Interspecific Comparison of the transformer Gene of Drosophila Reveals an Unusually High Degree of Evolutionary Divergence

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ABSTRACT

The transformer (tra) gene of Drosophila melanogaster occupies an intermediate position in the regulatory pathway controlling all aspects of somatic sexual differentiation. The female-specific expression of this gene’s function is regulated by the Sex lethal (Sxl) gene, through a mechanism involving sex-specific alternative splicing of tra pre-mRNA. The tra gene encodes a protein that is thought to act in conjunction with the transformer-2 (tra-2) gene product to control the sex-specific processing of doublesex (dsx) pre-mRNA. The bifunctional dsx gene carries out opposite functions in the two sexes, repressing female differentiation in males and repressing male differentiation in females. Here we report the results from an evolutionary approach to investigate tra regulation and function, by isolating the tra-homologous genes from selected Drosophila species, and then using the interspecific DNA sequence comparisons to help identify regions of functional significance. The tra-homologous genes from two Sophophoran subgenus species, Drosophila simulans and Drosophila erecta, and two Drosophila subgenus species, Drosophila hydei and Drosophila virilis, were cloned, sequenced and compared to the D. melanogaster tra gene. This comparison reveals an unusually high degree of evolutionary divergence among the tra coding sequences. These studies also highlight a highly conserved sequence within intron one that probably defines a cis-acting regulator of the sex-specific alternative splicing event.

The transformer (tra) gene of Drosophila melanogaster is one of a set of major regulatory loci controlling somatic sexual differentiation (reviewed by Baker 1989; Steinmann-Zwicky, Amrein and Nöthiger 1990). Like most of the other genes that have been identified in this regulatory pathway, the tra gene is expressed in a sex-specific manner. In chromosomally male individuals (i.e., XY), the tra gene is functionally “off,” resulting in the fly following the default pathway of male differentiation. In chromosomally female flies (i.e., XX), the tra gene is functionally “on,” where it acts to direct proper female differentiation of all sexually dimorphic somatic tissues. Loss-of-function mutations of the tra gene are, therefore, female-specific in their effects, with XX; tra− individuals being transformed to maleness, and XY; tra− being unaffected.

Molecular genetic studies of the tra gene have given us insight into how this sex-specific expression is achieved (Butler et al. 1986; McKeown, Belote and Baker 1987; Belote et al. 1989). Although the tra gene is transcribed from the same promoter in both sexes, the pre-mRNA is subjected to sex-specific alternative splicing of the first intron (Boggs et al. 1987). In males, the tra pre-mRNA is spliced in such a way that the mature transcript contains no long open reading frame (ORF), and can encode only a truncated, inactive tra polypeptide. In females, about half of the tra pre-mRNA that is made is spliced in a different manner, to yield an mRNA with an ORF encoding a polypeptide of 197 amino acids, representing the functional tra gene product. The alternative splicing of tra pre-mRNA is regulated by the functioning of the Sex lethal (Sxl) gene (Nagoshi et al. 1988; Bell et al. 1991). This gene is also expressed in a female-specific manner, and it encodes an RNA binding protein that is thought to act directly on tra pre-mRNA to influence its splicing (Bell et al. 1988; Inoue et al. 1990). It does this by inhibiting the use of the non-sex-specific 3′ splice site and allowing the use of the downstream female-specific 3′ splice site (Sosnowski, Belote and McKeown 1989). In males, no Sxl protein is produced, and tra pre-mRNA is spliced using the preferred non-sex-specific 3′ splice site by default.

Genetic and molecular analyses of tra suggest that its gene product acts in conjunction with the transformer-2 (tra-2) gene product to control the sex-specific expression of the doublesex (dsx) gene (Baker and Ridge 1980; Nagoshi et al. 1988; McKeown, Belote and Boggs 1988). The dsx gene is a bifunctional locus that has active, but opposite, functions in males and females (Baker and Ridge 1980; Nöthiger et al. 1987; Baker and Wolfner 1988). In males, dsx acts...
to repress female-specific differentiation of the sexually dimorphic somatic tissues, thereby leading to normal male development, and in females it acts to repress male differentiation of the soma, resulting in normal female development. The sex-specific expression of \(\text{dsx}\) is achieved by sex-specific alternative processing of \(\text{dsx}\) pre-mRNA, yielding different \(\text{dsx}\) gene products in the two sexes (Burtis and Baker 1989).

It has been proposed that the \(\text{tra}\) and \(\text{tra-2}\) gene products affect \(\text{dsx}\) pre-mRNA splicing by activating the female-specific 3' splice site in the \(\text{dsx}\) transcript (Burtis and Baker 1988; Nagoshi and Baker 1990; Hoshijima et al. 1991; Hedley and Maniatis 1991; Ryner and Baker 1991).

Among the important questions concerning the \(\text{tra}\) gene's expression and function are (1) how is the sex-specific splicing of \(\text{tra}\) pre-mRNA regulated by factors such as the \(\text{Sxl}\) gene product? and (2) what is the exact function of the \(\text{tra}\) protein? With respect to the first question, one relevant topic of inquiry deals with the cis-acting sequences that are responsible for the sex-specific control of intron 1 splicing. Work to date has focused on using site-directed mutagenesis procedures to manipulate the \(\text{tra}\) gene in vitro, and then examining the effects of those changes on the sex-specific splicing of the pre-mRNA in either transgenic flies (Sosnowski, Belote and McKeown 1989), or in transgenic cell lines (Inoue et al., 1990). As an alternative approach, we have examined the \(\text{tra}\) gene in other Drosophila species, in order to identify conserved sequence elements within the regulated intron that might identify important regulatory sequences controlling the alternative splicing event. With respect to the second question stated above, it was hoped that the interspecific comparison of the \(\text{tra}\) gene's coding region would reveal conserved domains of the protein and help focus future studies of the \(\text{tra}\) protein on regions of functional significance. Because the species chosen for our study have been estimated to have diverged approximately 60 million years ago, sequence conservation should be limited to functionally relevant regions of the gene.

**MATERIALS AND METHODS**

**Fly strains:** *Drosophila hydei* flies were obtained from a stock provided by David Sullivan, Syracuse University, *Drosophila erecta* flies from a stock provided by Donal Hickey, University of Ottawa, and *Drosophila virilis* from a stock provided by Larry Salkoff, Washington University School of Medicine. Flies were maintained at 22° on cornmeal-molasses-yeast-agar media containing propionic acid as a mold inhibitor. Mutations of *Drosophila melanogaster* are described in Lindsley and Grell (1968) or in Lindsley and Zimm (1985, 1986, 1987, 1988, 1990).

**Genomic libraries:** The *Drosophila simulans* and *D. hydei* libraries were constructed by ligating size-fractionated genomic DNA that had been partially digested with *MboI* into the BamHI site of the bacteriophage \(\text{XEMBL4}\) vector (Frischauff et al. 1985). The recombinant DNA was packaged into phage particles using the Gigapack Bacteriophage \(\lambda\) Packaging Kit from Stratagene according to the supplier's protocol, and plated on *Escherichia coli* strain Q589. An additional *D. hydei* library, similar to the one described above, but amplified, was provided to us by David Sullivan, Syracuse University.

The *D. erecta* and *D. virilis* libraries were amplified libraries constructed by Donal Hickey, University of Ottawa, and Ronald Blackman, Harvard University, respectively, using the bacteriophage \(\text{XEMBL3}\) vector (Frischauff et al. 1985).

Screening of the recombinant \(\lambda\) phage libraries was done essentially as described in Maniatis, Fritsch and Sambrook (1982). For each library, 15 plates containing 10⁴ plaque-forming units (pfu)/plate were prepared, and plaques lifted onto nitrocellulose filters (Millipore) and prepared for hybridization according to the method of Benton and Davis (1977). Probes were made by random-primed labeling of agarose gel-purified restriction fragments of cloned *D. melanogaster* transformer gene sequences using the Boehringer Mannheim Random Primed Labeling Kit. The \(\text{tra}\) clones used as probes in this study included pBlItra3.9R, a 3.9-kb EcoRI fragment, derived from a recombinant \(\lambda\) phage isolated from the Canton-S genomic library of Maniatis et al. (1978), cloned into the plasmid vector pBlI76 (International Biotechnologies, Inc.), and two cDNA clones, pBlI6.5.1 and pBlItra3.1P containing inserts derived from recombinant phage isolated from embryonic and pupal cDNA libraries, respectively, of Poole et al. (1985), and subcloned into the pBlI76 vector. pBlI6.5.1 represents a full-length female-specific \(\text{tra}\) transcript and pBlItra3.1P represents a full length non-sex-specific transcript. See McKeown, Belote and Baker (1987) and Boggs et al. (1987) for the molecular map and sequences of the *D. melanogaster* \(\text{tra}\) gene region and its transcripts. Hybridization was carried out overnight at 42° in 5 × SSPE, 0.1% sodium dodecyl sulfate (SDS), 1 × Denhardt's solution, 0.1 mg/ml sheared salmon sperm DNA, and 50% formamide (for the normal stringency hybridizations), or either 29% or 39% formamide (for the reduced stringency hybridizations).

Filters were washed at 50° in three changes of wash buffer (0.1 × SSPE, 0.1% SDS for normal stringency hybridizations, or 0.5 × SSPE, 0.1% SDS for reduced stringency hybridizations). Identification of positive clones, plaque purification, preparation of phage DNA, DNA fragment isolation, Southern blot analysis, identification of cross-hybridizing fragments, subcloning of the restriction fragments into the plasmid vector pBlI76 and isolation of plasmid DNA was carried out using the protocols described in Maniatis, Fritsch and Sambrook (1982).

**Southern blot analysis of fly DNA:** Drosophila genomic DNA was isolated as described in Bender, Spieler and Hogness (1983). Restriction digestion, agarose gel electrophoresis and transfer of the DNA to GeneScreen Plus membrane (NEN, Du Pont) was carried out as described in Maniatis, Fritsch and Sambrook (1982). Hybridization and wash conditions were the same as those described above for screening of the libraries.

**DNA sequencing:** For each of the cloned \(\text{tra}\)-homologous genes, a series of nested deletions were generated by EcoRI digestion according to the method of Henikoff (1987) using the Erase-A-Base kit from Promega. Plasmid DNA was purified by the ammonium acetate method of Morell (1989). Double stranded DNA templates were sequenced by the dideoxynucleotide chain termination method of Sanger, Nicklen and Coulson (1977) using [\(\text{32P}\)]dATP (New England Nuclear). Primers for the sequencing reactions
were either the T7 or SP6 promoter oligonucleotides purchased from Promega. Reaction products were electrophoresed according to the electroyte gradient method of SHERN and SEED (1988). DNA sequence data was managed using the programs of DNA STAR (Madison, Wisconsin). Comparisons of nucleotide sequences were performed by the algorithm of WILBUR and LIPMAN (1983).

**In situ hybridization:** Salivary glands from late third instar larvae were dissected in 0.7% saline, incubated for 10 sec in 45% acetic acid, and placed in a drop of 1 part lactic acid, 2 parts H2O, and 1 part acetic acid for 5 min. A siliconized coverslip was then applied and the glands were squashed. After freezing the slide in liquid nitrogen, the coverslip was removed and the chromosomes dehydrated through an ethanol series. Hybridization of the chromosomes was carried out according to the procedure of PLELEY, FARMER and JEFFREY (1986). Probes were prepared for in situ hybridization by random primed labelling of gel purified restriction fragments. The modified deoxynucleotide used was either biotinylated dUTP (Bethesda Research Laboratories) or digoxigenin-substituted dUTP (Boehringer Mannheim). Signal detection was done using a Detek-1-hrp Kit (ENZO) following the procedure of ASHURKAN (1989), for the biotinylated probes, or using the Genius Kit (Boehringer Mannheim) detection procedure for the digoxigenin-labelled probes (TAUTZ and PFEIFLE 1989).

**Germline mediated germ line transformation:** D. melanogaster embryos were collected within 90 min of egg deposition, dechorionated manually and injected with DNA as described in SPRADLING (1986). The injected DNA was cesium banded plasmid DNA consisting of a 2.8 PstI fragment containing the D. virilis tra-homologous gene region inserted into the pW8 transformation vector of KLEEMZ, WEBER and GERING (1987) which carries the white* (a*) eye color gene as a marker. Recipient flies were from the w; y*75Cv y*75Bb y(w*) strain of ROBERTSON et al. (1988). Since this strain carries a genomic dose of tra, and a helper plasmid was used, Co survivors were mated to w; TM3, rpha sep Sb bx* e/TM6B, Hu Tb e ca flies, and progeny scored for pigmented eyes to identify transformants. Such transformants were then crossed to w; Df(3L)jjar, Ki roe pha/TM6B, Hu Tb e ca mates and the Df(3L)jjar, Ki roe pha/Balancer progeny with pigmented eyes were mated to w; th st tra cp in ri pha/TM6B, Hu Tb e ca or w; st tra cp in ri pha/TM3, ri pha sep Sb bx* e. From this and subsequent crosses, flies were generated that were hemizygous or homozygous for the tra mutation and carried one or two copies of the virilis tra-homologous gene. In all of these crosses, males carried the B Y chromosome that allowed us to distinguish XX and XY individuals on the basis of the dominant phenotype associated with B*. Flies of interest were mounted between cover slips according to the method of SZABAD (1978) and examined under bright field optics using a Zeiss Axiosplan microscope.

**Transcript analysis:** Preparation of poly(A*) RNA and Northern blot analyses were carried out as described in MCKEOWN, BELOTE and BAKER (1987). DNA probes were made by random primed labelling of gel purified restriction fragments as described above. The procedure used for the generation of strand-specific RNA probes (i.e., riboprobes) was a modification of MELTON et al. (1984). Template DNA was linearized by restriction digestion and T7 RNA polymerase was used to create the run-on transcripts from the T7 promoter of plasmid vector pBP76. The [α-32P]CTP (NEN, Du Pont)-labeled transcripts were electrophoresed through a 5% native polyacrylamide gel in 0.5 X TBE buffer (89 mM Tris-base, 89 mM boric acid, 2 mM Na2EDTA). After electrophoresis, the gel was exposed to Kodak X-OMAT AR film for 15 min at room temperature. After development, the film was aligned with the gel with the positive exposure labeling the position of the riboprobe transcript. This region was then cut out of the gel, placed in a 1.5 ml Eppendorf tube containing 0.38 ml 2 M NaOHAc, 0.1% SDS and rocked for 2 hr at 37°. The supernatant was then precipitated with 10 μg yeast tRNA and 2.5 X volume of absolute ethanol for 15 min at -70°. The RNA pellet was resuspended in 25 μl of 0.2 M NaPipes, pH 6.4, 2 mM NaCl, 5 mM Na2EDTA.

For the amplification of reverse-transcribed RNA by polymerase chain reaction poly(A*) RNA was prepared as follows. Flies (0.5–1.0 g) were ground to a powder in liquid nitrogen and added to 4.0 ml of 5 mM guanidine isothiocyanate, 10 mM EDTA, 50 mM HEPES, 5% β-mercaptoethanol and homogenized in a Dounce tissue homogenizer. After brief centrifugation at 4° the supernatant was transferred to 1 ml of 5.7 M CsCl in HE buffer (10 mM HEPES, 1 mM EDTA). Dry Sarkosyl was added to 4% and the samples heated to 60° for 5 min. This solution was then layered over 1 ml of 5.7 M CsCl and centrifuged at 20° for 36 hr at 36,000 rpm in an AH650 rotor (Du Pont) to pellet the RNA. The RNA pellet was resuspended in 8 μl water in HE and then extracted several times with an equal mixture of phenol/chloroform. The RNA was then precipitated with a 1.0 X volume of 2.5 M NaAcO and a 2 X volume of absolute ethanol at -20°. The RNA was resuspended in diethylpyrocarbonate-treated H2O, and poly(A*) RNA was selected by passage over an oligo-dT-cellulose column as described in MANIATIS, FRTSCH and SAMBROOK (1982).

Polymerase chain reaction amplification of cDNA (cDNA-PCR) was carried out as described by KAWASAKI and WANG (1989). cDNA was synthesized in a 20-μl total volume reaction mixture of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 1 μM each dNTP, 1 unit/μl RNasin (BRL), 100 pmol random hexamers (Pharmacia), 0.5 μg poly(A*) RNA isolated from D. virilis adult males or females, and 200 units MuLV reverse transcriptase (BRL). Samples were incubated 10 min at room temperature, then 1 hr at 42°, and reactions stopped by heating to 95° for 5 min. Polymerase chain reaction amplification was carried out using the GenAmp DNA Amplification Reagent Kit (Perkin Elmer Cetus). Reaction mixtures consisted of 10 μM each dNTP, 0.1 μM Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 1 μl of cDNA template, prepared as above, 0.5 unit AmpliTag polymerase (Perkin Elmer Cetus), and 20 μM of each primer, in a total volume of 20 μl. The primers used for the amplification of D. virilis cDNAs were: primer Dvir2-5’ GACGCACATAGCTTCCGGT3’, and primer Dvir5’ ATGGACGCCGACCGTATC. Primers were synthesized by the Syrcause University DNA/Protein Core Facility using a Coder 300 DNA Synthesizer (Du Pont). The polymerase chain reaction was carried out using a Technne thermal cycler for 50 cycles, each cycle consisting of 1 min at 94°, 50 sec at 48°, and 2 min at 72°. PCR products were electrophoresed through 2% agarose gels, transferred to Gene Screen membrane filters (Du Pont), and probed with random primed 32P-labeled restriction fragments of the D. virilis tra-homologous gene. Autoradiograms were developed after about 1 or 2 weeks of exposure.

**RESULTS**

**Isolation of tra-homologous genes from D. simulans, D. erecta, D. kydei and D. virilis:** As a preliminary step toward the isolation of tra-homologous genes...
Having thus defined the hybridization conditions that should allow the isolation of tra-homologous genes from the other Drosophila species, we next screened genomic λ phage libraries of these four species under the appropriate conditions, using the tra region genomic probe pIBItra3.9R. For each library screened, the recombinant λ phage that were isolated by molecular cloning were then restriction mapped and aligned. The phage DNAs were then subjected to Southern blot analysis using a melanogaster tra cDNA clone as probe to further delimit the tra cross-hybridizing regions. For each species, two or more overlapping recombinant phage were isolated. Figure 2 shows the restriction maps of representative phage corresponding to the tra-homologous region of each species.

**DNA sequences of the tra-homologous genes of the Sophophoran species, *D. simulans* and *D. erecta*: Because the *simulans* and *erecta* tra-homologous sequences were isolated by screening under normal stringency conditions, and because only a single genomic region is detected under those same conditions in the Southern blot analysis, it is assumed that the recombinant phage that were isolated in these screens contain the tra-homologous genes. Southern blot analysis of the recombinant λ phage clones λDs24, from *D. simulans*, and λ De108.3, from *D. erecta*, using the *D. melanogaster* tra cDNA clone pIBItraP3.1 as a probe, revealed that the tra-homologous genes were contained within a 2.0-kb EcoRI/BamHI fragment of the *simulans* phage, and within a 3.0-kb BamHI fragment of the *erecta* phage. These fragments were therefore subcloned into plasmid vectors and a portion, corresponding to the tra transcribed region, of each was sequenced. Figure 3 shows the nucleotide sequence comparisons of the Sophophoran species examined.

The structure of the tra gene has been extensively characterized in *D. melanogaster* (McKenown, Belote and Baker 1987; Boggs et al. 1987). The tra transcription unit is relatively small, about 1100 base pairs in length, with two introns. The first intron exhibits sex-specific alternative RNA splicing. One type of splice is seen in both sexes and involves the removal of a 73-nucleotide long intron, resulting from this splice has a long ORF encoding the functional tra protein. Thus, this sex-specific splicing event provides the basis for the female-limited function of the tra gene.

The nucleotide sequences of *D. simulans* and *D. erecta* can be aligned with that of *D. melanogaster* to give the same gene structure. The initiation and ter-
mination codons are in similar positions, although the termination codon is UAA in D. erecta, and UGA in D. simulans and D. melanogaster. All three species have two in-frame methionine codons at the beginning of the ORF. It is not known which of these is used as the initiation codon in vivo. The exon/intron arrangement has been preserved: the splice junction sequences are conserved, and they occur in the same reading frame positions in all three species. While the occurrence of the alternative splice of intron 1 has not been directly demonstrated in these other species, the conservation of the 5′ and 3′ splice junctions, and the conserved female specific ORF, strongly suggest that the expression of tra in these species is very similar to that in D. melanogaster. Northern blot analysis of tra expression in D. erecta shows that there is a sex-specific pattern of tra transcripts indistinguishable from that seen in D. melanogaster (data not shown). Additional Northern blot analyses using strand specific probes, also show that the arrangement of the flanking transcription units is similar to that in D. melanogaster; i.e., the 5′ flanking gene is transcribed from the same strand as tra and has its 3′ end near the start of the tra transcription unit, and the 3′ neighboring gene is transcribed from the opposite strand as tra and has its 3′ end very near the 3′ end of the tra gene (data not shown). In D. melanogaster, the 3′ neighboring gene, (3)73Ah, corresponding to a complementation group defined by a late pupal lethal mutation, has been shown to actually overlap the tra transcription unit by about 75 bases at the 3′ ends of both genes (Boggs et al., 1987). Given the degree of sequence conservation in the 3′ untranslated region of tra (e.g., 59 continuous bases identical among all three species, the longest stretch of nucleotide sequence identity in the entire sequenced region), it is likely that this overlapping gene organization is present in these other species, as well.

Table 1 shows the nucleotide sequence conservation among these three species. As expected from the known phylogenetic relationships, the degree of similarity is higher when melanogaster and simulans are compared (93.6% identity) than when either of these species is compared to erecta (86.0% and 85.6% identity). The higher degree of conservation of sequences in intron 1 than in intron 2 is not unexpected, since intron 1 exhibits sex-specific alternative splicing while the splicing of intron 2 is presumably not regulated. In D. melanogaster it has been shown that the sex-specific splicing of intron 1 is controlled by sequences contained within the intron itself (Sosnowski, Belote and McKeown 1989), and these regulatory sequences are likely conserved. The close relationship between these members of the melanogaster subgroup, however, precludes using these comparisons to accurately delimit any well defined region of the intron that might be implicated in this regulation, since not enough time has passed for the nonimportant sequences to have diverged.

One obvious difference between the coding region of tra in D. melanogaster and that of the other two species is the presence of a 39-base pair sequence in the central exon of D. melanogaster that is missing in D. simulans and D. erecta. Given the phylogenetic relationships of these three species, the most parsimonious explanation is that there has been a duplication of this sequence in the lineage leading to D. melanogaster rather than two independent deletions.
occurring in the *D. simulans* and *D. erecta* lineages. This is supported by the fact that in *D. melanogaster*, these extra 39 bases exist as an almost perfect tandem repeat (37/39 identity) of the adjacent sequence. This duplication occurs in the very arginine-serine-rich region of the *tra* gene (see below, Figure 8), and its presence in either one or two copies is presumably not critical for the functioning of the *tra* protein.

Other smaller deletions/insertions also are seen when the coding regions of these three species are aligned (Figures 3 and 8). For example, *D. erecta* has an extra three nucleotides, encoding a cysteine, in exon 1 that are not seen in *D. melanogaster* and *D. simulans*. In exon 2 there are two sites where an extra six nucleotides, encoding R S, and an extra 15 nucleotides, encoding R E/G S R H, are present in *D. melanogaster* and *D. simulans*, but not in *D. erecta*. These differences among the coding regions yield different size *tra* proteins in the three species: 197, 184 and 178 amino acids, for *D. melanogaster*, *D. simulans* and *D. erecta*, respectively.

Because of the relatively close relationship among these three species, information about what regions of the *tra* gene are conserved and what regions are not conserved is limited. To better examine this, comparisons of these Sophophoran species with the Drosophila group species, *D. hydei* and *D. virilis*, was carried out.

Characterization of the *tra*-homologous genes of *D. hydei* and *D. virilis*: The reduced stringency conditions that were used for the isolation of the *tra*-homologous regions of *D. hydei* and *D. virilis* allow cross-hybridization of more than one genomic region, although in each of these cases there is one predominant cross-hybridization signal that is noticeably stronger than the others. Characterization of the three recombinant phage isolated from the *virilis* library showed that they all corresponded to the same genomic region. Restriction mapping of these clones yielded a map that matched our expectations based on the pattern of the most strongly hybridizing restric-
Interspecific Comparison of *tra*

**TABLE 1**

Interspecific comparisons of the *transformer* gene

<table>
<thead>
<tr>
<th>Gene region</th>
<th>Percentage</th>
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<tbody>
<tr>
<td></td>
<td>melanogaster</td>
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<tr>
<td>Entire gene</td>
<td>93.6</td>
</tr>
<tr>
<td>Coding region</td>
<td></td>
</tr>
<tr>
<td>Intron 1</td>
<td>93.1</td>
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<tr>
<td>Intron 2</td>
<td>92.7</td>
</tr>
<tr>
<td>Protein sequence</td>
<td></td>
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</tbody>
</table>

All DNA sequence comparisons were done using the algorithm of Wilbur and Lipman (1983). Percentages refer to the nucleotide sequence similarity index.

The entire gene includes coding regions, the two introns, 100 base pairs of sequence 3' to the end of the coding region, and 100–150 base pairs of DNA 5' to the start of the coding region.

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**i.h. indicates that there was insufficient homology for a meaningful comparison to be made.**

**Protein sequence comparisons were done using the algorithm of Needleman and Wunsch (1970). Percentages refer to the amount of amino acid identity in the aligned sequences.**

Isolated using reduced stringency hybridization conditions, and since such conditions permit cross-hybridization to more than one gene region in genomic Southern blots, it is conceivable that the primary candidates for the *tra*-homologous genes were not really the true *tra* homologs. To provide supporting evidence for our assumptions that the *λ* Dh107.1 clone and the *λ* Dv3 clone represent the *tra*-homologous regions from *D. hydei* and *D. virilis*, respectively, we mapped their chromosomal positions by in situ hybridization to salivary gland chromosomes. The chromosomal position of the *tra* gene in *D. melanogaster* is 73A9.10 on the left arm of chromosome three (Butler et al. 1986; McKeown, Belote and Baker 1987). If chromosome linkage arrangements are conserved among these three species, as is suggested by several studies involving in situ hybridization of cloned genes to polytene chromosomes of related species (Brock and Roberts 1983; Loukas and Kafatos 1986; Whiting et al. 1989; Tonzetich, Hayashi and Grigliatti 1990), then we would expect the *tra*-homologous gene of *D. virilis* to map to chromosome three (Alexander 1976) and that of *D. hydei* to map to chromosome four [using Berendes' (1963) chromosome numbering system].
When a 2.8-kb *PstI* fragment containing the *tra* cross-hybridizing sequences from *D. virilis* was used as probe to *D. virilis* salivary gland chromosomes, hybridization was detected at a single site, band 30F3.4 of chromosome 3, from the salivary gland chromosomes of *D. virilis* (Lerebeff 1939; Gubenko and Evgen’ev 1984), and the *D. hydei* map, showing the tip of chromosome 4, is redrawn from Berek (1935) and Ananiev and Barsky (1982). The approximate map position of the *D. virilis* intersex gene, *ix*, as determined by recombinational mapping experiments (Lerebeff 1939; Gubenko and Evgen’ev 1984), is also indicated.

![Figure 4](image-url)

**Figure 4.** — In situ hybridization of *tra*-homologous genomic clones to third instar larval salivary gland chromosomes from (a) *D. virilis* and (b) *D. hydei*. Sketches above the photomicrographs show the map positions as determined by the hybridization signal. The *D. virilis* map, showing the tip of chromosome 3, is drawn from the photomicrographs of Gubenko and Evgen’ev (1984), and the *D. hydei* map, showing the tip of chromosome 4, is redrawn from Berek (1935) and Ananiev and Barsky (1982). The approximate map position of the *D. virilis* intersex gene, *ix*, as determined by recombinational mapping experiments (Lerebeff 1939; Gubenko and Evgen’ev 1984), is also indicated.

Hybridization of a probe containing the putative *D. hydei* *tra*-homologous gene (i.e., p3.0H1.7S, a subclone of the 3.0-kb *Hind*III fragment from λ Dh107.1) to *D. hydei* salivary gland chromosomes resulted in a single hybridization signal at position 93A on chromosome 4 (Figure 4b). As with the *D. virilis* *tra*-homologous clone, the chromosome linkage of the *D. hydei* clone is consistent with our expectations based on the assumption that it represents the *tra*-homologous gene.

While these experiments do not prove that these clones represent the *tra*-homologous genes, they do provide good supporting evidence that this is the case, and taken together with the results presented below, it is almost certain that these genes from *D. hydei* and *D. virilis* represent the *tra*-homologous genes.

**P element-mediated germline transformation of *D. virilis* *tra* DNA into *D. melanogaster***: As an additional test to confirm that the *tra*-cross-hybridizing clones from the Drosophila subgroup species represent the *tra*-homologous gene, we used *P* element germline transformation methods to introduce the putative *tra* gene from *D. virilis* into *D. melanogaster*, and then tested the transduced gene for its ability to provide *tra* + function in *tra* mutant flies. The 2.8-kb *PstI* fragment containing the *tra* cross-hybridizing region from *D. virilis* was subcloned into the *P* element transformation vector pW8 (Klemenz, Weber and Gehring 1987) and introduced into the *D. melanogaster* genome by germline transformation. One transgenic line was obtained, which was then crossed to the appropriate stocks to construct chromosomally female flies that were homozygous for the *tra* mutant allele. This allele is a deletion of the *tra* coding region (Mckeeon, Boggs and Belote 1988). These transgenic flies should be phenotypically male unless the transduced *virilis* *tra* DNA is able to supply *tra* + activity, and thus shift the flies’ phenotypes towards femaleness. As shown in Figure 5, the *D. virilis* *tra*-homologous transgene is able to supply *tra* + function. This is most evident in the genital region of the adult, where there is a marked reduction in the development of male genital structures, such as parts of the penis apparatus and the claspers, and the appearance of female-specific structures, such as vaginal plates and thorn bristles. The shift toward femaleness is also apparent in the altered pigmentation patterns of the abdominal tergites, as well as in the structure of the anal plates. Additional evidence of a shift toward...
female development is seen in the sex-comb region of the foreleg, where the transgenic flies have bristles resembling what is seen in females, rather than the thick, blunt bristles characteristic of the male sex comb. While the shift toward femaleness is not complete in this transgenic line, it has been observed that even the D. melanogaster tra gene is frequently unable to completely rescue the tra- mutation when transduced into the genome by P element-mediated germ-line transformation. In fact, in most of the reported experiments where transgenic flies carrying transduced tra+ genes have been produced, only a minority of the lines showed complete phenotypic rescue of tra mutants (Butler et al. 1986; McKeown, Belote and Baker 1987; Feng, Schiff and Cavener 1991; J. Belote and M. McKeown, unpublished observations). This lack of complete rescue in these experiments is likely due to the tra transgene being especially sensitive to chromosomal position effects. Regardless of the reason for the failure of complete female development of XX; tra/tra flies in our transgenic line, the observation that there is substantial female differentiation shows that the tra-homologous gene from D. virilis is able to function quite well in D. melanogaster. The fact that chromosomal males carrying this transduced virilis tra gene are normal and fertile further suggests that the sex-specific alternative splicing of the first intron is being properly carried out, since other studies on the D. melanogaster tra gene show that the loss of the non-sex-specific splice site most frequently leads to default splicing of the tra pre-mRNA at the female-specific 3′ splice site in XY individuals, leading to male sterility and/or sex transformation toward femaleness (Sosnowski, Belote and McKeown 1989; B. A. Sosnowski, R. T. Boggs and M. McKeown, personal communication).

**DNA sequences of the tra-homologous genes of the Drosophila species, D. hydei and D. virilis:** Southern blot analysis of the recombinant λ phage clones λDh107.1, from D. hydei, and λDv5, from D. virilis, using the D. melanogaster tra cDNA clone pBltraP3.1 (Boggs et al. 1987) as a probe indicated that the tra-homologous genes were located within a 3.0-kb HindIII fragment of the hydei phage and within a 2.8-kb PstI fragment of the virilis phage. These fragments were therefore subcloned into plasmid vectors and a portion, corresponding to the tra transcribed region, of each was sequenced. Figure 6 shows the nucleotide sequences of these two species, and Table 1 indicates the degree of similarity among all of the species examined. The alignment of the sequences with each other, and with that of D. melanogaster, suggests that the approximate size and structure of the tra gene is fairly well conserved among all of the species examined. The protein-coding regions and the positions of the introns were deduced by comparison with the melanogaster sequence. Unlike the case with the Sophophoran species, there is only one methionine codon at the beginning of the ORF in these two Drosophila species and it corresponds to the second ATG seen in the Sophophoran species. The exon/intron structure is similar in all species examined: the splice junctions of both introns are conserved at the 5′ and 3′ ends, and the introns interrupt the tra reading frame in similar ways. Intron 1 occurs between the G and the A of a conserved GAT aspartate codon, and intron 2 occurs just after a conserved tyrosine codon TAC, in all five species.

Northern blot analysis of D. hydei poly(A+) RNA shows a sex-specific pattern of tra transcripts similar to what is seen in D. melanogaster (data not shown). In order to confirm that there is sex-specific alternative splicing of the first intron in flies from the Drosophila subgenus, PCR primers flanking intron 1 were synthesized, and a cDNA-PCR analysis was carried out on RNA isolated from male and female D. virilis
sequences have been aligned using the algorithm of Wilbur and Lipman (1983). The complete nucleotide sequence is shown only for the translation start and translation stop (with asterisk) sites. The boxed sequence is the consensus polyadenylation signal. The shaded region shows what is operative in D. melanogaster.

**Figure 6.** Comparison of the DNA sequences of the *tra* genes from the Drosophila subgenus species *D. virilis* and *D. hydei*. These sequences have been aligned using the algorithm of Wilbur and Lipman (1985). The complete nucleotide sequence is shown only for *D. virilis*. For *D. hydei* only those bases that differ from the *D. virilis* sequence are given. Dashes represent deletions in the sequence relative to *D. virilis*.

Adults. As shown in Figure 7, *D. virilis* shows a sex-specific pattern of *tra* transcripts consistent with their having an alternative splicing event at intron 1 similar to what is operative in *D. melanogaster*. While the exact splice sites have not been determined for the *D. virilis* gene, comparison of the sequence with that of *D. melanogaster* strongly suggests that the non-sex-specific and female specific splice sites are as shown in Figure 7. The most striking aspect of the *tra* interspecific sequence comparison is the high degree of divergence shown by this gene (see Table 1). This is especially apparent when the Drosophila subgenus species are compared with the Sophophorans. Because of the high degree of divergence between these two groups of species, an unambiguous alignment of the sequences is impossible. At the level of nucleotide sequence comparisons, the *tra* gene region similarity among these species is only about 60% identity, too low for the DNA sequences of all five species to be reliably aligned. When the noncoding regions are discounted, the sequence similarity is still only about 60%. In fact, the two longest stretches of nucleotide sequence identity are in intron 1, 23 base pairs corresponding to the sequence immediately upstream and flanking the non-sex-specific 3' splice site (see below), and in the 3' untranslated region, 21 base pairs surrounding the sequence similarity is only about 60% identity, too low for the DNA sequences of all five species to be reliably aligned. When the noncoding regions are discounted, the sequence similarity is still only about 60%. In fact, the two longest stretches of nucleotide sequence identity are in intron 1, 23 base pairs corresponding to the sequence immediately upstream and flanking the non-sex-specific 3' splice site (see below), and in the 3' untranslated region, 21 base pairs surrounding the
sequence ATTTATTTA, a sequence which has been suggested to play a role in RNA turnover (Shaw and Kamen 1986) (Figure 6). While the comparison of D. hydei and D. virilis shows a few stretches of sequence identity in the 5' upstream region, comparison with the Sophophoran species fails to reveal any obvious conserved sequence elements in this region of the gene. One remarkable feature of these sequence comparisons is the high level of deletion/insertion changes that are observed when the protein coding sequences are compared. For example, when D. hydei and D. virilis are aligned, there are ten regions within the coding regions in which a deletion or insertion is postulated to have occurred. This suggests that there is an unusual degree of flexibility in the protein's structure that still allows function, since if there was not this flexibility, such deletions or insertions would be selected against.

Given the high degree of divergence at the level of the DNA, it is not surprising to see a correlative high level of divergence when the protein sequences are compared (Table 1 and Figure 8). When the tra proteins of all five species are aligned, there are only a few limited stretches of amino acid sequence identities. The longest stretches of conservation among all five species are two regions of six contiguous amino acids: MDADSS in the first exon and PYFADE in the second exon. The functional significance of these two conserved regions is not known. A search of the GenBank database for proteins with such regions failed to turn up any meaningful homologies. In addition to these regions, the tra genes in all species were found to contain extensive regions of arginine-serine, or serine-arginine, dipeptides interspersed throughout the central exon. While the high arginine-serine content of the tra gene is conserved, the number and spacings of these dipeptides is quite variable among the species examined. It has been observed that several proteins that play roles in pre-mRNA splicing, e.g., the gene products of the su(w) and tra-2 genes of D. melanogaster, and the 70K U1 snRNP and SF2/ASF splicing factors of humans, exhibit similar arginine-serine rich domains (Bingham et al. 1988; Amrein, Gorman and Nöthiger 1988; Goralski, Edström and Baker 1989; Query, Bentley and Keene 1989; Ge, Zuo and Manley 1991; Krainer et al. 1991). Since the tra gene product is thought to interact with the tra-2 gene product to influence the female-specific splicing of the doublesex gene's pre-mRNA, this common feature of these gene products may relate to a general role in pre-mRNA splicing. In fact, recent experiments by Li and Bingham (1991) have suggested that such arginine-serine domains help target proteins to a subnuclear compartment involved in RNA splicing.

Another interesting feature revealed by the interspecies comparisons of tra is that the carboxy-terminal one-third of all five proteins are very rich in proline residues (16/67 = 24% for D. melanogaster, 17/67 = 25% for D. simulans and D. erecta, 26/74 = 35% for D. hydei, and 20/68 = 29% for D. virilis), while the primary sequences of this part of the protein are not all that well conserved. Inspection of Figure 8 reveals that throughout most of the coding sequence, the only parts of the tra protein that can be aligned among all five species are short and scattered regions consisting of only a few contiguous amino acids. This lack of conservation among the different species' tra proteins represents one of the most extreme cases of rapid protein evolution, and is made more remarkable by our observation that the D. virilis tra-homologous gene can function reasonably well when expressed in D. melanogaster transgenic flies.

Conservation of a presumptive regulatory sequence within the alternatively spliced first intron: As mentioned above, the longest stretch of nucleotide sequence conservation occurs within intron 1, flanking the regulated non-sex-specific 3' splice site (Figure 9). This conserved sequence is contained within the
Figure 8.—The predicted amino acid sequences for the tra proteins of *D. melanogaster*, *D. simulans*, *D. erecta*, *D. hydei* and *D. virilis*. The dashes represent deletions in the sequence relative to the other species. Nonshaded boxes indicate amino acid identity among the five species. Shaded boxes highlight the arginine-serine, or serine-arginine, dipeptides found in all of the species. For some regions, especially in the central exon, the alignment shown is only one of many possible alignments consistent with the interspecific comparisons. The arrows indicate the sites of the two introns. It should be noted that it is not known whether translation is initiated at the first or the second methionine codon in the Sophophoran species.

![Diagram of introns and amino acid sequences](image)

**Figure 9.**—Comparison of the non-sex-specific 3′ splice sites of the alternatively spliced intron 1 of *D. melanogaster*, *D. simulans*, *D. erecta*, *D. hydei* and *D. virilis*. The arrow indicates the position of the 3′ splice site. The upper case letters represent nucleotides that are conserved among all five species. The boxed sequence represents the region of tra that shows the highest degree of conservation (38/43 = 88% identity) as compared to the rest of the gene.

One point that needs to be emphasized here is that the conserved intron sequences do not necessarily identify only those sequences of *tra* that interact with Sxl to control the female-specific splicing event (i.e., by interfering with the use of the non-sex-specific 3′ splice site). While the sequences that interact with Sxl should be among the conserved sequences, it is also critical for normal sexual differentiation and fertility that the non-sex-specific splice site be highly favored over the female-specific splice site in the absence of
regulation by Sxl (i.e., in males). It has been shown that even partial use of the female-specific splice site in males leads to partial sex transformation, with an accompanying sterility phenotype (Sosnowski, Belote and McKeown 1989). Therefore, it is important that the female splice site be used in preference to the downstream female-specific splice site. Any change in the sequence that reduces the ability of the non-sex-specific splice site to be used in preference to the downstream female-specific splice site would likely be selected against. Thus, the conserved sequences highlighted in Figure 9 should include not only (1) sequences that interact with Sxl protein to cause blockage of the non-sex-specific 3' splice site in females, but also (2) sequences that ensure that this non-sex-specific 3' splice site is highly favored over the downstream female-specific 3' splice site in the absence of Sxl function.

**Interspecific comparison of the non-sex-specific transcript's ORF:** In *D. melanogaster*, a deletion of the *tra* gene has no detectable phenotypic effect in XY males, suggesting that the non-sex-specific transcript has no necessary function in males (McKeown, Belote and Boggs 1988). Additional studies in this species have further suggested that the non-sex-specific transcript may not be required in females either, and that it might represent a truly nonfunctional transcript. This conclusion is based on experiments involving XX female flies that were deleted for their endogenous *tra* loci but that carried minigene constructs consisting of the female specific *tra* cDNA sequence driven by the *hsp70* heat shock promoter (McKeown, Belote and Boggs 1988). Such flies constitutively express the female specific *tra* transcript, but are missing the non-sex-specific *tra* transcript. The observation that these flies develop as females with normal sexual morphology demonstrates that the non-sex-specific transcript plays little if any role in causing female differentiation (McKeown, Belote and Boggs 1988). However, the observation that these transgenic flies were sterile left open the possibility that the non-sex-specific gene product is responsible for some subtle function necessary for female fertility. An alternative explanation for the above result is that the female-specific minigene construct is not being expressed in the proper develop-
interpretation of the extreme lack of conservation of the non-sex-specific ORF is that the non-sex-specific transcript is either not translated, or if it is, it encodes a nonfunctional polypeptide.

**DISCUSSION**

The most surprising finding to come out of this study was the unusually high degree of divergence shown by the tra coding region, when compared with other genes that have been examined by interspecific sequence comparisons among these Drosophila species. For example, published values of amino acid sequence identity between genes of *D. melanogaster* and their homologs from species of the Drosophila subgenus (e.g., *D. viridis*, *D. hydei* and *D. funebris*) range from about 55% for the *period* gene (Colot, Hall and Rosbash 1988; Thackeray and Kyriacou 1990) to 97% for the *hsp82* (Blackman and Meselson 1986) and *sina* (Neufeld, Carthew and Rubin 1991) genes, with typical values in the range of 80–90% (see Table 2). In contrast, the *tra* gene coding region shows only 31–36% amino acid identity when the genes of these two subgenera are compared.

While there are examples of genes that exhibit rapidly evolving domains interspersed within more highly conserved domains, for example, the *period* (Colot, Hall and Rosbash 1988; Thackeray and Kyriacou 1990), *sevenless* (Michael, Bowtell and Rubin 1990), *mastermind* (Newfield, Smoller and Yedvobnick 1991), *engrailed* (Kassis et al. 1986), and *hunchback* (Treier, Pfeifle and Tautz 1989) loci, in these cases the rapidly evolving sequences make up a small (i.e., <30%) part of the overall coding region, and they frequently are regions of relatively simple sequence motifs. Moreover, within the conserved portions of these genes there are reasonably long stretches (e.g., 20–90, or more, in a row) of identical amino acids. The *tra* gene is remarkable in that the rapidly evolving sequences constitute most of the coding region, and the longest contiguous stretch of identity is only nine amino acids, when the *D. melanogaster* and *D. viridis* genes are aligned, or six amino acids, when *D. melanogaster* and *D. hydei* are compared.

Because of the rapidly evolving nature of the *tra* coding region, and since transformation studies show that the *D. viridis* gene can function in transgenic *D. melanogaster*, this interspecific comparison is particularly useful in delimiting domains of the *tra* gene that are of functional significance. Molecular genetic analyses in *D. melanogaster* have suggested that the *tra* gene product acts in concert with the *tra-2* gene product, perhaps involving a physical interaction between these two proteins, in order to activate the female-specific splicing of *dsx* pre-mRNA, thereby causing female differentiation. In the experiments reported here, the *D. viridis* *tra* transgene is able to act in a reasonably normal manner to direct female development, implying that this transgene encodes a *tra* protein that is able to act together with the *D. melanogaster* *tra-2* protein to direct the female-specific splicing of the *melanogaster* *dsx* pre-mRNA. Thus, the rather limited sequence conservation of the *tra* coding region highlights the elements of the protein that are sufficient for these functions, and this information should provide guidance for further studies aimed at a more detailed dissection of the *tra* protein's function.

While the present study was confined to comparisons within the genus Drosophila, future studies of a more practical nature might involve the isolation of the *tra*-homologous genes from other insect species (e.g., certain insect pests), with the long-range goal of using them as agents for the manipulation of sexual development, or in schemes for the generation of unisexual laboratory populations. The results presented here suggest that strategies involving cross-hybridization might be problematic for the isolation of this particular gene, due to its unusually high evolutionary divergence. However, this interspecific sequence comparison does highlight a few sequences within the *tra* gene that are conserved, and that can therefore be used for designing primers that might facilitate the isolation of the *tra* homologous genes from other, more distantly related diptera, by PCR amplification methods.

We are indebted to Karen Kaczman Daniel and Mary Miller.

### Table 2

Amino acid sequence identity between genes of *D. melanogaster* and their homologous genes in species of the *Drosophila* subgenus

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species compared with <em>D. melanogaster</em></th>
<th>Amino acid identity (%)</th>
<th>Referencesa</th>
</tr>
</thead>
<tbody>
<tr>
<td>transformer</td>
<td><em>D. hydei</em></td>
<td>31</td>
<td>1</td>
</tr>
<tr>
<td>transformer</td>
<td><em>D. viridis</em></td>
<td>36</td>
<td>1</td>
</tr>
<tr>
<td>period</td>
<td><em>D. viridis</em></td>
<td>~55</td>
<td>2</td>
</tr>
<tr>
<td>sevenless</td>
<td><em>D. viridis</em></td>
<td>63</td>
<td>3</td>
</tr>
<tr>
<td>E74</td>
<td><em>D. viridis</em></td>
<td>65</td>
<td>4</td>
</tr>
<tr>
<td>hunchback</td>
<td><em>D. viridis</em></td>
<td>~80</td>
<td>5</td>
</tr>
<tr>
<td>engrailed</td>
<td><em>D. viridis</em></td>
<td>~80</td>
<td>6</td>
</tr>
<tr>
<td>(3)Ai</td>
<td><em>D. viridis</em></td>
<td>~80</td>
<td>7</td>
</tr>
<tr>
<td>Adh</td>
<td><em>D. hydei</em></td>
<td>82</td>
<td>8</td>
</tr>
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<td>Ultrabithorax</td>
<td><em>D. funebris</em></td>
<td>85</td>
<td>9</td>
</tr>
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<td><em>D. viridis</em></td>
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</tr>
<tr>
<td>Rh4</td>
<td><em>D. viridis</em></td>
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<tr>
<td>sina</td>
<td><em>D. viridis</em></td>
<td>97</td>
<td>11</td>
</tr>
<tr>
<td>hsp82</td>
<td><em>D. viridis</em></td>
<td>97</td>
<td>12</td>
</tr>
</tbody>
</table>

a The references are (1) this report; (2) Colot, Hall and Rosbash (1988); (3) Michael, Bowtell and Rubin (1990); (4) Jones, Dalton and Townley (1991); (5) Treier, Pfeifle and Tautz (1989); (6) Kassis et al. (1986); (7) K. Saville and J. M. Belote, unpublished; (8) Sullivan, Atkinson and Starmer (1990); (9) Wilde and Aram (1987); (10) Neufeld, Smoller and Yedvobnick (1991); (11) Neufeld, Carthew and Rubin (1991); (12) Blackman and Meselson (1986).
Interspecific Comparison of tra


Li, H. and P. M. Bingham, 1991 Arginine/serine rich domains for their excellent technical assistance, and to Shannon Irving for assistance with the photography. We thank Donal Hickey, Cynthia Smith and David Sullivan for providing some of the fly strains and/or genomic libraries used in this study. We especially thank David Sullivan, Shoko Yokowama and Tom Starmer for their helpful comments. This work was supported by U.S. Public Health Service grant GM36579 from the National Institutes of Health.

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