

TECHNOLOGY REPORT

Temperature-Dependent Gene Silencing by an Expressed Inverted Repeat in *Drosophila*

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Summary: Posttranscriptional gene silencing (PTGS) induced by double-stranded RNA (dsRNA) is an intriguing phenomenon that has been observed in a variety of organisms, including *Drosophila melanogaster*. Although PTGS in *Drosophila* is typically observed following direct injection of the dsRNA into embryos, it is theoretically possible that the in vivo transcription of an inverted repeat transgene might also produce a dsRNA “hairpin” that is capable of triggering PTGS. Here we test this idea, and show that an expressed inverted repeat of a portion of the sex differentiation gene, *transformer-2*, (*tra-2*), driven by a GAL4-dependent promoter, does genetically repress the endogenous wild-type *tra-2* function, producing a dominant loss-of-function mutant phenotype. Remarkably, this effect is temperature-sensitive, with phenotypic consequences seen at 29°, but not at 22°. Moreover, by altering the dosage of either the transgenes or the endogenous *tra2*⁺ loci, one can vary the effect over a wide range of mutant phenotypes. *genesis* 26:240–244, 2000. © 2000 Wiley-Liss, Inc.

Key words: RNA; UAS/GAL4

The discovery that injected double-stranded (ds) RNA can trigger posttranscriptional gene silencing (PTGS) in a number of species, including *Caenorhabditis elegans* and *Drosophila melanogaster*, has given geneticists a powerful tool to investigate the function of unknown genes and to manipulate the expression of known genes without the need for mutants (Alvarado and Newmark, 1999; Fire, 1999; Fire *et al.*, 1998; Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999; Montgomery and Fire, 1998). There are, however, some aspects of this technique that limit its usefulness. For example, although dsRNA injected into preblastoderm *Drosophila* embryos is a strong inhibitor of the corresponding gene's function during early development, it does not produce a very robust effect at later stages (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999). To overcome this limitation, we sought to develop a system in which the dsRNA is produced in vivo, by targeted expression of an inverted repeat transgene. The underlying hypothesis for these experiments is that an inverted repeat's transcript, because of its self-complemen-

tarity, might fold into a hairpin of dsRNA and trigger an RNA-mediated genetic interference (RNAi) response, as has been suggested to occur in some cases of PTGS in plants (Stam *et al.*, 1997; Waterhouse *et al.*, 1998).

The *tra-2* gene was chosen as the subject for these experiments because the null mutant is completely viable, and it has an easily scorable adult phenotype. That is, homozygous *tra-2* chromosomal females (XX) develop as phenotypic males, with male-like segmentation and pigmentation patterns, sex combs on their forelegs, and male genitalia (Watanabe, 1975). Leaky alleles of *tra-2* also give easily recognizable phenotypes, ranging from male-like intersexes with slight differences from the usual male phenotype, through more female-like intersexes that show simultaneous differentiation of male and female genital structures, abnormal anal plates, partial male-like pigmentation, and abnormal sex combs (Belote and Baker, 1982; Fujihara *et al.*, 1978).

The *tra-2* gene is complex, with alternative splicing events giving rise to four major mRNAs that are produced in a sex- and germline-specific manner (Mattox *et al.*, 1990, 1996; McGuffin *et al.*, 1998). To create the *tra-2* inverted repeat, a 700 bp DNA fragment, encompassing most of exons 6 and 7, was PCR-amplified using a *tra-2* cDNA clone as a template (Fig. 1). This fragment, containing about half of the *tra-2* coding region and 297 bp of the 3' UTR, was selected because it is included in all *tra-2* transcripts, and it contains no ATG codon that might allow inappropriate translation initiation of the inverted repeat's transcript and produce a truncated TRA-2 protein that could complicate the interpretation of the results.

Three constructs were made (Fig. 1). The first, *UAS-tra2-IR*, contains two copies of the 700 bp *tra-2* fragment in an antisense-sense inverted repeat arrangement. The two copies are separated by 42 bp of vector polylinker sequence and flanked by a GAL4-directed promoter on one side and a polyadenylation signal and

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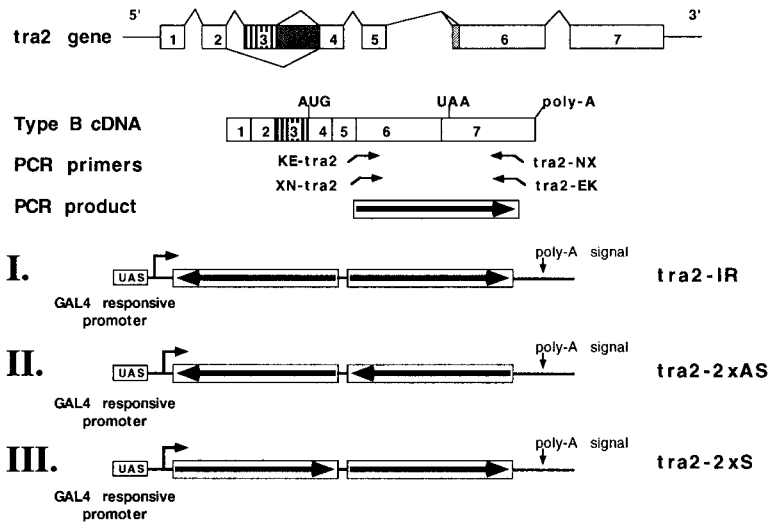


FIG. 1. Construction of the *tra-2* transgenes. At the top is the structure of the *tra-2* gene (taken from Mattox *et al.*, 1990). The open boxes are exons, with filled regions representing alternatively spliced segments. Shown are the positions of the start and stop codons, and the polyadenylation site, in the Type-B cDNA used as a template for PCR. I, II, and III represent the three constructs in which two copies of the 700 bp PCR fragment have been cloned into the pUAST vector in various orientations. UAS is a regulatory sequence that confers GAL4 dependence on the transcription of the inserted sequences.

transcription terminator on the other. The two control constructs were *UAS-tra2-2xAS*, consisting of two copies of the 700 bp *tra2* fragment in an antisense-antisense arrangement, and *UAS-tra2-2xS*, having two copies of this fragment in the sense-sense orientation. These constructs were introduced into the *Drosophila* genome by P-element transformation, and several independent transgenic lines were established for each.

These experiments utilize the UAS/GAL4 binary system to control expression of the *tra-2* transgenes (Brand and Perrimon, 1993; Brand *et al.*, 1994). In the absence of the yeast transcriptional activator GAL4, the *UAS-tra2* transgenes are transcriptionally silent. They can be activated by crossing the *UAS-tra2*-bearing flies to another transgenic line that expresses GAL4 under the control of a *Drosophila* gene promoter. Thus, depending on the particular GAL4 "driver" line that is used, the *UAS-tra2* transgene is activated in the progeny either constitutively, or in a targeted, spatially restricted manner.

In the first series of experiments, carried out at 22° and 29°, *UAS-tra2-IR* transgenic lines were crossed to flies carrying the *Act5C-GAL4* driver, which expresses GAL4 under the control of the constitutive *Actin5C* promoter. Genetic markers were used to distinguish the XX and XY progeny, and to identify those that receive the *UAS-tra2-IR* and/or *Act5C-GAL4* transgenes. As expected, at both temperatures the XX flies that carry *UAS-tra2-IR* but not *Act5C-GAL4* develop as normal, fertile females, as do those that carry *Act5C-GAL4* alone. In contrast, while XX flies with both *UAS-tra2-IR* and *Act5C-GAL4* develop as normal, fertile females at 22°, they develop as sexual intermediates (intersexes) when raised at 29° (Fig. 2, Table 1). The temperature-sensitive nature of this effect is not owing to a specific *UAS-tra2-IR* transgene because all of the lines tested showed sex transformation phenotypes at 29° but not at 22°, nor is it specific for the *Act5C-GAL4* driver, because crosses with other GAL4 drivers showed similar temperature-dependence. In each cross with a given *UAS-tra2-IR* line,

the sexual phenotype of all XX, *UAS-tra2-IR/Act5C-GAL4* offspring was fairly uniform, although there was variation in the strength of the effect among the different *UAS-tra2-IR* lines (Table 1, lines b, k-q).

The sex transformation phenotypes seen here were significant and dramatic, but in no case did they mimic the *tra-2* null mutant phenotype. We therefore sought to enhance the strength of the expressed inverted repeat (EIR) effect in three ways. First, we reduced the endogenous *tra-2*⁺ gene dosage by making the fly heterozygous for *Df(2R)trix*, a deficiency of the *tra-2* region. As shown in Table 1 (compare b and c, or i and j), this shifted the sexual phenotype of the *UAS-tra2-IR-61X/Act5C-GAL4* flies toward maleness when compared to similar flies carrying two copies of *tra2*⁺. A second way to shift the phenotype toward maleness was to increase the dosage of the *UAS-tra2-IR* transgene. For example, one of our original lines, *UAS-tra2-IR/86-2/3*, carried two unlinked transgenes. When this line was crossed to the *Act5C-GAL4* driver, offspring receiving both *UAS-tra2-IR* constructs showed a stronger male phenotype than those receiving only one (Table 1, compare i with k and l). Finally, we used a different GAL4 expressing line, *T80-GAL4*, that expresses GAL4 at high levels in all imaginal discs. Flies carrying the *UAS-tra2-IR-86-2/3* transgenes and *T80-GAL4* develop as phenotypic males, with male segmentation, pigmentation, anal plates, genitalia, and sex combs (Fig. 2, Table 1 m), thus resembling strong loss-of-function *tra-2* mutants.

As controls, the *UAS-tra2-2xAS* and *UAS-tra2-2xS* constructs were also expressed under the control of the *Act5C-GAL4* driver. In neither case was there any detectable effect on the sexual phenotype of the XX flies, and they developed as normal, fertile females at both temperatures (Table 1, r-w). This is consistent with what is known about the RNAi phenomenon, because in both flies and worms injected sense or antisense RNAs by themselves produce a much weaker effect than the dou-

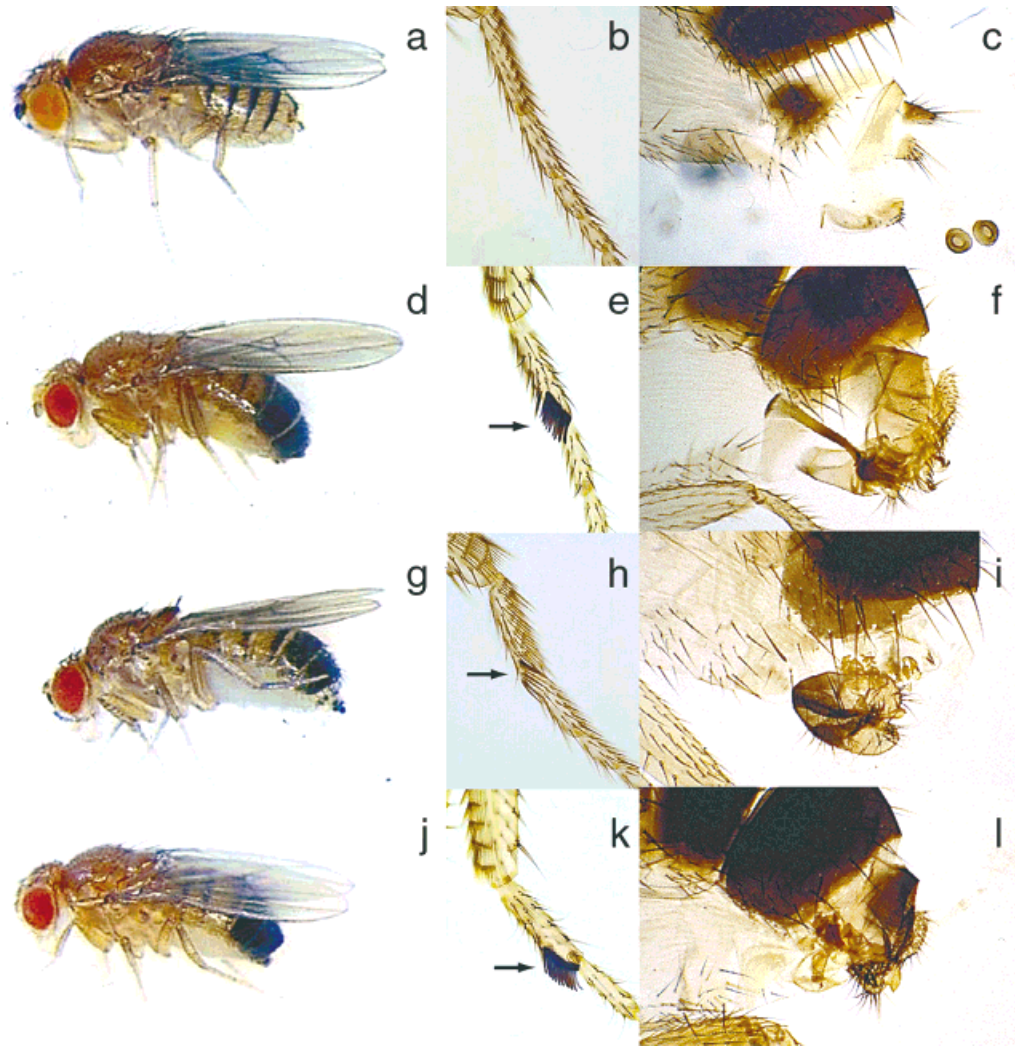


FIG. 2. Sexual phenotypes of transgenic flies. (a) Normal female. (b) Foreleg and (c) genital region of a normal female. (d) Normal male. (e) Foreleg (with sex comb) and (f) genital region of a normal male. (g) Intersex of genotype *XX,UAS-tra2-IR-61A-X; Act5C-GAL4*, raised at 29°. (h) Foreleg and (i) genital region of the intersex showing partially developed male structures. (j) Male of genotype *XX, UAS-tra2-IR-86-2/3; T80-GAL4*. (k) Foreleg and (l) genital region of the *XX, UAS-tra2-IR-86-3; T80-GAL4* male showing male structures.

ble-stranded RNA (Fire *et al.*, 1998; Kennerdell and Carthew, 1998).

Although the *UAS-tra2-2xAS* and *UAS-tra2-2xS* transgenes had no detectable effects when individually expressed under the control of the *Act5C-GAL4* driver, it was of interest to see if there would be an effect if both were simultaneously expressed in the same fly. That is, expression of both sense and antisense transcripts in the same cells might allow significant levels of dsRNA to form, and thus trigger the RNAi effect. To test this, flies carrying *UAS-tra2-2xAS-8X* and *UAS-tra2-2xS-4C* were crossed to the *Act5c-GAL4* line to produce flies expressing both the sense and antisense *tra-2* transcripts. At both 22° and 29° these XX flies develop as normal, fertile females. Thus, having the sense and antisense sequences present on the same RNA transcript produces a stronger

gene silencing effect than expressing them as separate molecules.

One potential problem with using this EIR method to suppress expression of specific genes is that some inverted repeat constructs are very difficult, if not impossible, to clone. For example, although the *UAS/tra2-IR* antisense-sense inverted repeat was readily cloned, we were unable to generate any clones with the sense-antisense arrangement, despite numerous attempts.

Because expression of the inverted repeat transgene is controlled by the *GAL4*-directed promoter, it might be possible to target the EIR effect using different *GAL4* drivers that are expressed in a spatially restricted manner. To test this, the *UAS-tra2-IR-61X* line was crossed to flies carrying the *e22C-GAL4* driver. In this case, the XX flies that receive both transgenes develop with male-like

Table 1
Effect of *tra-2* Transgenes on XX Sexual Phenotype*

UAS transgene	<i>tra2</i> ⁺ dosage	GAL4 driver	Temperature	Sexual phenotype of XX flies					
				SC	P	7T	6S	AP	G
I. <i>tra2</i> -IR/									
(a) 61-X	2	Act5C-GAL	22°	f	f	f	f	f	f
(b) 61-X	2	Act5C-GAL	29°	f	m	m	m	i	i
(c) 61-X	1	Act5C-GAL	29°	i	m	m	m	m	mi
(d) 61-X	2	T80-GAL	22°	f	f	f	f	f	f
(e) 61-X	2	T80-GAL	29°	mi	i	m	m	m	mi
(f) 61-X	2	e22c-GAL	22°	f	f	f	f	f	f
(g) 61-X	2	e22c-GAL	29°	f	m	m	m	f	fi
(h) 86-2/3	2	Act5C-GAL	22°	f	f	f	f	f	f
(i) 86-2/3	2	Act5C-GAL	29°	i	m	m	m	m	mi
(j) 86-2/3	1	Act5C-GAL	29°	mi	m	m	m	m	m/mi
(k) 86-2	2	Act5C-GAL	29°	f	i	f	f	i	fi
(l) 86-3	2	Act5C-GAL	29°	f	m	i	i	mi	i
(m) 86-2/3	2	T80-GAL	29°	m	m	m	mi	m	m/mi
(n) 71-3	2	Act5C-GAL	29°	f	i	f	f	i	fi
(o) 72-3	2	Act5C-GAL	29°	f	m	m	mi	i	i
(p) 82A-3	2	Act5C-GAL	29°	f	m	m	m	mi	mi
(q) 82B-2	2	Act5C-GAL	29°	f	m	f	f	fi	fi
II. <i>tra2</i> -2xAS/									
(r) 8-X	2	Act5C-GAL	22°/29°	f	f	f	f	f	f
(s) 21A-3	2	Act5C-GAL	22°/29°	f	f	f	f	f	f
(t) 21B-3	2	Act5C-GAL	22°/29°	f	f	f	f	f	f
III. <i>tra2</i> -2xS/									
(u) 4C-2	2	Act5C-GAL	22°/29°	f	f	f	f	f	f
(v) 23-2	2	Act5C-GAL	22°/29°	f	f	f	f	f	f
(w) 25-2	2	Act5C-GAL	22°/29°	f	f	f	f	f	f

*SC, sex comb region of foreleg; P, pigmentation of the 5th and 6th tergites; 7T, 7th tergite; 6S, 6th sternite; AP, anal plates; G, external genitalia; f, female; m, male; i, intersex; mi, strong male-like intersex; fi, weak female-like intersex.

abdominal segmentation and pigmentation patterns, but with female-like genitalia and no sex combs, consistent with the notion that the EIR effect is targetable, at least to some extent (Table 1, compare b, e, and g).

The molecular basis of the temperature-sensitivity observed here is not known, nor is it known if this is a general feature of EIR-induced RNAi in flies. One possibility is that the higher temperature allows a more dynamic dissociation-reassociation of the nascent inverted repeat transcript with RNA-binding proteins, and that this allows the stable dsRNA hairpin to form more readily. It is also possible that the temperature effect is owing to some downstream component of the RNAi pathway. Another possibility is that the temperature-sensitivity is related to the observation that the GAL4 protein is a stronger activator of transcription in flies raised at 29° (Brand *et al.*, 1994). Finally, it might not be a general feature of this system but, instead, it might be related to an inherent temperature-sensitivity of the *Drosophila* sex determination pathway, given the observation that hypomorphic alleles of several sex differentiation genes show temperature effects. Future experiments should clarify this question.

Regardless of the nature of this temperature dependence, it is a valuable feature of this system because it gives the experimenter temporal control over the gene silencing effect. That is, by shifting the developing flies from the permissive to the restrictive temperatures, or vice versa, one can target the RNAi effect to a specific

developmental period. The use of the UAS/GAL4 system to govern expression of the inverted repeat transgene provides a second dimension of control. Finally, the ease with which one can vary the strength of the gene silencing effect, from mild to severe, by altering gene dosage makes this method a powerful system to finely manipulate phenotypes without conventional mutant alleles. Given these properties, this approach might be a very useful tool for the functional dissection of newly identified gene sequences that are now becoming available as the sequencing of the *Drosophila* genome nears completion.

MATERIALS AND METHODS

General Molecular Procedures

All standard techniques (e.g., restriction digestions, ligations, bacterial transformations, PCR amplifications, etc.) were done as described (Sambrook *et al.*, 1989).

PCR and Plasmid Construction

For PCR amplification of the *tra-2* fragment, a *tra-2* cDNA of the B-Type (Mattox *et al.*, 1990) was used as template. The primers XN-*tra2* = 5' CCTCGAGGCGGC-CGCTTGTTTCATTTACTTTGAGAACTC 3' and *tra2*-EK = 5' GGGTACCGAATTCATATCACAATCCATGCG-GACCTCTGTATG 3' were used to amplify a 700 bp fragment containing the last 121 codons of *tra2* and 297

bp of 3' UTR sequence, flanked by the restriction sites *Xba*I and *Not*I on the 5' side, and *Eco*RI and *Kpn*I on the 3' side. The PCR product was cloned into pBlueScript KS+ plasmid (Stratagene) to yield the plasmid pBS/0.7 AB. The 0.7 kb *tra-2* fragment was then cut out of this plasmid by digestion with *Eco*RI and *Not*I, and ligated to *Eco*RI-*Not*I double-digested pUAST vector, to give pUAST/0.7 EN-AB. This clone was cut with *Kpn*I, treated with Shrimp Alkaline Phosphatase (US Biochemicals), and ligated to the 0.7 kb *Kpn*I fragment isolated from pBS/0.7 AB, to produce the inverted repeat construct pUAST/0.7 EN-AB/0.7K(I), and the 2x antisense construct pUAST/0.7 EN-AB/0.7K(II). Similar procedures were used to construct the 2X sense construct pUAST/0.7 EN-CD/0.7K(III), except the primers used for the PCR were KE-*tra2* = 5' GGGTACCGAATTCTTGTTCATTTACTTTGAGAACTC 3' and *tra2*-NX = 5' CCTCGAGGCGGCCGCATATCACAAATCCATGCGGACCTCTGTATG 3'. The PCR product was cloned into pBluescript KS+ to give pBS/0.7-CD, and then the *Not*I-*Eco*RI fragment cut out and ligated into pUAST to yield pUAST/0.7 EN-CD. The 0.7 kb *Kpn*I fragment from pBS/0.7 AB was ligated into the *Kpn*I site of this plasmid to give the 2x sense construct pUAST/0.7 EN-CD/0.7K(III).

Phenotypic Analyses

The initial tests used the X-linked *UAS-tra2-IR-61X* transgene and a chromosome 2-linked "driver" that expresses GAL4 constitutively from the *Actin5C* promoter. Males of the genotype *w* *P(UAS-tra2-IR-61X)*, *w*⁺ were crossed to *y w*; *P(Act5C-GAL4)*, *w*⁺/*CyO* females. Chromosomally female (XX) and male (XY) offspring could be distinguished on the basis of the yellow body color marker. The non-yellow, Curly winged offspring represented XX flies with the *UAS-tra2-IR* transgene but without the *Act5C-GAL4* driver, whereas the non-yellow, non-Curly flies are XX individuals carrying both the *UAS-tra2-IR* and *Act5C-GAL4* transgenes. Control crosses, using males from the original *w*¹¹⁸ stock, were also carried out to generate XX and XY flies that carry the *Act5C-GAL4* driver without the *UAS-tra2-IR* construct. To assess the effects of the autosomally-linked *UAS-tra2-IR* transgenes, males of the genotype *y*⁺ *w*; *P(UAS-tra2-IR)*, *w*⁺/*T(2;3)ap*^{Xa} were mated to *y w*; *P(Act5C-GAL4)*, *w*⁺/*CyO* females. Offspring receiving both the *Act5C-GAL4* and *UAS-tra2-IR* transgenes were identified by their normal wings (i.e., non-Curly, non-apterous Xasta). As in the previous cross, XX and XY progeny could be distinguished as non-yellow and yellow flies, respectively.

Similar crosses were carried out using other GAL4 driver stocks, including *P(GawB)T80/CyO* (expressed ubiquitously in imaginal discs), *P(e22C)/SM5,Cy* (expressed ubiquitously in the embryo, later expression patterns not described), and *P(Act5C-GAL4)/TM6B*, *Tb* (constitutively expressed from the *Actin5C* promoter).

Flies were raised on standard medium at the temperature indicated (22°, 25° or 29°). All *Drosophila* stocks

were obtained from the Bloomington Stock Center, and descriptions of markers, GAL4-expressing lines, and chromosomes can be accessed using the Flybase database (Flybase Consortium 1999).

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