PCR products from *D. virilis* females (F) or males (M) obtained by using either of two different primer combinations were loaded in each lane. Results for controls without reverse transcriptase are also shown. DNA in each of the indicated bands was isolated and sequenced to identify the RNA types present. (C) Five alternative Dv*tra-2* transcript types (VA, VB, VC, VE, and VF) deduced from the sequences of RT-PCR products, 5′ RACE amplification products (not shown), and products obtained in RNase protection assays (Fig. 7). The sex (F, female; M, male) in which each RNA transcript was detected, the protein isoform encoded by each transcript, and the respective DmTRA-2 homologs are also indicated. Only the start codons that give rise to TRA-2-homologous proteins are shown; therefore, the AUG in the first exons of VA and VF is not depicted, as it gives rise to prematurely truncated proteins.

The Dv*tra-2* gene can regulate somatic sexual differentiation and *dsx* splicing. To test whether the Dv*tra-2* gene has conserved the ability to regulate splicing of pre-mRNAs involved in sexual differentiation, we inserted a 7.8-kb fragment containing the entire Dv*tra-2* gene and flanking sequences (Fig. 1C) into the P-element vector pCaSpeR4 (42) and microinjected this construct (pCSPDvTRA2) into *D. melanogaster* embryos of the genotype $w^{1118}/B^{s}Y$; $tra\text{-}2^{B}/CyO$. The $tra\text{-}2^{B}$ mutation contains a nonsense codon within the RRM that truncates translation of all TRA-2 isoforms, deleting critical regions of the RRM as well as the entire essential RS2 domain (34). The $tra\text{-}2^{B}/tra\text{-}2^{B}$ individuals generated from the resulting transgenic strains were then examined to determine whether the Dv*tra-2* gene is able to replace Dm*tra-2* in directing sexual differentiation and alternative RNA splicing. Without an introduced transgene, chromosomally female $tra\text{-}2^{B}/tra\text{-}2^{B}$ individuals develop into somatic males as illustrated by the differentiation of their sex combs (compare Fig. 4A, B, and C). In transgenic lines with a single introduced copy of the Dv*tra-2* gene, female differentiation was restored to various extents. In two of six independent transgenic strains examined, a single dose of Dv*tra-2* was sufficient to restore female somatic differentiation to chromosomally female $tra\text{-}2^{B}/tra\text{-}2^{B}$ individuals (Fig. 4D). Two lines produced single-dose individuals with very female-like but slightly intersexual cuticular characteristics (Fig. 4E). Individuals from the same lines having two doses of the transgene had fully female cuticles (Fig. 4F). Another line produced only partially rescued individuals with two doses of the Dv*tra-2* transgene (not shown). In only one line was the inserted gene observed to have no effect on sexual differentiation. The line-to-line variations in this experiment are probably due to position effects resulting from different P-element insertion sites, since similar variation is observed when transgenic strains are similarly produced by using the Dm*tra-2* gene

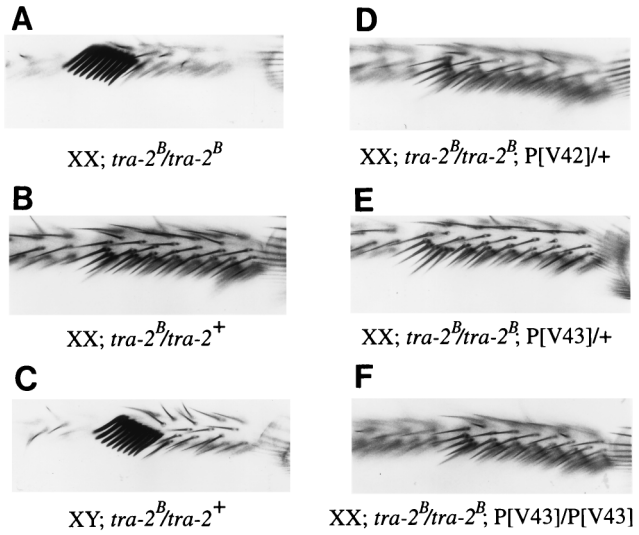


FIG. 4. Rescue of female sexual differentiation by pCSPDvTRA2. Sex comb bristles from various control and transgenic genotypes are shown. Sex combs from chromosomally female (XX) homozygous *tra-2*^{B} individuals (A) differ from those of heterozygous females (B) but closely resemble those of heterozygous chromosomal males (XY). Rescue of female sexual differentiation in homozygous *tra-2*^{B} chromosomally female individuals by a single P element insert in line 42 (D) and either one (E) or two (F) inserts in line 43 are shown. The sex comb bristles in panel E are female in number and thickness but have a more male-like orientation.

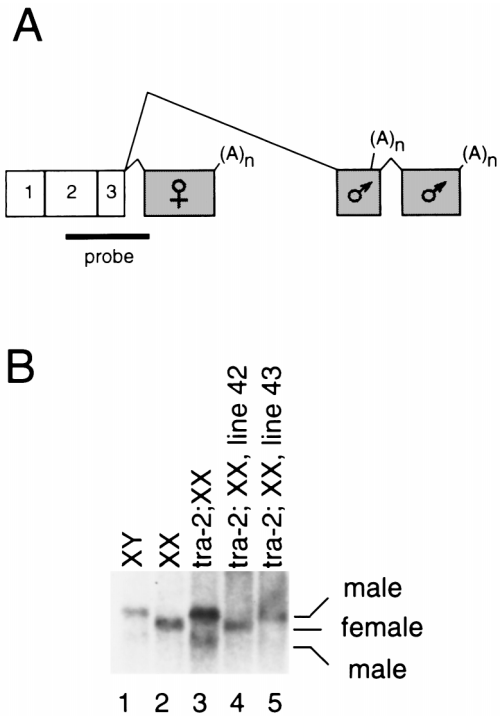


FIG. 5. Rescue of *doublesex* female-specific splicing by pCSPDvTRA2. (A) Schematic diagram showing the alternative splicing of *dsx* pre-mRNA. Note that two differently sized male-specific transcripts are produced through the use of two alternative polyadenylation sites. (B) Northern blot hybridization with the probe indicated in panel A shows the expected patterns of bands in wild-type male-specific (lane 1) and female-specific (lane 2) poly(A)$^+$ RNAs. The positions of the male- and female-specific RNAs are indicated to the right of the blot. Chromosomally female w^{1118}/w^{1118}; *tra-2*^{B}/*tra-2*^{B} individuals have a male-like splicing pattern due to the absence of functional TRA-2 protein (lane 3). Adult individuals of the same background genotype carrying a single dose of the pCSPDvTRA2 transgene produced *dsx* RNAs spliced in the female pattern in the case of line 42 (lane 4) and in the male pattern in the case of line 43 (lane 5).

(21). The ability of several transgenic lines to undergo female somatic sexual differentiation under the direction of DvTRA-2 shows that this gene can functionally substitute for DmTRA-2 in the soma.

Since female sexual differentiation normally requires expression of the female form of *dsx* mRNA, the results described above suggest that the DvTRA-2 protein can regulate processing of the *D. melanogaster dsx* pre-mRNA. To test this, we examined *dsx* mRNAs produced in chromosomally female *tra-2*^{B}/*tra-2*^{B} adults. Results from two transgenic strains are shown in Fig. 5. Line 42, which gave complete rescue of female cuticular differentiation with a single transgene dose, produced exclusively female-specific *dsx* transcripts, as expected. This result shows that DvTRA-2 can affect the splicing pattern of *dsx* RNA. Surprisingly, line 43, which gave only slightly less phenotypic rescue of cuticular differentiation than line 42, produced only male-specific *dsx* mRNA. This apparent inconsistency between the rescue of cuticular phenotype observed and splicing patterns in adults must be considered in light of the fact that cuticular differentiation of adult tissues occurs at earlier developmental stages than those used for RNA isolation. Since it is known that *dsx* female splicing is required for female cuticular differentiation during larval and pupal development (4), we suggest that the Dv*tra-2* gene insertion in line 43 affects *dsx* splicing during these stages but is expressed at lower levels in later stages and therefore fails to affect *dsx* splicing in adults. Nevertheless, the results described above are consistent with the idea that DvTRA-2 proteins have maintained the ability to regulate *dsx* splicing in the soma.

DvTRA-2 can support spermatogenesis and repress splicing of the *tra-2* M1 intron. In addition to its role in somatic tissues, DmTRA-2 is also needed in the male germ line for normal spermatogenesis. To determine if DvTRA-2 can restore this function to *tra-2* mutants, the fertility of chromosomally male (XY) *tra-2*^{B}/*tra-2*^{B} individuals carrying a single dose of the DvTRA-2 gene was compared to those of both wild-type individuals and *tra-2*^{B}/*tra-2*^{B} individuals lacking a transgene. As shown in Table 1, five of the six independent transgene insertions restored fertility to *tra-2*^{B}/*tra-2*^{B} males. To determine if fertility in males expressing a single copy of the Dv*tra-2* gene

TABLE 1. Phenotypic rescue of sexual differentiation in XX and XY *tra-2*^{B}/*tra-2*^{B} individuals carrying a single dose of pCSPDvTRA2[a]

Transgenic line	No. fertile (n = 10)[b]		Characteristics of XX flies		
	XY flies	XX flies	Pigmentation	Sex combs	Genitalia
No insert	0	0	Male	Male	Male
V12	0	0	Male	Male	Male
V42	10	10	Female	Female	Female
V43	10	10	Female	Fl. intersex[c]	Female
V44	10	0	Fl. intersex	Female	Female
V66	9	0	Intersex	Intersex	Intersex
V71	10	10	Female	Female	Female

[a] XX and XY flies were distinguished by using the B^{s}Y chromosome. The complete genotypes of individuals scored here are w^{1118}/w^{1118}; *tra-2*^{B}/*tra-2*^{B}; P[Dv *tra-2*$^+$]/+ (XX flies) and w^{1118}/B^{s}Y; *tra-2*^{B}/*tra-2*^{B}; P[Dv *tra-2*$^+$]/+ (XY flies).

[b] Flies were scored as fertile if a single individual produced more than three progeny. In nearly all cases that were denoted fertile, substantially higher numbers of progeny (>20) were generated.

[c] Fl. intersex, female-like intersex.

TABLE 2. Fertility of *tra-2*^{B} males carrying the Dv*tra-2* gene

Genotype[a]	No. of fertile males/no. of males tested	Avg progeny/ male tested	Avg progeny/ fertile male[b]
tra-2^B/*tra-2*^{B}	0/9	0	NA
tra-2^B/*tra-2*$^+$	14/15	148	159
P[*tra-2*$^+$]; *tra-2*^{B}/*tra-2*^{B}	12/12	165	165
P[V42]; *tra-2*^{B}/*tra-2*^{B}	13/13	159	159
P[V43]; *tra-2*^{B}/*tra-2*^{B}	12/12	137	137
P[V71]; *tra-2*^{B}/*tra-2*^{B}	11/14	101	128

[a] Sex chromosome markers are like those described for Table 1. P[*tra-2*$^+$] denotes a P element containing the 4-kb genomic *Eco*RI fragment that encompasses the Dm*tra-2* gene and its promoter.

[b] Males producing no progeny are excluded from the average. NA, not applicable.

was restored to the same level observed in flies with a single dose of the endogenous wild-type *tra-2* gene, in a separate experiment we counted the number of progeny produced by individual males from three of the transgenic strains. As shown in Table 2, the number of progeny produced by transgenic line 43 was similar to that in flies expressing the Dm*tra-2* gene. Lines 42 and 71 produced slightly lower numbers of progeny. These results indicate that DvTRA-2 can support all functions needed for male fertility.

One function of TRA-2 in the male germ line is to regulate its own expression by repressing splicing of the M1 intron in about 60% of *tra-2* mRNA (34). However, it has remained unclear whether such regulation is in fact needed for normal male fertility. Since we might expect a function critical for fertility to be conserved during evolution, it was of interest to determine whether DvTRA-2 expressed in transgenic *D. melanogaster* strains can repress M1 splicing in the endogenous *tra-2* RNA. Figure 6 shows an RNA blot on which poly(A)$^+$ mRNA from such lines was hybridized with a probe from within the M1 intron. While no M1-containing RNAs were detected in nontransgenic, chromosomally male *tra-2*^{B}/*tra-2*^{B} individuals (Fig. 6, top panel, lane 2), similarly homozygous mutant males from two transgenic lines examined (lanes 4 and 7) showed levels of M1-containing RNA that were similar to those observed in *tra-2*^{B}/+ males (lanes 5 and 8). These results suggest that the DvTRA-2 protein has conserved the ability to autoregulate splicing and can recognize *cis*-regulatory signals in Dm*tra-2* pre-mRNA.

The VM1 intron of Dv*tra-2* and the M1 intron of Dm*tra-2* are similarly positioned and regulated. While the above-described result indicates that the DvTRA-2 protein is capable of affecting the splicing of *tra-2* pre-mRNA from *D. melanogaster*, it does not address the issue of whether Dv*tra-2* mRNA processing is itself autoregulated. We therefore wished to examine how splicing of DvTRA-2 pre-mRNA is affected by TRA-2 proteins. The fourth intron of Dv*tra-2* (hereafter referred to as VM1) is a strong candidate as a target of autoregulation. Analogous to the M1 intron, the VM1 intron splits the initiation codon for DvTRA-2^{225}. The VM1 intron is fully retained in VC RNA, and part of the intron remains present in VF RNA. Both of these RNAs encode a 179-amino-acid isoform lacking RS1, as is true for M1-containing RNAs expressed in *D. melanogaster* (Fig. 3). The VM1 intron is thus in a similar position with respect to protein-coding sequences as the M1 intron in Dm*tra-2* and is subject to alternative splicing. Since both VC and VF RNAs were found in cloned RT-PCR products from males but not females, we wished to test whether the expression of these RNAs is male specific. To do so, we performed an RNase protection assay with *D. virilis* male and female

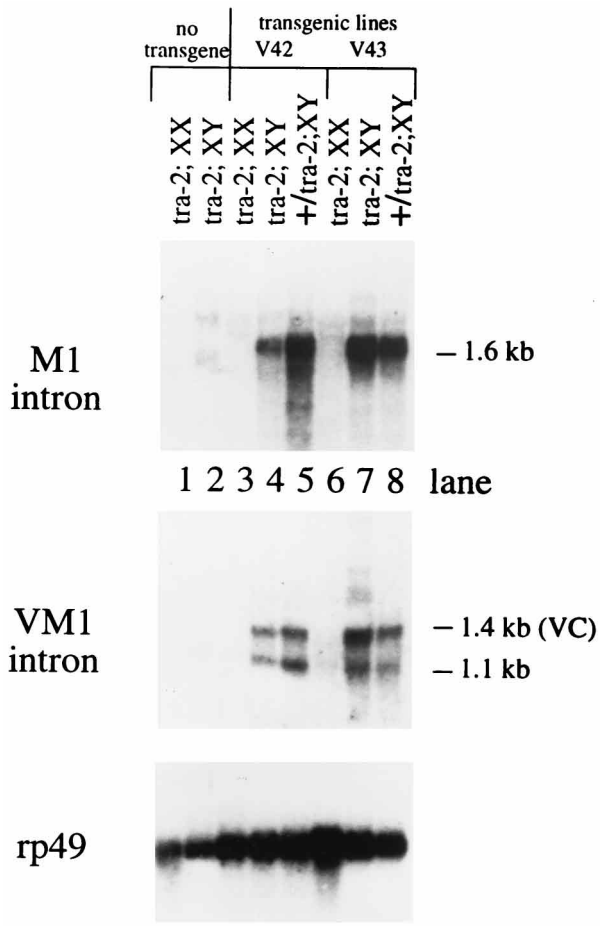


FIG. 6. Repression of M1 and VM1 splicing by Dv*tra-2*. Northern blots of poly(A)$^+$ RNAs from transgenic *D. melanogaster* fly strains and controls are shown hybridized with a probe from within either the Dm*tra-2* M1 intron (top), the Dv*tra-2* VM1 intron (middle), or the ribosomal protein 49 gene (bottom). RNA in lane 1 was from XX; *tra-2*^{B}/*tra-2*^{B} individuals, and that in lane 2 was from XY; *tra-2*^{B}/*tra-2*^{B} individuals. RNAs in lanes 3 to 8 were derived from various transgenic genotypes carrying a single dose of the Dv*tra-2* gene introduced by using pCSPDvTRA2. Line V42 (lanes 3 to 5) and line V43 (lanes 6 to 8) are two independent P insertions. The genotypes of these transgenic flies are XX; *tra-2*^{B}/*tra-2*^{B} (lanes 3 and 6), XY; *tra-2*^{B}/*tra-2*^{B} (lanes 4 and 7), and XY; *tra-2*$^+$/*tra-2*^{B} (lanes 5 and 8). All flies used, including controls, carry the *w*^{1118} marker on their X chromosomes to allow scoring of the *w*$^+$ marker in the pCSPDvTRA2 P-element. The *B*^{s}*Y* chromosome was used in all cases to distinguish XX and XY individuals. The 1.6-kb band detected in the top panel is of the size normally observed for M1-containing RNAs. The 1.4-kb band in the middle panel (VM1 probe) corresponds to VC RNA and is the same size as a band detected in RNA from *D. virilis* males (not shown). The 1.1-kb band detected in these transgenic strains is not found by using the same probe with *D. virilis* RNA and is of unknown origin.

poly(A)$^+$ RNAs. As shown in Fig. 7A, bands representative of VC RNA (355 and 301 nt) were observed predominantly in male *D. virilis* RNA and only at a very low level in female *D. virilis* RNA (compare lanes 3 and 7 to lanes 4 and 8). In the same experiment the 242-nt protected fragment, representative of VF RNA, was also detected at much higher levels in males than in females.

To test the male gonad specificity of these RNAs, RT-PCR amplification of RNA from dissected gonads and carcasses of *D. virilis* adults was performed. Amplification products from VC and VF transcripts were found in testes but not in ovaries or in the remaining carcasses of dissected individuals from either sex (Fig. 8). We conclude that these RNAs are highly

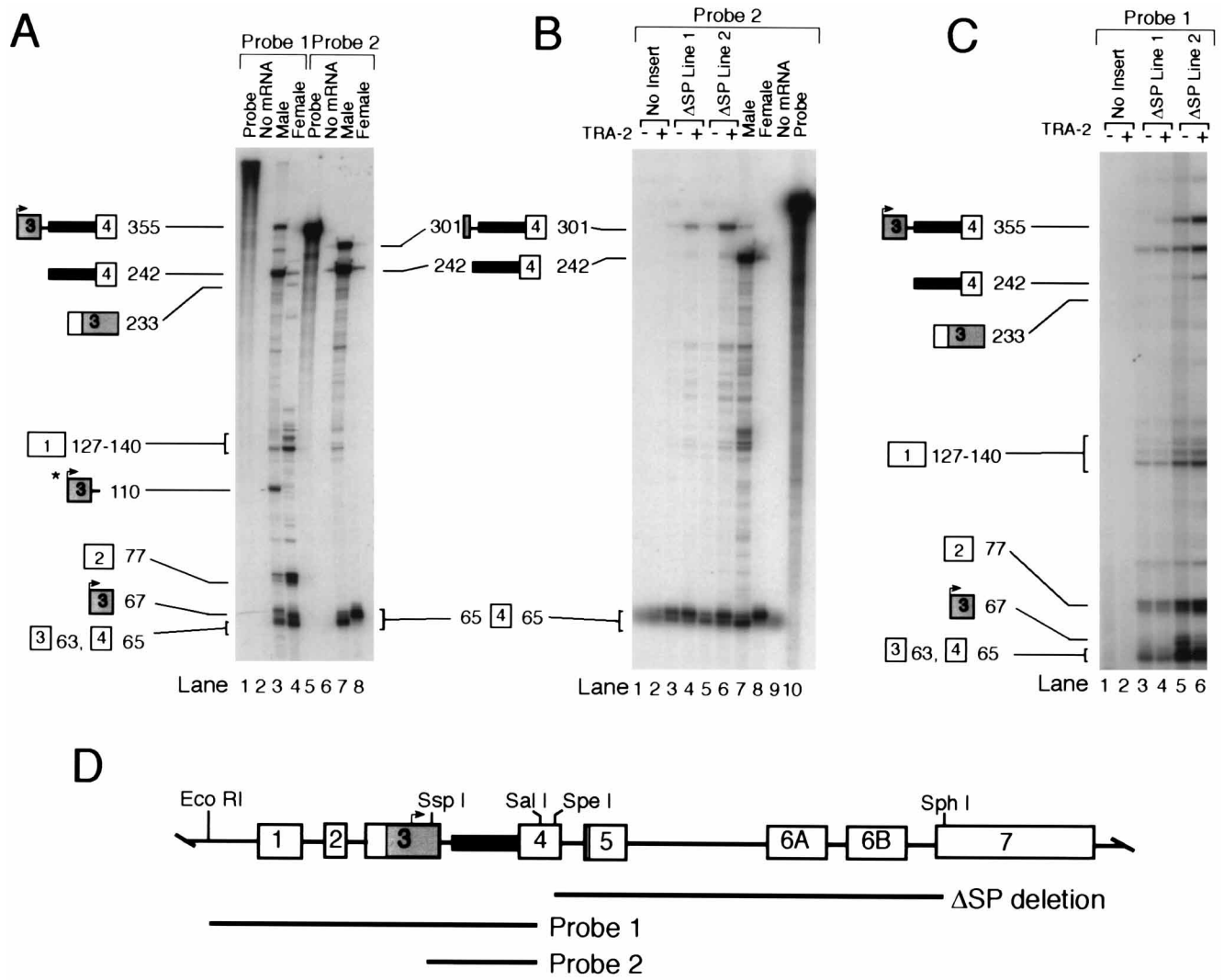


FIG. 7. RNase protection assays. (A) Dv*tra-2* splicing was examined by using *D. virilis* male and female poly(A)$^+$ RNAs by RNase protection with probe 1 (lanes 1 to 4) and probe 2 (lanes 5 to 8) followed by electrophoresis on denaturing polyacrylamide gels. Schematic diagrams of protected fragments are indicated on either side of the gel. The 355-nt band protected by probe 1 and the 301-nt band protected by probe 2 represent VC RNA, while the 242-nt fragment represents VF RNA. The staggered bands between 127 and 140 nt (lanes 3 and 4) are likely to be protected fragments derived from exon 1. The 110-nt fragment observed with probe 1 is derived from partial cleavage 38 nt downstream of the VM1 5′ splice site at a single-base polymorphism between the probe RNA and mRNA (which are derived from different strains of *D. virilis*). This fragment extends from the polymorphism to the male-specific transcription start site in exon 3. The appearance of this band is highly dependent on digestion conditions. (B) Analysis of splicing in ΔSP transgenic lines by RNase protection analysis with probe 2. Lines 1 and 2 are independent insertions of the P element pCSPDvTRA2ΔSP. Protections carried out with nontransgenic males are shown in lanes 1 and 2. Similar experiments on RNAs from transgenic males that were $tra\text{-}2^B/tra\text{-}2^B$ in genotype (−) are shown in lanes 3 and 5, while lanes 4 and 6 contain products from protections with RNAs from $tra\text{-}2^+/tra\text{-}2^B$ males (+). Lanes 7 and 8 show protections of RNAs from wild-type *D. virilis* males and females, respectively. (C) Analysis of splicing in ΔSP transgenic lines by RNase protection analysis with probe 1. Lanes are as lanes 1 to 6 of panel B. The 67-nt band visible in lanes 5 and 6 uniquely represents VE RNA, which is the form of Dv*tra-2* RNA lacking the VM1 intron produced in the male germ line. (D) Schematic diagram indicating how probes and the deletion present in pCSPDvTRA2ΔSP relate to the exons and introns in the Dv*tra-2* gene (Fig. 3). Probe 1 includes all sequences between the *Sal*I site in the middle of exon 4 and the *Eco*RI site upstream of exon 1. Probe 2 extends from the *Sal*I site in the middle of exon 4 to the *Ssp*I site just upstream of the VM1 intron. The deletion in pCSPDvTRA2ΔSP removes all protein-coding sequences downstream of RS1.

enriched in male gonads, as would be expected if they were the by-products of male germ line-specific autoregulation.

To test whether the formation of VC and VF RNAs depends on the presence of functional TRA-2 protein, it was necessary to express Dv*tra-2* mRNA from a construct unable to produce functional protein. We therefore deleted from pCSPDvTRA2 a 1-kb *Spe*I-*Sph*I fragment that includes much of the protein-coding sequences but does not affect the VM1 intron or its surrounding splice junctions (Fig. 7C). The deleted region of the protein spans domains previously shown to be essential for TRA-2 function, including most of RS1 and RS2 as well as the entire RRM (1). This deleted version of pCSPDvTRA2 (called pCSPDvTRA2ΔSP) was used to produce transgenic *D. melanogaster* fly strains. The splicing of DvTRA2ΔSP mRNAs was then tested in both $tra\text{-}2^B/tra\text{-}2^B$ and $tra\text{-}2^B/+$ individuals by using an RNase protection assay. As shown in Fig. 7B (lanes 3 to 6) and C (lanes 3 to 6), the 242-nt band representative of VF RNA was absent from all of the transgenic genotypes examined. RT-PCR experiments (not shown) confirmed that the VF RNA was not produced in *D. melanogaster* transgenic strains produced with either pCSPDvTRA2 or pCSPDvTRA2ΔSP. This suggests that either the DmTRA-2 protein or the *D.*

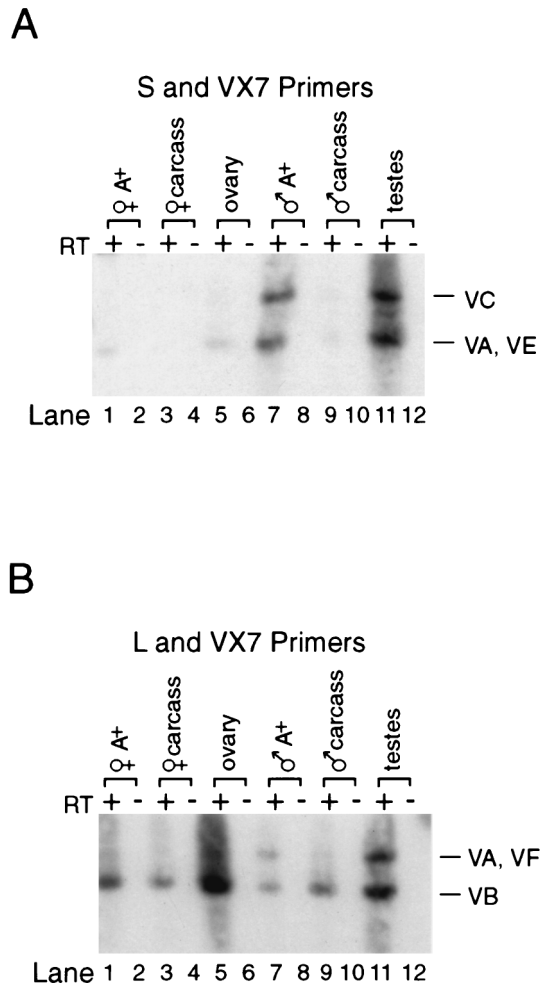


FIG. 8. Tissue distribution of Dv*tra-2* RNAs. (A) PCR was performed on cDNAs from whole *D. virilis* females (lane 1), female carcasses after dissection of gonads (lane 3), ovaries (lane 5), *D. virilis* males (lane 7), male carcasses lacking gonads (lane 9), or testes (lane 11) by using the S and VX7 primer set shown in Fig. 3. PCR amplification with material from mock reverse transcriptase (RT) reactions in which enzyme was omitted are shown in lanes 2, 4, 6, 8, 10, and 12. (B) PCR was performed on the same samples as in panel A, but the primer set used was L and VX7.

melanogaster general splicing machinery was unable to recognize *cis* elements in Dv*tra-2* RNA needed to generate this splicing alternative. The protected fragment derived from VC RNA, on the other hand, was readily observed in DvTRA2ΔSP strains (Fig. 7B and C). This RNA accumulated to higher levels in *tra-2*^{B}/+ males than in *tra-2*^{B}/*tra-2*^{B} males, indicating that splicing of the VM1 intron is repressed by the presence of functional DmTRA-2 protein. As expected, the accumulation of VE RNA (RNA resulting from VM1 splicing) in the male germ line is affected in the opposite way by the presence of functional TRA-2. This RNA is uniquely represented by a fragment at 67 nt in Fig. 7C that increases in amount in *tra-2*^{B}/*tra-2*^{B} males (compare lanes 5 and 6). These results indicate that Dv*tra-2* RNA contains *cis*-regulatory signals allowing autoregulation of VM1 splicing. Taken together with the observations that DvTRA-2 proteins have the ability to repress splicing of the analogous M1 intron from Dm*tra-2* and that VM1-containing RNAs accumulate in the *D. virilis* male gonad, the results described above suggest that the DvTRA-2 protein represses removal of the VM1 intron in the *D. virilis* male germ line. This idea is supported by the results shown in the middle panel of Fig. 6, where RNAs from transgenic lines expressing the intact Dv*tra-2* gene were found to accumulate a 1.4-kb VM1-containing RNA (the expected size of VC RNA) in the absence of functional endogenous DmTRA-2 protein.

DISCUSSION

Although sexual differentiation is a ubiquitous feature of metazoan organisms, the pathways regulating sex have been defined for only a small number of species (47). In *D. melanogaster* a cascade of genes has been identified in which sequential regulatory interactions at both the transcriptional and posttranscriptional levels ultimately result in the activation of male or female differentiation programs in somatic tissues. The splicing regulator *tra-2* plays a central role in this cascade and also affects sexual differentiation in the germ line, where it is required for the completion of spermatogenesis in males (23, 34). Here we have explored the issue of whether a *tra-2* gene with similar activities also exists in *D. virilis*. We find that the *D. virilis tra-2* homolog is conserved not only in its primary sequence but also in functional capabilities, being able to replace the Dm*tra-2* gene in both somatic and germ line sexual differentiation. The conservation of gene architecture and functional capabilities observed suggests that the TRA-2 proteins present in these two species play similar regulatory roles in sexual differentiation.

The expression of multiple TRA-2 isoforms is conserved during evolution. Analysis of *tra-2* expression in *D. melanogaster* has demonstrated that the gene produces alternative mRNAs that together encode three alternative isoforms of the TRA-2 protein that differ in their amino termini (2, 36). Individual isoforms expressed under normal developmental signals in transgenic fly strains were found to be largely redundant in function (2, 35). The fact that a single isoform (TRA-2^{226}) is sufficient for both normal sexual differentiation and fertility suggests the possibility that additional isoforms are superfluous (35). However, the analysis of the Dv*tra-2* gene presented here argues otherwise. This gene also produces mRNAs encoding three TRA-2 isoforms with similar variations at their amino termini. Thus, the ability to produce these alternative isoforms has been conserved through over 60 million years of evolution. This suggests that additional *tra-2* isoforms play a significant but unidentified role in the fitness of the organism.

Evolutionary conservation of a male germ line-specific autoregulatory strategy. The relative amounts of mRNAs encoding functional and nonfunctional TRA-2 isoforms in the male germ line of *D. melanogaster* are governed by an autoregulatory mechanism in which the TRA-2^{226} isoform specifically acts to repress splicing of the M1 intron (35). Our analysis of the Dv*tra-2* gene strongly indicates that a similar autoregulatory strategy is used by *D. virilis*, supporting the idea that autoregulation of TRA-2 expression is a conserved feature of male germ line differentiation. The VM1 intron is positioned similarly to M1 in Dv*tra-2*, disrupting the predicted initiation codon of the DvTRA-2^{225} isoform. When the Dv*tra-2* gene is expressed in *tra-2*^{B}/*tra-2*^{B} males, the splicing of both VM1 from Dv*tra-2* RNA and M1 from endogenous Dm*tra-2* RNA is repressed. Therefore, the DvTRA-2 protein has the ability to recognize *cis*-regulatory signals within *tra-2* pre-mRNAs from both species. Although the absence of available *D. virilis* mutants prevented us from demonstrating autoregulation within the context of the *D. virilis* male germ line, the observation that VM1-containing mRNAs are found specifically in the testes of

D. virilis males supports the idea that autoregulation occurs similarly in this species.

It is worth noting that cross-species autoregulation in both possible combinations (the DvTRA-2 protein affecting Dm*tra-2* RNA and the DmTRA-2 protein affecting Dv*tra-2* RNA) was observed within the context of *D. melanogaster* transgenic strains. The ability of components from different species to interact suggests that the mechanisms used for autoregulation in the two species must be quite similar.

While in *D. melanogaster* only a single functional TRA-2 isoform (TRA-2^{226}) is expressed in the male germ line, in *D. virilis* the situation is more complex. In this species RNAs encoding both TRA-2^{272} (VB) and TRA-2^{225} (VE) are present in the male germ line. While the expression of TRA-2^{225} can be blocked by TRA-2-dependent repression of VM1 splicing (which prevents formation of VE RNA), the expression of TRA-2^{272} appears to be regulated through a different splicing choice. The RNA encoding this isoform is produced by using an alternative 5′ splice site that is upstream from the VM1 5′ splice site. This site may be spliced to either of two 3′ splice sites. In VB RNA, splicing is to the 3′ splice site at the end of the VM1 intron, whereas in VF RNA, splicing is to a site in the middle of the VM1 intron. Notably, like RNAs retaining the complete VM1 intron, VF RNA is specific to the male germ line and encodes the DvTRA-2^{179} isoform, which lacks RS1. This raises the interesting possibility that the expression of DvTRA-2^{272} and DvTRA-2^{225} is regulated through two parallel alternative processing pathways, both of which result in the production of an RNA encoding DvTRA-2^{179}. Although we were not able to test the idea that, like TRA-2^{225}, the alternative splicing leading to TRA-2^{272} expression is subject to autoregulation, both splicing pathways could potentially be affected by a block in the recognition of the 3′ splice site at the end of the VM1 intron.

Conserved sequences in the M1 intron. Because noncoding sequences in *D. melanogaster* and *D. virilis* are sufficiently divergent, regulatory elements are frequently evident as conserved blocks of sequence within introns and untranslated regions. Comparison of the sequences of the VM1 intron and the noncoding sequences of exon 3 to the analogous segment of the Dm*tra-2* gene thus provides a useful way to identify *cis*-regulatory elements potentially necessary for regulation of M1 splicing. Such a comparison revealed three conserved noncoding elements (Fig. 9). The first of these is a region within exon 3 in which 11 of 12 nt are identical to the sequences surrounding the male-specific transcription start sites used in *D. melanogaster* (36). RNase protection experiments support the idea that this element corresponds to a male-specific transcription start site in *D. virilis*, since a protected fragment of exactly the predicted 67-nt distance between this site and the beginning of the VM1 intron is observed (Fig. 7). Also conserved are the polypyrimidine tract regions and suboptimal 3′ splice sites of the VM1 and M1 introns. The 3′ splice sites in both species differ significantly from the *Drosophila* consensus by the presence of an adenosine at the −3 position, which is typically occupied by a pyrimidine (38). A mutation in the Dm*tra-2* splice site region that makes it more closely match the *Drosophila* consensus resulted in constitutive splicing of the M1 intron, suggesting that suboptimal sequences in this region are required for repression of M1 splicing by TRA-2 (11a). Suboptimal splicing signals also play critical roles in other cases where regulated splicing occurs (8, 10, 17). A third conserved element is located 12 to 30 nt downstream of the 5′ splice site in the M1 intron of Dm*tra-2* and near the putative branchpoint region of Dv*tra-2*, where 15 of 18 nt are identical. Preliminary experiments suggest that this sequence is necessary for appropriate tissue-specific production of M1-containing RNAs in *D. melanogaster* (11a). Thus, the conserved elements within these noncoding regions appear to play important roles in splicing regulation and transcription.

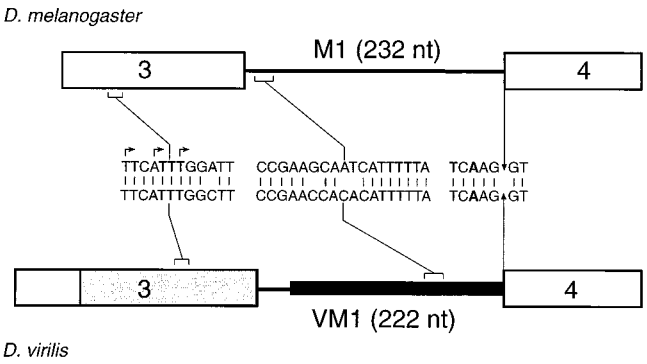


FIG. 9. Conserved sequences in exon 3 and the M1 and VM1 introns. Three conserved elements in noncoding sequences within or near the M1 intron are shown in relation to their positions in both Dm*tra-2* (top) and Dv*tra-2* (bottom). The positions of three male-specific transcription start sites mapped in *D. melanogaster* in the conserved element from exon 3 are indicated. The positions of the 3′ splice sites of M1 and VM1 are indicated by arrows, and the nonconsensus adenosine at position −3 is in boldface. The sizes of the M1 and VM1 introns are indicated.

Sequence divergence of RS domains. RS domains, which are characterized by their high content of arginine and serine and the presence of repeating RS dipeptides, are found in many splicing factors and have been shown to be essential for protein-protein interactions taking place both during spliceosome assembly and in the formation of protein complexes involved in the regulation of splicing (18, 32). The RS2 domain of *tra-2* is thought to be essential for all of the protein's known functions, while RS1 appears to be required only in the male germ line (1, 35). Comparison of Dm*tra-2* and Dv*tra-2* sequences revealed that RS regions in the two proteins have similar amino acid compositions but differ significantly in primary sequence. The fact that Dv*tra-2* functionally replaces Dm*tra-2* indicates that the observed differences in primary sequence do not substantially impair the function of this domain. This suggests either that the rather limited conserved regions of RS2 are sufficient for function or that only a high content of arginine and serine in these regions, rather than primary sequence conservation, is necessary. Interestingly, a similar situation has been observed for RS domains of the TRA protein, which has overall low identity with its *D. virilis* homolog but is partially functional when expressed in *D. melanogaster* (40).

The observed divergence of RS domains between *Drosophila* species contrasts with observations that such regions remain highly conserved when sequences from splicing factors in different vertebrates are compared (16, 48). Recently we have identified *tra-2* homologs from both humans and mice (15) and have observed that the amino acid sequences of the RS2 domains of the mouse and human *tra-2* proteins are identical to each other (data not shown). Thus, the primary sequence of RS domains may be more constrained in mammals than in drosophilids.

Sex determination in *D. virilis*. Several lines of evidence now suggest that sexual differentiation in *D. virilis* is regulated by a pathway of genes that is homologous to the *D. melanogaster* sex determination pathway. By using molecular approaches, homologs of the *Sex-lethal*, *tra*, *tra-2*, and *dsx* genes of *D. virilis* have now been identified and are each known to produce sex-specific products through alternative splicing, like their *D.*

melanogaster counterparts (9, 12, 25, 40). Moreover, the *cis*-regulatory sequences used to control splicing are generally conserved in the pre-mRNA targets of these factors. For example, sequences in the *tra* pre-mRNA through which SXL binds and affects selection of its 3′ splice sites are conserved in *D. virilis* (40, 50). Likewise, the 13-nt repeat elements of the *dsx* female-specific exon that are recognized by TRA and TRA-2 proteins are also conserved (12, 25). Thus, it is likely that *D. virilis* utilizes a splicing regulatory cascade like that found in *D. melanogaster* to control sexual differentiation.

This idea is further supported by the genetic analysis of *D. virilis*. Mutations in two genes causing sex transformations in this species have been reported. Dominant mutations in the *Intersex-of-Blanco* (Ix^B) gene on chromosome 2 cause sexual transformations like those of several *dsx* dominant mutations, suggesting that Ix^B may correspond to the *D. virilis dsx* homolog (33, 49). Mutations in the *intersex* (*ix*) gene on chromosome 3 cause recessive somatic sexual transformations from female to male like those observed in *D. melanogaster tra* and *tra-2* mutants (29, 35, 58). The recombinational map position of *ix* puts it in the same region of chromosome 3 where the *D. virilis tra* homolog was mapped, suggesting that these mutations affect this gene (58).

The apparent conservation of a portion of the sex determination regulatory pathway among distantly related drosophilids might seem surprising in view of the observation that multiple chromosomal sex determination mechanisms have been observed even within individual species of several insects (27, 28, 54, 57). However, it is probably important to draw a distinction between factors involved in primary sex determination (i.e., measurement of the X/A ratio) and subordinate factors in the sex-determining regulatory pathway (i.e., *tra*, *tra-2*, and *dsx*). It has recently been suggested that sex pathways may evolve in reverse order, with subordinate genes at the bottom of these pathways being the most ancient and therefore most widespread among related organisms (56). The conserved factors in the *Drosophila* sex pathway studied so far each function downstream from the primary X/A counting mechanism. Determination of whether a higher degree of variation will be observed in primary sex-determining mechanisms awaits analysis of the X/A counting mechanism used by *D. virilis* and of sex factors in more distant insect species.

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