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Alternatively spliced transcripts of the sex-determining gene *tra-2* of *Drosophila* encode functional proteins of different size

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The *Drosophila transformer-2* gene (*tra-2*) is required for female sex determination in somatic cells and for spermatogenesis in male germ cells. We studied the organization of the *tra-2* gene and characterized the transcripts in wild type and mutant animals. Two transcripts are detected in males and females; they differ in their abundance and in the presence (minor transcript T_{min}) or absence (major transcript T_{maj}) of one exon. Two other transcripts are present only in male germ cells. One of these is rare (msT_{min}) and represents a spliced form of the other, more abundant transcript (msT_{maj}). The transcript T_{maj} encodes a protein of 264 amino acids, whereas transcripts T_{min} and msT_{maj} encode proteins that are truncated at the N-terminus. All three putative proteins contain a stretch of ~90 amino acids, the ribonucleoprotein motif (RNP motif), which shows similarity to a variety of different ribonucleoproteins. Transformation studies reveal that a cDNA corresponding to the transcript T_{maj} can provide all the functions for female sex determination and male fertility. Surprisingly, a cDNA corresponding to the transcript msT_{maj} could only supply some female sex-determining function, but was unable to restore fertility in mutant males. Sequence analysis of two temperature-sensitive mutations provides evidence that the RNP motif represents an important functional domain of the *tra-2* protein.

Key words: alternative splicing/*Drosophila* sex determination/spermatogenesis

Introduction

transformer-2 (*tra-2*) is one of a few genes that determine sex in *Drosophila melanogaster* (reviewed by Baker, 1989; Steinmann-Zwicky *et al.*, 1990). The function of *tra-2* is required in somatic cells of females where it is essential, together with that of *transformer* (*tra*), for expression of the gene *doublesex* (*dsx*) in the female-specific mode. Lack of function of *tra-2* or *tra* leads to an alternative, male-specific expression of the *dsx* gene (Nagoshi *et al.*, 1988), resulting in sexual transformation of females (XX) into sterile males, so-called pseudomales. Null mutations in *tra* or *tra-2* have no effect on the sexual differentiation of somatic cells of males (XY). The alternative, male-specific expression of *dsx* is typical of wild type males which do not express *tra* protein (Boggs *et al.*, 1987).

The regulation of *dsx* occurs at the level of differential RNA processing. The *dsx* transcripts of males and females are identical within the first three exons, but differ in the 3′ most exons. Consequently, these different sex-specific *dsx* transcripts encode proteins that share a common N-terminus, but have different C-termini (Burtis and Baker, 1989). In females, the proteins encoded by *tra-2* and *tra* are probably directly involved in the processing of the *dsx* pre-mRNA to produce a female-specific *dsx* mRNA, as supported by three observations. First, *Sex-lethal* (*Sxl*), the only other gene known to affect *dsx* expression, acts indirectly on *dsx* by regulating alternative splicing of the *tra* pre-mRNA. In females, the presence of *Sxl* protein allows the splicing of a *tra* mRNA which encodes a functional protein. In males, the lack of *Sxl* protein leads to a default splice and the production of a non-functional *tra* mRNA with no long open reading frame (ORF; McKeown *et al.*, 1988; Nagoshi *et al.*, 1988; Sosnowski *et al.*, 1989). Second, mutations in *tra-2* do not affect female-specific splicing of the *tra* mRNA (Nagoshi *et al.*, 1988), suggesting that *tra-2* does not act through regulation of *tra* on the *dsx* pre-mRNA. Third, the *tra-2* protein contains a conserved domain of ~90 amino acids (Amrein *et al.*, 1988; Goralski *et al.*, 1989), the ribonucleoprotein motif (RNP motif), which has been found in many different RNA-binding proteins (reviewed by Bandziulis *et al.*, 1989). In several cases, namely the U1 70K protein (Query *et al.*, 1989), the U1A protein (Scherly *et al.*, 1989) and the U2B″ protein (Scherly *et al.*, 1990), the RNP motif and a few amino acids adjacent to it have been shown to be sufficient for specific RNA binding *in vitro*. The role of the RNP motif of *tra-2* in female-specific splicing of the *dsx* pre-mRNA remains to be established.

In addition to its regulatory role in female somatic sex determination, *tra-2* function is also required in male germ cells for spermatogenesis (Schüpbach, 1982; Belote and Baker, 1982, 1983). Since the function of *dsx* is dispensable in the male germ line (Schüpbach, 1982), the substrate of the *tra-2* protein must be different in this tissue.

Transcripts of the *tra-2* gene containing the RNP motif are present in somatic cells of females and in germ cells of males where *tra-2* function is required, but also in somatic cells of males and in germ cells of females (Amrein *et al.*, 1988) where lack of *tra-2* function has no effect (Watanabe, 1975; Schüpbach, 1982). Moreover, a functional *tra-2* protein appears to be present in somatic cells of males since constitutive expression of a cDNA encoding a functional *tra* protein results in sexual transformation of XY animals into pseudofemales (McKeown *et al.*, 1988).

Based on the sequence analysis of a *tra-2* cDNA and S1 nuclease mapping, we have previously not found any evidence for different forms of *tra-2* transcripts in males and females (Amrein *et al.*, 1988). Sequence analysis of different genomic and cDNA clones of *tra-2*, however, revealed that the first 28 nucleotides of the cDNA sequence shown in our initial study were the result of a double insert (Amrein *et al.*,

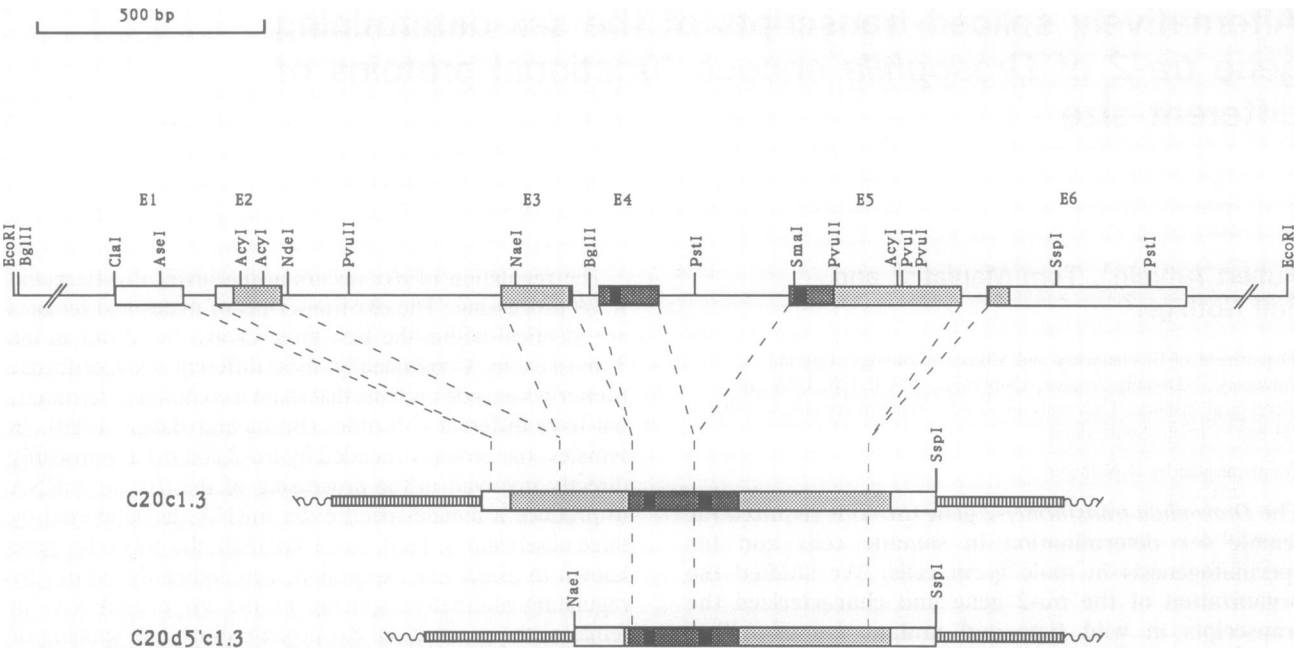

Fig. 1. Organization of the *tra-2* gene and representation of *tra-2* cDNAs used for transformation studies. The major *tra-2* transcript T_{maj} consists of six exons (E1−E6), shown as boxes, which map within a genomic *Eco*RI fragment of 3.9 kb that provides both functions of *tra-2* (Goralski *et al.*, 1989). Coding sequences are represented by light hatched boxes, the ribonucleoprotein motif (RNP motif; Bandziulis *et al.*, 1989) by dark hatched boxes, and the highly conserved region within this domain (RNP1 and RNP2) by black boxes. The structures of the two *tra-2* cDNAs used in transformation studies are shown below. Promoter and trailer sequences are derived from the *Drosophila hsp70* gene (see Materials and methods) and are shown as striped bars. Sequences from the Carnegie 20 transformation vector (Rubin and Spradling, 1982) are given as wavy lines.

1989); in addition, S1 nuclease mapping indicated that alternatively processed forms of *tra-2* transcripts are present in both males and females.

We have extended our previous molecular analysis of *tra-2* to elucidate the nature of these different transcripts and to determine their functional role in somatic cells of females and in germ cells of males. We determined the genetic organization and sequence of the *tra-2* gene and identified four different transcripts which are produced by differential splicing and/or the use of a different promoter. Three of these transcripts, T_{maj}, T_{min} and msT_{maj}, have been characterized in detail. The corresponding proteins differ in the length of their N-terminal sequences, but they all contain the RNP motif. Expression of a cDNA corresponding to the major non sex-specific transcript T_{maj} can completely rescue the somatic sexual transformation of females and, to a certain extent, the sterility of males in *tra-2* homozygous mutant flies. Surprisingly, a *tra-2* cDNA corresponding to the male germ cell-specific transcript msT_{maj} did not rescue the sterility of males, but did partially rescue the somatic sexual transformation of females.

Analysis of three *tra-2* mutations reveals that the RNP motif is essential for *tra-2* function. We find that a substitution of a single amino acid within the RNP motif has more severe phenotypic effects than a substitution or even a truncation outside this region.

Results

Genomic organization of the tra-2 gene

P-mediated transformation experiments demonstrated that both *tra-2* functions are provided by a 3.9 kb DNA fragment (Goralski *et al.*, 1989). Comparison of the DNA sequence of the *tra-2* gene with the sequence of c1.3, a *tra-2* cDNA clone (Amrein *et al.*, 1988, 1989), revealed that the gene consists of 6 exons (Figures 1 and 2). The ORF, defined by stop codons in the second and in the last exon, is preceded by an untranslated first exon and followed by 400 nucleotides of 3′ noncoding sequences.

To define the 5′ ends of the transcripts in the two sexes, we performed RNase protection experiments using the two genomic probes 1A and 1B (Figure 3A). Both probes protect one major fragment of 144 nucleotides in RNA from males and females (Figure 4A) indicating that this fragment represents the first exon (E1). The abundance of the protected fragment is much lower in wild type males than in females; it is also low in females and males derived from *osk* mothers and thus lacking germ cells, and in animals homozygous for the weak mutation $tra\text{-}2^{P}$. A higher level of transcripts detected in wild type females than in females lacking germ cells suggests that the germ cells provide the majority of *tra-2* transcripts; this observation is consistent with the finding that early embryos contain high levels of *tra-2* transcripts (Amrein *et al.*, 1988, and data not shown).

Alternative splicing of the tra-2 transcripts occurs in the second intron

Transcripts containing E1 (see above), E2 (Figure 4B; probe 2) and the coding region beginning with the third exon (E3, E4, E5 and E6) are present in both sexes and correspond to cDNA c1.3 (Amrein *et al.*, 1988). RNase protection assays using various probes derived from the remaining region of the *tra-2* gene (Figure 3A; probes 3A, 3B, 3C and 3D), however, reveal that distinct forms of *tra-2* transcripts are present in both males and females. The most abundant fragment protected with probe 3A corresponds to E3, and we designated transcripts consisting of E1, E2, E3, E4, E5 and E6, also represented by cDNA c1.3, as the major non

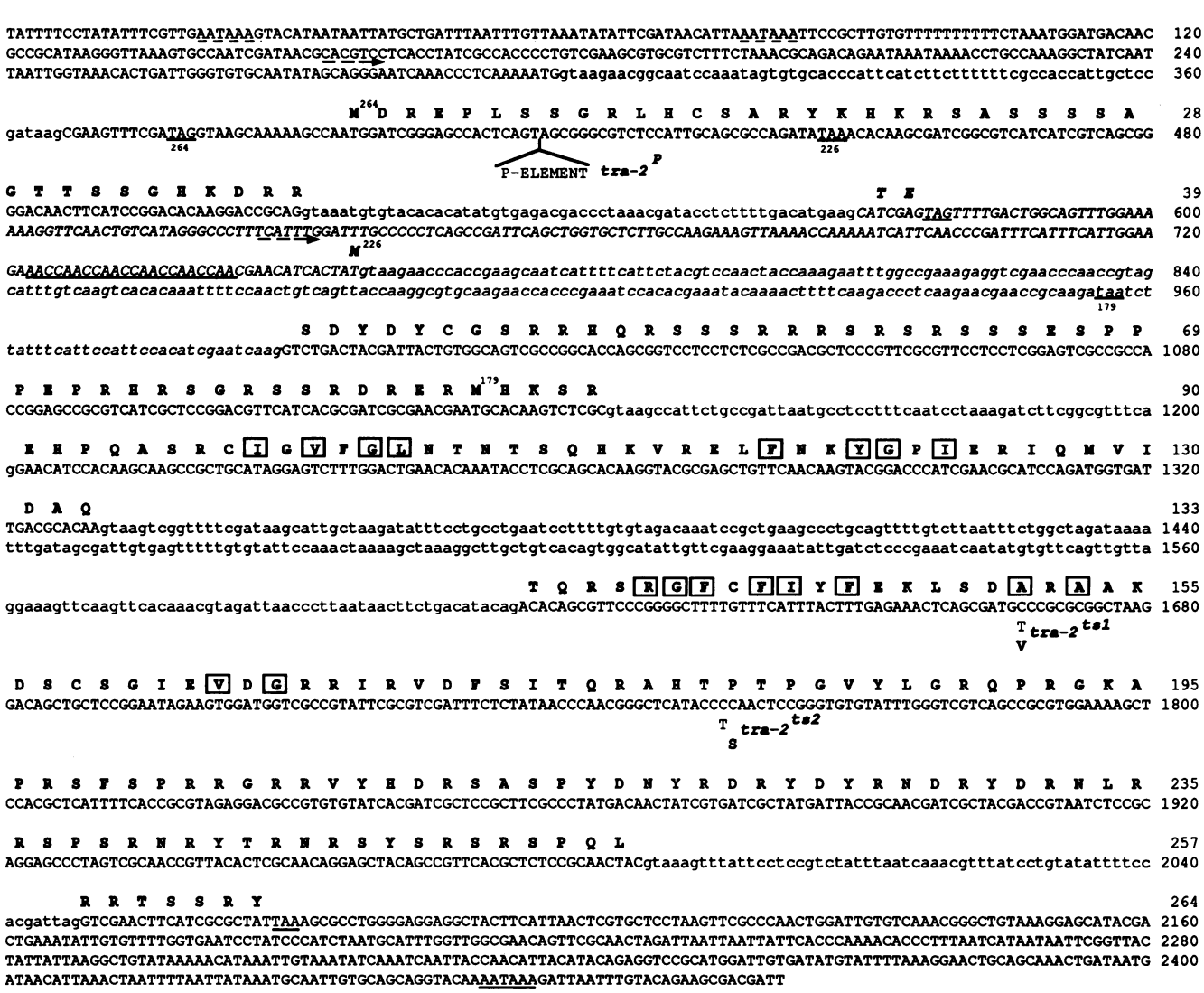

Fig. 2. Genomic sequence of *tra-2* wild type and mutant genes. Exon sequences and sequences preceding the first intron are shown in capital, intron sequences in lower case letters. Exon E2′ which is present only in the minor non sex-specific *tra-2* transcript (T_{min}) is shown in capital italic letters. The 5′ ends of the male-specific transcripts (~nucleotide 630) and the non sex-specific transcripts (~nucleotide 155) are indicated by arrows (see also Figure 3B). The 5′ stop codons defining the ORFs of each of the transcript species are underlined; the numbers below the stop codons indicate the numbers of amino acids encoded by the corresponding ORFs. The stop codon that interrupts the major ORF of T_{min}, the common 3′ stop codon and the polyadenylation signal are underlined. The 5.5 tetranucleotide repeats (nucleotides 723–744) in which a deletion polymorphism was observed in one cDNA clone are underlined. The amino acid sequence is shown in bold capitals above the corresponding exon sequences. The three methionine residues corresponding to the translation initiation codons of the three putative *tra-2* proteins are indicated with the number of amino acids. Highly conserved amino acid residues (18 or more conserved residues in 32 RNP motifs examined; Bandziulis *et al.*, 1989) within the RNP motif are shown as boxes. The positions of the single substitutions found in the two temperature-sensitive mutations *tra-2*^{ts1} and *tra-2*^{ts2} and the P-element insertion site in *tra-2*^{P} are indicated below the nucleotide sequence. Nucleotide residue 500 is C in the wild type *tra-2* gene and in *tra-2*^{ts2}, whereas it is T in cDNA c1.3 and in *tra-2*^{ts1}; this substitution, however, has no consequence at the amino acid level. The P-element insertion site of the allele *tra-2*^{P} was derived from sequence analysis of plasmid bsSB3.8 (Amrein *et al.*, 1988). Direction of transcription of the P-element is the same as for the *tra-2* gene. The sequence between the junction of the P-element (putative translation initiation codon is shown in bold letters) and the *tra-2* gene (underlined) is: ...ACC′TTA′TGT′TAT′TTC′ATC′ATG′AGC′GGG′CGT′CTC...

sex-specific *tra-2* transcript T_{maj}. In addition, another non sex-specific, but less abundant fragment of 190 nucleotides (E2′) and several male-specific fragments (msE3^{u}, msE3^{d} and msE3^{p}) are also protected with this probe (Figure 4C, probe 3A). It should be noted that the male-specific fragments do not represent several exons, but only one represented by the 510 nucleotide fragment msE3^{u} (male-specific exon, uncleaved). The fragments msE3^{d} and msE3^{p} (~110 + ~400 = ~510 nucleotides) are cleaved due to a DNA polymorphism within our wild type strain and represent the distal and the proximal portion of virtually the same exon (see below). Interestingly, the male-specific transcripts are only found in fertile males such as wild type and homozygous *tra-2*^{P} males, but not in animals that lack a functional male germ line such as females, *XX;tra-2*^{P}/*tra-2*^{P} intersexes or males derived from *osk* mothers (Figure 4C). Thus, we conclude that these fragments represent part of a *tra-2* transcript (msT$_{maj}$) that is expressed, perhaps exclusively, in male germ cells. This conclusion is supported by *in situ* hybridization experiments of probe c1.3 to the testes of adult males which show high levels of transcripts in spermatocytes of different maturation stages (data not shown).

The non sex-specific fragment of 190 nucleotides,

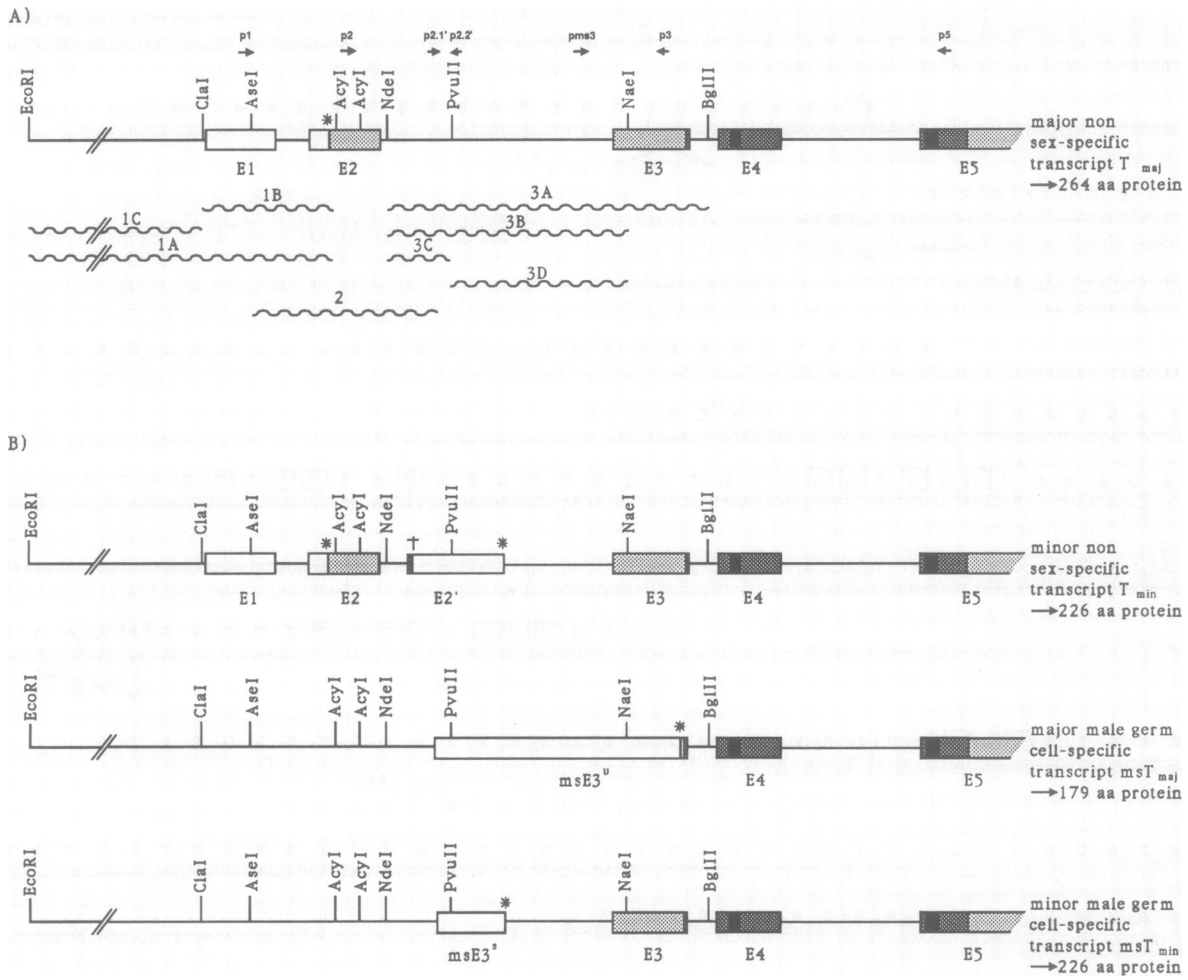

Fig. 3. Structures of the different *tra-2* transcripts. The organization of the exons and introns is represented as in Figure 1. The ORF in the 3′ region of all transcripts is identical and not shown. Only restriction sites that have been used for preparing the different RNA probes are shown. * indicates translation start sites; + indicates the stop codon of the main ORF in transcript T_{min}. (**A**) shows the structure of the major non sex-specific transcript (T_{maj}). The wavy lines below indicate the antisense RNA probes used in the RNase protection assays shown in Figure 4. (**B**) shows the structure of the minor non sex-specific (T_{min}) and the male germ cell-specific transcripts (msT_{maj} and msT_{min}). Transcript T_{min} consists of the same six exons as transcript T_{maj} plus E2′ which is joined to the adjacent exons E2 and E3. Primers used in PCR for amplification of transcript T_{min} were p2.2′ and p1, and p5 and p2.1′. From the male-specific transcripts only the structure of msT_{maj} has been determined by PCR of RNA from males using primers p5 and pms3. The structure of msT_{min} is based on the assumption that $msE3^s$ is spliced regularly to its next downstream exon E3.

protected with probe 3A, is also protected with probe 3B (Figure 4C, left lanes), indicating that this fragment represents an additional exon E2′. The abundance of E2′ varied between different RNA preparations so that this exon was sometimes barely detectable in males due to the DNA polymorphism (see below and Figure 5). The 5′ and 3′ ends of E2′ have been determined by two probes representing sequences either upstream (probe 3C) or downstream (probe 3D) of the *Pvu*II site (see Figure 3). These probes protect fragments of 90 and 100 nucleotides, respectively (data not shown). The presence of splice sites at both ends of this exon and a polypyrimidine stretch preceding the putative 3′ splice site predict a size of 188 nucleotides (Figure 2). Due to its low abundance, transcripts containing E2′ are designated as the minor non sex-specific transcript T_{min}.

With probe 3B the sizes of $msE3^u$ and $msE3^p$ are reduced to 385 and 275 nucleotides, respectively (Figure 4C and 5A), indicating that these fragments represent an exon of msT_{maj} which consists of all the sequences of E3 and 355 nucleotides of the adjacent intron 2 (Figure 2 and 3).

The male-specific transcript msT_{maj} and the minor non sex-specific transcript T_{min} encode 5′ truncated tra-2 proteins

To determine the putative protein sequences that might be encoded by the transcripts msT_{maj} and T_{min}, we cloned and sequenced corresponding cDNAs using the polymerase chain reaction (PCR). Analysis of cDNAs amplified from male RNA using oligonucleotide primers p5 and pms3 (Figure 3A) revealed that msE3 is spliced to the next downstream exon E4. This is consistent with our previous observation that all detectable transcripts downstream of E3 correspond to cDNA c1.3 (Amrein *et al.*, 1988). Thus, transcript msT_{maj} consisting of at least $msE3^u$, E4, E5 and E6 contains an ORF which is defined by a stop codon at position 955 in $msE3^u$ and the stop codon at position 2069. The putative male-specific protein corresponds to the 179 C-terminal amino acids of the major *tra-2* protein.

In similar experiments we determined the structure of transcript T_{min}. Sequence analysis of cDNAs, amplified from RNA with primers p2.2′ and p1 or primers p5 and

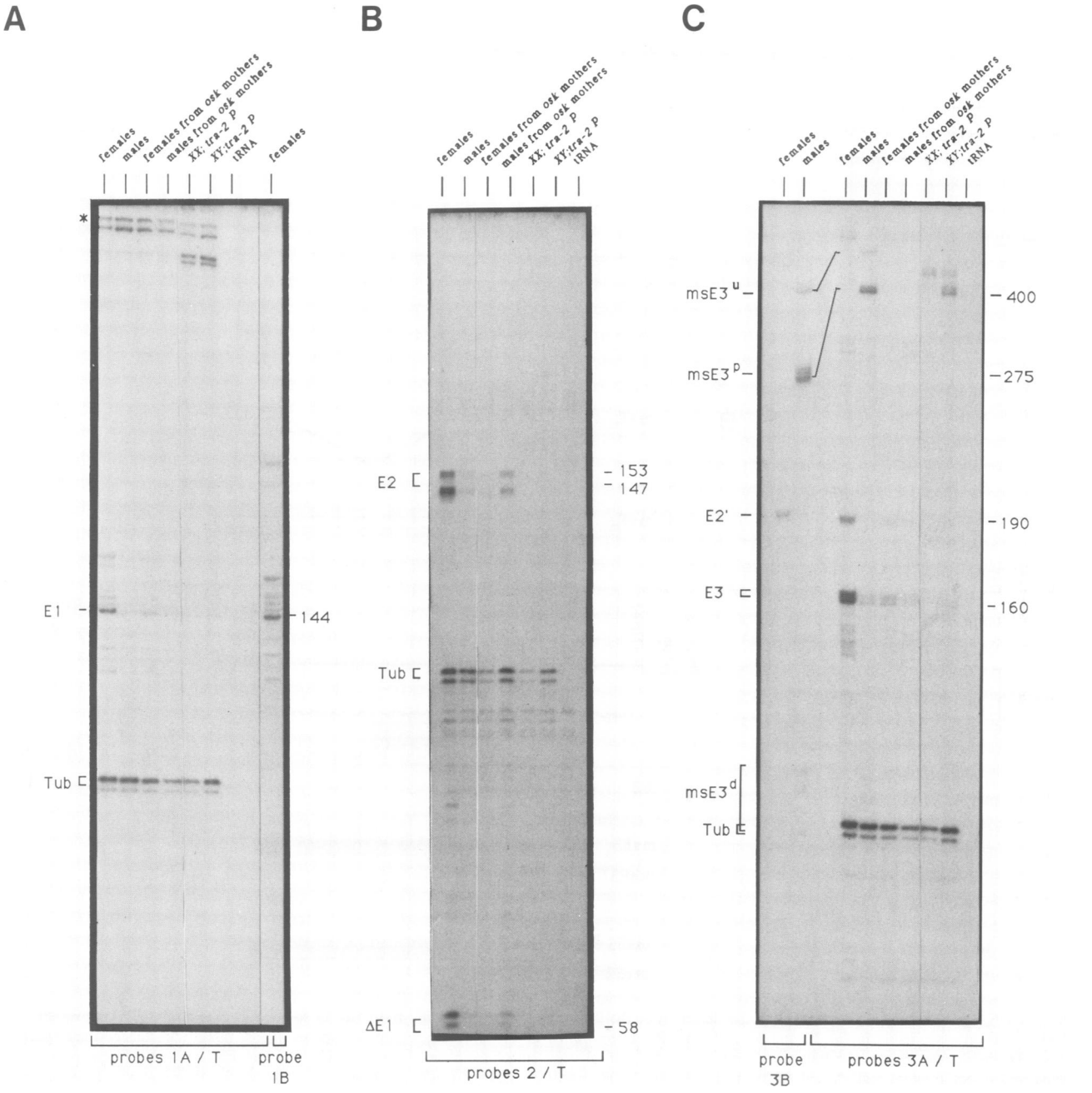

Fig. 4. Analysis of alternatively spliced exons in wild type and mutant *tra-2* flies. 10–20 μg of total RNA were hybridized with uniformly labelled RNA probes, treated with RNase A and separated on denaturing polyacrylamide gels. The size and nature of the specifically protected fragments are shown alongside the gels. In the reactions with probes 1A, 2 and 3A, an antisense RNA probe (probe T) from a part of the first exon of the α-tubulin gene was included to compare loading of RNA in the different genotypes. Animals lacking germ cells were obtained from females homozygous for the mutation osk^{301} (Lehmann and Nüsslein-Volhard, 1986). (**A**) E1 is protected with both probes 1A and 1B. Slightly longer and shorter, but much less abundant fragments are due to improper RNase cleavage and/or might reflect different transcription start sites. The fragments only protected with RNA from $tra\text{-}2^P$ animals represent fragments most likely derived from aberrantly spliced *tra-2* transcripts. * indicates two large fragments (>450 and >500 nucleotides) which were also protected with probe 1C (data not shown). These two fragments represent the 3′ ends of a gene upstream of *tra-2* and do not belong to the *tra-2* transcription unit, as indicated by the following observations. First, sequence analysis revealed the presence of three putative polyadenylation signals 421, 513 and 570 nucleotides downstream of the *Eco*RI sites (see also Figure 2); the size of the two fragments protected with probes 1A and 1C is consistent with fragments defined by the *Eco*RI site and cleavage sites following the first two polyadenylation signals. Second, the levels of these transcripts are very similar in all different RNAs analysed, unlike those of the *tra-2* transcripts. And third, the *Eco*RI site lies within the ORF of these transcripts (H.Amrein, unpublished data), whereas a fragment of 3.9 kb of genomic DNA downstream of this *Eco*RI site is sufficient for complete *tra-2* function (Goralski *et al.*, 1989). (**B**) E2 is protected with probe 2 and represented by two fragments, probably due to use of two 3′ splice sites of intron 1 (nucleotides 365/366 and 368/369). The cryptic splice site six nucleotides downstream of the regular 5′ splice site of intron 2 does not seem to account for the longer fragment since the sequences of ten different cDNAs amplified from RNA of females using primers p2 and p3 (see Figure 3) were identical and correspond to the sequence of cDNA c1.3. The 3′ part of exon 1 which is also protected with this probe is indicated with ΔE1. (**C**) Probes 3A and 3B were used to define exons E2′, E3 and $msE3^u$. Due to the DNA polymorphism within our wild type as well as the $tra\text{-}2^P$ strain, the male-specific exon is represented by either one long fragment ($msE3^u$) or a distal ($msE3^d$) and a proximal ($msE3^p$) set of fragments (see text and Figure 5). $msE3^u$ and $msE3^p$ are shortened for ~125 nucleotides in both wild type (second lane from the left) and homozygous $tra\text{-}2^P$ males (not shown) if probe 3B is used; the difference in size represents the 125 nucleotides of $msE3^u$ that are present in 3A but absent in 3B.

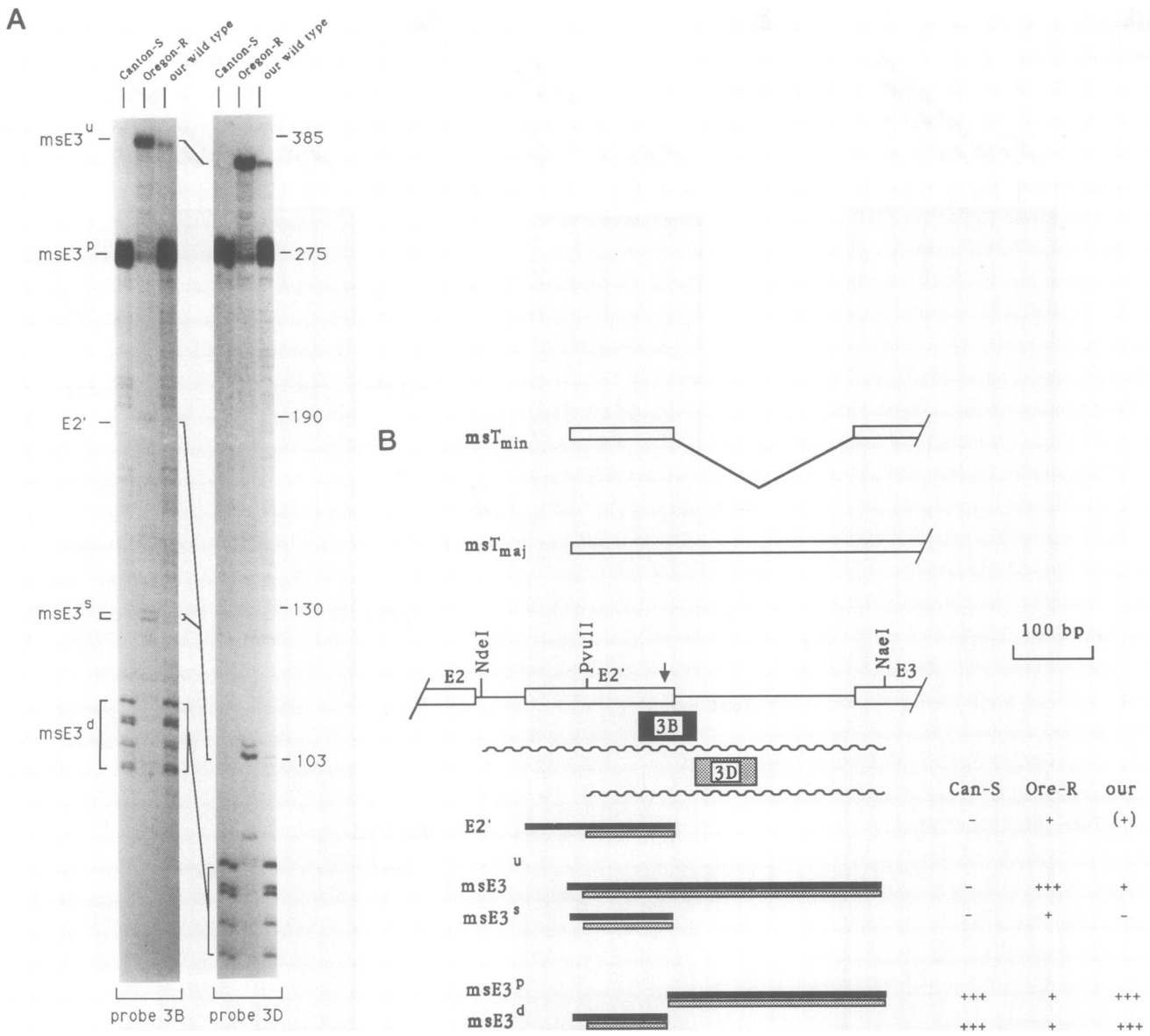

Fig. 5. A DNA polymorphism interferes with the detection of the low abundant male-specific transcript in some wild type strains. (**A**) shows an RNase protection assay using probe 3B and 3E and male RNA from three different wild type strains. Abbreviations: msE3^u, uncleaved male-specific exon of the major non sex-specific transcript (msT$_{maj}$); msE3^d, distal fragment of the cleaved male-specific exon; msE3^p, proximal fragment of the cleaved male-specific exon. msE3^s, spliced exon of the minor, male-specific transcript (msT$_{min}$). Due to the polymorphism in our wild type and in the Canton-S strain, the rare fragments msE3^s as well as E2′ are only visible as distinct bands in the Oregon-R males. Note the different sizes of fragments corresponding to msE3^d and msE3^p suggesting the presence of slightly different polymorphisms. The size of the protected fragments of msE3^u, E2′ and msE3^d is reduced as expected if probe 3E is used. (**B**) Localization of the protected fragments shown in (A) within intron 2 (thin line). E2, E2′ and E3 are shown as open boxes. The two male-specific transcripts msT$_{maj}$ and msT$_{min}$ are shown above. Protected fragments with either 3B (black bars) or 3D (hatched bars) are shown below. The panels on the right show the abundance of the corresponding fragments in the three different strains; +++ high abundance; + low abundance; (+) very low abundance; − not detectable. The arrow indicates the tetranucleotide repeats in which the DNA polymorphism occurs.

p2.1′, revealed that E2′ is spliced to the adjacent exons E2 and E3. Therefore, transcript T$_{min}$ consists of seven exons, E1, E2, E2′, E3, E4, E5 and E6. The major ORF of this transcript is interrupted by a UAG stop codon at the beginning of E2′. However, reinitiation of translation at the AUG created by ligation of E2′ to E3 (Figure 2 and 3) could encode a protein consisting of the N-terminal methionine and the 225 C-terminal amino acids also present in the major *tra-2* protein.

The two cDNA clones obtained in our PCR experiment with primers p5 and p2.1′ differed slightly from each other in the region between nucleotides 723 and 744. This region consists of the tetranucleotide motif AACC (22 nucleotides = 5.5 repeats; underlined region in Figure 2). One of the cDNA clones corresponds to the genomic sequence ('Oregon-R' type), whereas the other cDNA clone lacks two repeats ('Canton-S' type), suggesting the presence of at least two different *tra-2* wild type genes in our strain. This idea is supported by the observation that the male-specific exon is frequently cleaved in our RNase protection experiments within this region (Figure 4C and 5; msE3^d and msE3^p), but not completely (msE3^u) and indicates the existence of a DNA polymorphism within our wild type strain. We believe that the cDNA clone containing this deletion represents just one of probably several different polymorphisms as indicated by the slightly different sizes of

Table I. Ability of different *tra-2* cDNAs to rescue the *tra-2* mutant phenotype

Transformed line	cDNA construct	Copy number	Heat shock	Phenotype in XX	Phenotype in XY
T22	C20c1.3	1 or 2	no	pseudomale[1]	sterile (0/>30)[4]
T22	C20c1.3	1 or 2	yes	female[2], $n=27$	fertile (4/>40)[4]
T14 and T15	C20d5'c1.3	1	no	pseudomale	sterile (0/>35)[4]
T14 and T15	C20d5'c1.3	1	yes	intersex[3], $n=30$	sterile (0/>35)[4]
T14/T14	C20d5'c1.3	2	yes	female-like intersex[3], $n = 30$	–

C20c1.3 encodes the major, non sex-specific *tra-2* protein (264 aa), C20d5'c1.3 encodes the male-specific *tra-2* protein (179 aa; Figures 1 and 3). Heat shock treatment (36±0.5°C) was performed for 25 min every 6 h from early embryogenesis until late pupal stage. Between heat shocks, the animals were raised at 25°C. *n* indicates the number of XX animals examined.

[1]These pseudomales show occasional malformations in the genitalia due to basal heat shock promoter activity.

[2]Two females containing two copies of the Tp were fertile.

[3]Variable degree of rescue was observed in these animals (see also Figure 6).

[4]Numbers in parentheses indicate number of fertile males and total number of males tested, respectively.

Genotypes were (Tp denotes a transposon containing the respective cDNA as shown in Figure 1):

T22: One copy of Tp: *X/X* or *X/B^{s}Y; cn tra-2/tra-2 bw; Tp(C20c1.3ry$^+$) ry^{42}/ry^{42}*;
Two copies of Tp: *X/X* or *X/B^{s}Y; tra-2 bw/tra-2 bw; Tp(C20c1.3ry$^+$) ry^{42}/Tp(C20c1.3ry$^+$) ry^{42}*;

T14 and T15: *X/Tp(C20d5'c1.3ry$^+$)* or *B^{s}Y/Tp(C20d5'c1.3ry$^+$); cn tra-2/tra-2 bw; ry^{42}/ry^{42}*;

T14/T14: *Tp(C20d5'c1.3ry$^+$)/Tp(C20d5'c1.3ry$^+$); tra-2 bw/tra-2 bw; ry^{42}/ry^{42}*.

fragments msE3^d and msE3^p, respectively. It is noteworthy that all wild type strains seem to contain polymorphic 'Canton-S' type *tra-2* genes and that the Canton-S strain seems to be the only one lacking the 'Oregon-R' type *tra-2* gene (Figure 5).

A rare male-specific transcript msT$_{min}$ might encode the 226 amino acid protein

After submitting this paper, we became aware of a second male-specific transcript of low abundance (msT$_{min}$; Figure 3 and 5B). The detection of this transcript in our wild type strain was not possible due to the DNA polymorphism. RNA from Oregon-R males, however, protects in addition to the more abundant uncleaved fragment msE3^u (Figure 5) a rare doublet (msE3^s: male-specific exon, spliced) of ~128 nucleotides if 3B is used as a probe. These fragments were not observed in RNA from Oregon-R females or any other females (data not shown). The size of msE3^s corresponds well to an exon defined by the 5' end of msE3^u (~nucleotide 630; Figure 2) and a 3' end identical with that of E2' (nucleotide 757). This was confirmed with probe 3D (Figure 5B). Although other possibilities cannot be excluded, it is reasonable to assume that msE3^s is spliced to E3, and that the corresponding transcript msT$_{min}$ encodes the same protein as T$_{min}$ (226 amino acids).

A cDNA encoding the major tra-2 protein can rescue the tra-2 mutant phenotype

The high abundance of transcript T$_{maj}$ in females and the presence of a male germ cell-specific transcript msT$_{maj}$ suggest that the corresponding proteins provide the sex-determining function of females and the fertility function of males, respectively. To test this hypothesis, we transformed flies with corresponding *tra-2* cDNAs under the control of the heat shock hsp70 promoter. Flies homozygous for *tra-2* mutations carrying one or two copies of either construct were analysed (Table I). Expression of cDNA C20c1.3 encoding the major *tra-2* protein of 264 amino acids could rescue the mutant phenotypes of both sexes. XX animals differentiate female structures (Figure 6A) and can be fertile. Without heat shocks, such animals were almost perfect pseudomales (data not shown). A few abnormalities in the male genital structures suggest that some residual *tra-2* function is provided from basal hsp70 promoter activity. Similarly, the male sterility could only be rescued if *tra-2* expression was induced by heat shocks (Table I).

Unexpected results were obtained with C20d5'c1.3 encoding the male germ cell-specific *tra-2* protein of 179 amino acids. Expression of this cDNA did not restore male fertility (Table I). The same construct, however, was able to rescue XX animals, at least partially, from the sex-transforming effect, and the rescue was significantly improved when two copies were present (Figure 6B and 6C). Thus, the male germ cell-specific cDNA is able to provide some female sex-determining function, but cannot restore male fertility.

The substitution of a single amino acid within the RNP motif severely reduces the function of the tra-2 protein

The results of our cDNA transformation studies showed that the major, non sex-specific and the male germ cell-specific protein of *tra-2* are able to provide complete or partial levels of the sex-determining function. Both proteins encoded by these cDNAs contain the RNP motif suggesting that this motif is important. The sequence analysis of three leaky *tra-2* mutations, *tra-2^{ts1}*, *tra-2^{ts2}* (Belote and Baker, 1982, 1983) and *tra-2^P* (Amrein *et al.*, 1988) provides more direct evidence for the functional significance of the RNP motif. As shown in Figure 2, both temperature-sensitive mutations are changes of a single nucleotide, resulting in the substitution of one amino acid. In *tra-2^{ts1}*, alanine 151 is replaced by valine and in *tra-2^{ts2}*, proline 181 is replaced by serine. The mutation *tra-2^P* is a P-element insertion into the translated part of the second exon.

Of these three mutations, *tra-2^{ts1}* is the strongest allele, causing intersexuality of mutant XX animals already at 18°C. At this temperature, *tra-2^{ts2}* still allows female development (Table II). Interestingly, the mutation *tra-2^{ts1}* substitutes a rather highly conserved amino acid (alanine 151 is conserved in 18 out of 32 RNP motifs examined; Bandziulis *et al.*, 1989) and is the only one that maps within the RNP motif.

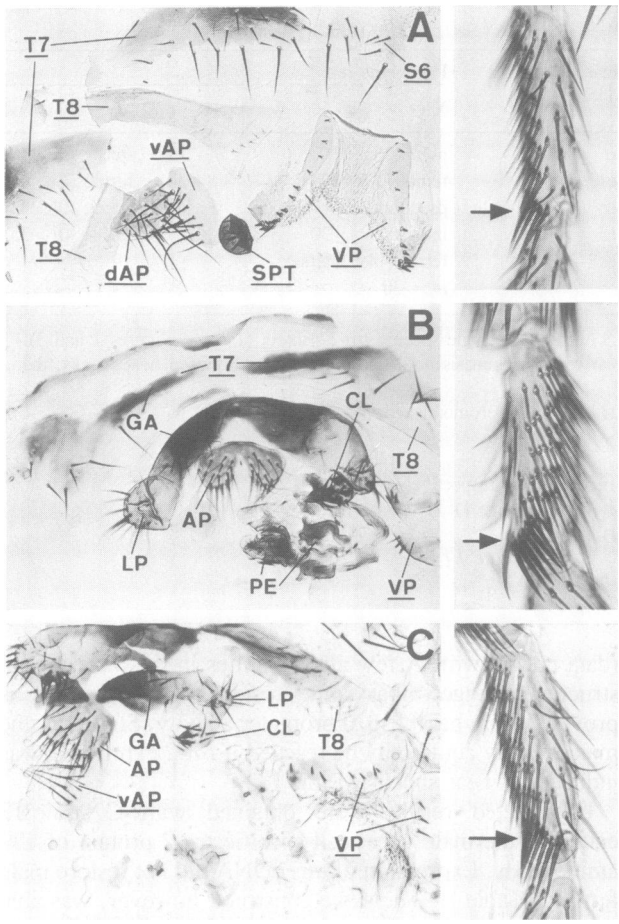

Fig. 6. Ability of different cDNA constructs to rescue the *tra-2* mutant phenotype of XX flies. All flies are chromosomal females, homozygous for *tra-2* and carrying one (A and B) or two copies (C) of construct C20c1.3 and C20d5'c1.3, respectively (for detailed genotypes see Table I). The flies were kept at 25°C, interrupted by heat shocks every 6 h for 25–30 min at 36.5°C from egg laying to eclosion. The figure shows the postabdomen, mainly genitalia and analia (left side), and basitarsi of the forelegs (right side). Arrows point to sex comb region on basitarsi. Abbreviations: Female structures (underlined): dAP, vAP, dorsal and ventral anal plates; S6, sixth sternite; SPT, spermatheca; T7, T8, seventh and eighth tergites; VP, vaginal plate. Male structures: AP, anal plate: CL, clasper; GA, genital arch; LP, lateral plate; PE, penis apparatus. (**A**) complete rescue is achieved by one copy of construct C20c1.3. (**B**) and (**C**) show flies carrying either one (B) or two (C) copies of construct C20d5'c1.3. Note the increased number and quality of female structures and the concomitant reduction of male structures in (C) versus (B), indicating a dosage effect of the construct on the rescuing ability.

Discussion

Alternative RNA processing has emerged as the major principle in the regulation of the three sex-determining genes *Sxl*, *tra* and *dsx*. In the cases of *Sxl* and *tra*, pre-mRNAs of females are spliced so that the resulting mRNAs contain long ORFs and can encode proteins. In males, the same pre-mRNAs are spliced in such a way that the mRNAs retain sequences that introduce stop codons early in the long ORF (Bell *et al.*, 1988; Boggs *et al.*, 1987). Sex-specifically processed mRNAs are also found for *dsx*, but in contrast to *Sxl* and *tra*, the transcripts of both females and males are functional and contain long ORFs which encode proteins with an identical N-terminus but different C-termini (Burtis and Baker, 1989). These findings are consistent with genetic data which demonstrate that the functions of *Sxl* and *tra* are required only in females but not in males (Cline, 1978; Baker and Ridge, 1980; Sanchez and Nöthiger, 1982; Wieschaus and Nöthiger, 1982), and that *dsx* contains functions required in both sexes as well as male- and female-specific functions (Nöthiger *et al.*, 1987).

In this report we show that *tra-2* transcripts are also alternatively spliced. The major transcript T_{maj} is present in both sexes, consists of six exons (E1, E2, E3, E4, E5 and E6) and encodes a protein of 264 amino acids. Transcript T_{min} is an alternatively spliced form of transcript T_{maj}, contains one additional exon (E2′) between E2 and E3 and encodes an N-terminally truncated *tra-2* protein of 226 amino acids. The major male-specific transcript msT_{maj} is only present in males with a functional germ line and consists of four exons $msE3^u$, E4, E5 and E6. The level of male-specific transcripts is not significantly affected by the P-element insertion in $tra\text{-}2^P$ (Figure 4C), in contrast to the non sex-specific transcripts, which are either remarkably reduced or not detectable in animals homozygous for the mutation $tra\text{-}2^P$ (Figure 4B and C). This observation, taken together with the failure of PCR to amplify male RNA with a primer specific for msE3 and p1 or p2 and the isolation of a new mutation ($tra\text{-}2^{Pd4}$; see below) strongly suggests that the transcript msT_{maj} does not contain E1 and E2, but derives from a different promoter within intron 2. This transcript, however, encodes a truncated protein of the 179 C-terminal amino acids that are also present in the non sex-specific *tra-2* proteins. Finally, a rare male-specific transcript msT_{min} has been detected. Although msT_{min} has not yet been characterized completely, it presumably encodes the same protein as T_{min}.

The transcript T_{maj} encodes a protein of 264 amino acids and can provide both known functions of tra-2

Based on the male germ cell-specific expression of the transcript msT_{maj}, it was reasonable to assume that this transcript can provide the function for male fertility, whereas the transcripts encoding the non sex-specific *tra-2* proteins could provide the female sex-determining function. Our transformation studies, however, show that both *tra-2* functions were supplied by the major non sex-specific *tra-2* cDNA, whereas a cDNA corresponding to transcript msT_{maj} failed to rescue the male sterility. Surprisingly, this cDNA was able to provide a remarkably high female sex-determining function (Figure 6B and C). These results might suggest that the major *tra-2* protein (264 amino acids) is responsible in wild type flies for both male fertility and female sexual differentiation, and that the truncated male germ cell-specific protein is dispensable. The ability to rescue the male fertility with construct C20c1.3 is only observed in a low percentage of males examined (Table I) and this rescue was obtained by ectopically expressing a protein most likely not required for male fertility (see below). Nevertheless, the major *tra-2* protein of 264 amino acids can provide all known function of *tra-2* under certain conditions.

We have recently isolated a new *tra-2* mutation, $tra\text{-}2^{Pd4}$, that completely transforms females into pseudomales but does not affect male fertility. This mutation which was obtained by remobilization of the P-element in the mutation $tra\text{-}2^P$, does not affect the structure and abundance of $msE3^P$, but drastically reduces non sex-specific transcripts in both sexes (H.Amrein, unpublished results). Taken together, these data suggest that the fertility of males does

Table II. Effects and molecular basis of *tra-2* leaky mutations

Allele		Reference	Phenotype in XX	XY	Molecular basis	Putative *tra-2* proteins
$tra\text{-}2^{P}$		1	very female-like intersex	fertile	P-element insertion into the second exon	$tra\text{-}2^{P258}$ $tra\text{-}2^{226}$? $tra\text{-}2^{179}$
$tra\text{-}2^{ts1}$	16°C	2	nearly normal, but sterile female	sterile		$tra\text{-}2^{ts1\ 264}$
	18°C	3	intersex	sterile	$ala^{151} \rightarrow val^{151}$	$tra\text{-}2^{ts1\ 226}$
	29°C	2	pseudomale	sterile		$tra\text{-}2^{ts1\ 179}$
$tra\text{-}2^{ts2}$	16°C	2	fertile female	fertile		$tra\text{-}2^{ts2\ 264}$
	18°C	3	sterile female	sterile	$pro^{181} \rightarrow ser^{181}$	$tra\text{-}2^{ts2\ 226}$
	29°C	2	pseudomale-like intersex	sterile		$tra\text{-}2^{ts2\ 179}$

The references for a more detailed description of the phenotypes are: (1) Amrein *et al.* (1988), (2) Belote and Baker (1982) and (3) R.Nöthiger and H.Amrein (unpublished results). The level of the sex-nonspecific transcripts in pseudomales homozygous for either $tra\text{-}2^{ts1}$ or $tra\text{-}2^{ts2}$ raised at the restrictive temperature (29 ± 1°C) roughly corresponds to that of wild type males (data not shown).

not require the presence of transcripts encoding the 264 amino acid protein.

The identification of a rare, male-specific transcript by Mattox *et al.* (1990) and its confirmation in this study (Figure 5) might explain the failure of C20d5'c1.3 (Figure 1, table 1) to rescue male sterility. This transcript presumably encodes the 226 amino acid protein and would contain 47 additional amino acids not present in the 179 amino acid protein encoded by C20d5'c1.3, suggesting that this portion of the protein might be important for the male-specific fertility function.

Alternatively, the 179 amino acid protein encoded by the more abundant transcript msT_{maj} might be sufficient for the male fertility function; if so, the failure of C20d5'c1.3 to rescue male sterility could be explained by differences in the 5' and 3' untranslated sequences between this construct and the endogenous transcript msT_{maj} (Figure 1 and 3B). The transcripts derived from the cDNA may be less stable or less efficiently translated in male germ cells than the endogenous transcript msT_{maj}.

Sequence analysis of tra-2 mutations provides evidence for the functional significance of the RNP motif

The RNP motif including a few adjacent amino acids of three proteins have been shown to be sufficient for RNA binding *in vitro* (Query *et al.*, 1989: U1 70K protein; Scherly *et al.*, 1989, 1990: U1A protein and U2" protein). In *Drosophila* female sex determination, the RNP motif of *tra-2* might be important for interaction of the *tra-2* protein with the *dsx* pre-mRNA by binding to specific RNA sequences and promoting female-specific processing of the *dsx* transcript. This may explain why ectopic expression of the truncated male germ cell-specific *tra-2* protein, which contains the complete RNP motif, is able to rescue the female mutant phenotype to a certain extent. It is even possible that this truncated *tra-2* protein can bind to the *dsx* pre-mRNA with the same affinity as the full length protein. The failure for complete function might be due to inefficient interaction with other proteins involved in *dsx* pre-mRNA splicing, for instance with the *tra* protein. The fact that the rescue of the *tra-2* mutant phenotype can be significantly improved by a second copy of this cDNA suggests that this inefficient interaction can be partially compensated by the amount of the protein (Figure 6B and C).

The sequence analysis of leaky *tra-2* mutations provides more direct evidence for the functional importance of the RNP motif. The most severe alteration at the molecular level is found in $tra\text{-}2^{P}$ in which the insertion of a P-element into exon E2 interrupts the long ORF of the major *tra-2* transcript (Figure 2). Furthermore, $tra\text{-}2^{P}$ animals do not show detectable levels of E2' (this finding, however, could also be explained by multiple DNA polymorphisms within the tetranucleotide repeat; see above) and substantially reduced levels of transcripts containing exon E3 (Figure 4C). The detection of *tra-2* transcripts containing exons downstream of the P-element insertion site is consistent with the observation that a significant level of P-element transcripts are read-through products resulting from leakiness of the P-element polyadenylation site (Karess and Rubin, 1984; Levis *et al.*, 1984). Thus, the *tra-2* function of $XX;tra\text{-}2^{P}/tra\text{-}2^{P}$ animals is provided by proteins with a truncated N-terminus. One of these proteins could be defined by the AUG in E3, which then would correspond to the male germ cell-specific protein. Another protein could consist of the methionine encoded by the last three nucleotides of the P-element (Figure 2) and 257 out of 264 amino acids from the major *tra-2* protein. In any case, the very weak phenotype of this mutation, i.e. the relatively high level of *tra-2* function in homozygous mutant $tra\text{-}2^{P}$ animals, shows that at least one of these proteins must exist.

The temperature-sensitivity of the proline−serine exchange in $tra\text{-}2^{ts2}$ suggests that this substitution causes a structural instability of the protein at higher temperature. No phenotypic effects of this mutation, however, are observed up to 18°C, relatively high levels of *tra-2* function are still provided up to 25°C, and some residual function is even left at 29°C (Belote and Baker, 1982; Table II).

The mutation $tra\text{-}2^{ts1}$ clearly has more severe phenotypic effects on both sexes than $tra\text{-}2^{P}$ or $tra\text{-}2^{ts2}$ (Table II). On the other hand, the substitution of alanine by valine in $tra\text{-}2^{ts1}$ represents a more conservative change, compared with the substitution of proline by serine in $tra\text{-}2^{ts2}$ or the alteration of the N-terminus in $tra\text{-}2^{P}$. It is intriguing that $tra\text{-}2^{ts1}$ affects a rather highly conserved residue within the RNP motif (Bandziulis *et al.*, 1989) that maps close to the

RNP1 (residues 138–145), the most highly conserved region among RNA-binding proteins. It is possible that the substitution in *tra-2^{ts1}* affects part of a catalytic domain of the protein, or that this substitution has severe effects on such a domain nearby, for instance on the RNP1. Conversely, the substituted residue in *tra-2^{ts2}* and the truncated N-terminus in *tra-2^{P}* map in regions in which alterations have less severe effects on the function of the protein. Although we do not yet know how the *tra-2* protein interacts with other proteins and the *dsx* pre-mRNA to achieve female-specific processing of the *dsx* transcript, our data suggest that the RNP motif represents an important domain for *tra-2* function.

Materials and methods

Library construction, cloning and sequence analysis

Genomic DNA of homozygous *tra-2^{ts1}* and *tra-2^{ts2}* flies was isolated according to McGinnis *et al.* (1983), digested with *Eco*RI and cloned into the λ ZAP vector (Stratagene). Library screening and cloning methods were done as described in Maniatis *et al.* (1982) and Sambrook *et al.* (1989). Sequence analysis was performed as described by Sanger *et al.* (1977) and according to the procedure of the supplier (USB). The sequence encompassing the 3 kb *Eco*RI–*Hin*dIII fragment, containing the whole *tra-2* gene, was determined by sequence analysis of the inserts of the following plasmids: pbSB2 (4.5 kb *Sal*I–*Bam*HI fragment), pbPE1 (1.9 kb *Pst*I–*Eco*RI fragment), pbAcE1 (0.95 kb *Eco*RI–*Acy*I fragment), pSP73CB (0.95 kb *Cla*I–*Bgl*II fragment), pbSC1 (1.7 kb *Sma*I–*Cla*I fragment), pbSC2 (1.5 kb *Sma*I–*Cla*I fragment) and pbSH1 (0.95 kb *Sma*I–*Hin*dIII fragment). The 3.9 kb *Eco*RI inserts containing the DNA from the *tra-2* temperature sensitive mutations were cloned into bluescript plasmid KS$^+$ (Stratagene) and the sequence was determined for the ORFs of all three putative *tra-2* proteins. (The introns of the mutant genes were not completely sequenced.) Several regions of the wild type DNA and all the sequences from mutant *tra-2* genes were determined using primers identical to sequences from within the *tra-2* gene. The region of the P-element insertion in the mutation *tra-2^{P}* was determined by sequence analysis of the insert of plasmid bsSB3.8 (Amrein *et al.*, 1988) using a primer identical to sequences from within the P-element (O'Hare and Rubin, 1983).

Preparation of RNA and RNase protection assays

Total RNA from 200–500 adult flies was isolated according to Chirgwin *et al.* (1979) and recovered through guanidinium–$CsCl_2$ centrifugation (45 000 r.p.m. for 12 h). RNA protection experiments were performed as described by Zinn *et al.* (1983), but the probes were gel purified and extracted in 0.5 M ammonium acetate. RNase digestions were carried out with 40 μg/ml RNase A.

Reverse transcription, polymerase chain reaction (PCR) and cloning of PCR products

Amplification of total RNA was performed according to Kawasaki (1990) with the following modifications: reverse transcription reactions (AMV reverse transcriptase, Promega) were carried out at 43°C for 1h in reaction volumes of 20 μl in the presence of 1 pmol specific downstream primer. 5–10 μl of such a reaction were used to amplify the cDNA products in the presence of specific primers (2 pmol each). The reaction volume was either 50 or 100 μl, and 30–35 PCR cycles were carried out. The following pairs of primers (see below and Figure 3) were used to specifically amplify the cDNAs corresponding to different transcripts: T_{maj}: p2 and p3; 5′ region of T_{min}: p1 and p2.2′; 3′ region of T_{min}: p2.2′ and p5; and msT_{maj}: pms3 and p5. The oligonucleotide primers were synthesized by a 391 DNA synthesizer (Applied Biosystems) and were designed to bear convenient restriction sites (underlined):

p1:5′ CGGGATCCAAAGGCTATCAATTAATTGG 3′ (*Bam*HI)

p2.1′:5′ CATAGGGCCCTTTCATTTGGAT 3′ (*Apa*I)

p2.2′:5′ AAGAGCACCAGCTGAATCGG 3′ (*Pvu*II)

p3:5′ GGAATTCCGAGGAGGAACGCGAA 3′ (*Eco*RI)

pms3:5′ GCAGATCTTTTCAAGACCCTCAAGAACG 3′ (*Bgl*II)

p5:5′ GCGAATTCATCGCTGAGTTTCTCAAAG 3′ (*Eco*RI)

One third of each PCR was analysed by agarose gel electrophoresis. The remaining portion of the reaction was purified using Geneclean or Mermaid DNA purification kits (Bio 101) and the DNAs were digested with the appropriate restriction enzymes, cloned into corresponding Bluescript KS$^+$ plasmids (Stratagene) and sequenced.

Plasmid construction and P-element transformation

Plasmid construction of C20d5′c1.3: a plasmid BShsd5′c1.3 was created by insertion of the *Nae*I–*Ssp*I fragment from the cDNA clone c1.3 into the *Eco*RV polylinker site of plasmid BShs. BShs was obtained by insertion of the *Xba*I–*Pst*I fragment (promoter element) from the *hsp70* gene of *Drosophila* (Karch *et al.*, 1981) into plasmid Bluescript KS$^+$ (Stratagene). The orientation of the insert was such that the 5′ end of the *tra-2* insert (*Nae*I) was preceded by the *hsp70* promoter. The *Xba*I–*Sal*I fragment of BShsd5′c1.3 (whole insert) was cloned into a modified Carnegie 20 transformation vector (Hiromi *et al.*, 1985) resulting in plasmid C20d5′c1.3 which consists of the *hsp70* promoter, the coding region of the *tra-2* transcript msT_{maj} and the hs trailer of the *hsp70* gene. Plasmid construction of C20c1.3: plasmid BShsc1.3 was created by insertion of the *Eco*RI–*Ssp*I fragment from the cDNA clone c1.3 into the *Eco*RI and *Eco*RV sites of plasmid BShs. The cloning steps to create C20c1.3 were identical to that of C20d5′c1.3. As a result, plasmid C20c1.3 contained the coding region of the *tra-2* transcript T_{maj}. Injection of *cn;ry^{42} Drosophila* embryos were done as described by Rubin and Spradling (1982). Transformed flies were selected by their *ry*$^+$ eye colour. For genetic symbols see Lindsley and Grell (1968) and Lindsley and Zimm (1985).

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