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# A genetic pathway for regulation of *tra-2* translation

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## SUMMARY

**In *Caenorhabditis elegans*, the *tra-2* sex-determining gene is regulated at the translational level by two 28 nt direct repeat elements (DREs) located in its 3′ untranslated region (3′UTR). DRF is a factor that binds the DREs and may be a *trans*-acting translational regulator of *tra-2*. Here we identify two genes that are required for the normal pattern of translational control. A newly identified gene, called *laf-1*, is required for translational repression by the *tra-2* 3′UTR. In addition, the sex-determining gene, *tra-3*, appears to promote female development by freeing *tra-2* from *laf-1* repression. Finally, we show that DRF activity correlates with translational repression of *tra-2* during development and that *tra-3* regulates DRF activity. We suggest that *tra-3* may promote female development by releasing *tra-2* from translation repression by *laf-1* and that translational control is important for proper sex determination – both in the early embryo and during postembryonic development.**

Key words: translational control, sex determination, development, *Caenorhabditis elegans*, *tra-2*, *tra-3*, *laf-1*, 3′UTR, DRE, DRF

## INTRODUCTION

Translational controls are critical for numerous developmental decisions (for review see Wickens et al., 1996). In a variety of organisms, *cis*-acting regulatory elements in the 3′UTR govern such major developmental events as embryonic axis formation, maternal mRNA expression and sex determination (Wharton and Struhl, 1991; Evans et al., 1994; Standart., 1993; Ahringer and Kimble, 1991; Goodwin et al., 1993). Most translational controls identified to date rely on *cis*-acting regulatory elements located within the 3′ untranslated region (3′UTR). However, few *trans*-acting factors have been identified and little is known about the regulation of translational regulators themselves. In this paper, we focus on translational regulation of the *Caenorhabditis elegans* sex determination gene, *tra-2* and its genetic control.

Sex determination in *C. elegans* is controlled by a cascade of regulatory genes that specify one of two sexual fates (Fig. 1; for reviews see Hodgkin, 1990; Villenueve and Meyer, 1990; Kuwabara and Kimble, 1992). The primary signal for sex determination is the ratio of the number of X chromosomes to sets of autosomes (Fig. 1). Animals that contain two X chromosomes (XX) develop as hermaphrodites, while animals with one X chromosome (XO) develop as males. Hermaphrodites are essentially female animals that first produce sperm and then switch to oogenesis.

The *tra-2* gene directs female cell fates (Hodgkin and Brenner, 1977). *tra-2* has been cloned and is predicted to encode a large transmembrane protein (Okkema and Kimble, 1991; Kuwabara et al., 1992). The TRA-2 protein is thought to function by inhibiting downstream male determinants and by coordinating neighboring cells to adopt the same fate (Kuwabara et al., 1992). In the male soma, *tra-2* is inactive and male development ensues.

Development of both hermaphrodites and males depends on negative regulation of *tra-2*. In hermaphrodites, spermatogenesis requires that *tra-2* be repressed during L2 and L3, the second and third larval stages of development (Hodgkin, 1986). Dominant gain-of-function (*gf*) mutations of *tra-2* express increased *tra-2* activity, resulting in transformation of hermaphrodites into ‘females’ (Doniach, 1986; Schedl and Kimble, 1988). ‘Females’ and hermaphrodites are identical in somatic tissues but differ in the germ line: whereas hermaphrodites make both sperm and oocytes, females produce only oocytes. In males, repression of *tra-2* is required for both somatic and germ-line sexual development. Of particular importance for this paper, XO animals carrying the strongest *tra-2(gf)* mutation produce yolk in the intestine and make oocytes in the germ line (Doniach, 1986).

The *tra-2(gf)* mutations map to a 60 nt direct repeat located in the *tra-2* 3′UTR. The direct repeat consists of two identical 28 nt elements (DREs) that are separated by a 4 nt spacer (Goodwin et al., 1993). The DREs control *tra-2* activity by inhibiting the translation of *tra-2* mRNA (Goodwin et al., 1993). A factor, called DRF, specifically binds to the DREs (Goodwin et al., 1993). Our working model is that DRF binding to the DREs represses *tra-2* translation and thereby inhibits female development.

No good candidate for a translational repressor of *tra-2* has previously been described. Although the *her-1* and *fog-2* genes are necessary for repressing *tra-2* activity, their loss-of-

function phenotypes do not mimic that of the *tra-2(gf)* mutations. Loss-of-function *her-1* mutations have no apparent effect on XX animals, but transform XO animals from males to hermaphrodites (Hodgkin, 1980; Trent et al., 1988). Loss-of-function *fog-2* mutations, on the contrary, transform XX animals into females, but have no effect on XO animals (Schedl and Kimble, 1988). Therefore, it is likely that some previously undescribed gene may encode the *tra-2* translational repressor.

The sex-determining gene *tra-3*, like *tra-2*, is necessary for female development (Hodgkin and Brenner, 1977). Genetic analysis has not separated the activities of *tra-3* and *tra*-2 in the hierarchy of sex-determining genes, though it has been proposed that *tra-3* may promote female development by either inhibiting the *fem* genes or by potentiating *tra-2* activity (Hodgkin and Brenner, 1977; Hodgkin, 1986).

In this paper, we identify two genes that affect the *tra-2* 3′UTR control. First, we describe the genetic identification and characterization of a new gene, *laf-1*, which is required for inhibition of *tra-2* translation by its 3′UTR, and may in fact encode DRF. Second, we show that *tra-3* activity alleviates the translational repression of *tra-2*. Third, we find that DRF activity correlates with translational repression of *tra-2* during development and that *tra-3* is required to reduce DRF activity in embryos. We suggest that *tra-3* may promote female development by releasing *tra-2* from translational repression by *laf-1*.

## MATERIALS AND METHODS

### General procedures and strains

Routine maintenance was as described by Brenner (1974). All strains were raised at 20°C unless otherwise indictated. Males were generated by use of *him-8*, which causes a high incidence of males (Hodgkin et al., 1979).

The following mutations were used in this study. LGII, *tra-2(e1095), unc-4(e120)*; LGIII, *dpy-1(e1), laf-1*(*q80, q217, q267, q349*), *daf-2(e1370) unc-32(e189)*; LGIV, *fem-3(e1996), him-8(e1489), tra-3(e1107)*; LGV, *fog-2(q71)*. In addition, we used two balancer chromosomes: *mnC1[dpy-10(e128) unc-52(e444)]*, which suppresses recombination over the right half of chromosome II, and *qC1[dpy-19(e1259) glp-1(q339)]*, which suppresses recombination over much of chromosome III. Finally, we used a deficiency that removes the *laf-1* locus, *saDf1*. Most mutations are described in Swanson et al (1984); balancers are described in Edgley et al. (1995); *tra-2(e1095)* and *tra-3(e1107)* in Hodgkin and Brenner (1977).

### Isolation and characterization of laf-1 alleles

Four *laf-1* mutations were isolated by selecting dominant supressors of *fem-3(gf)* sterilility as described (Barton and Kimble, 1990). At restrictive temperature, *fem-3(gf)* homozygotes produce only sperm, whereas *laf-1/+; fem-3(gf)* animals produce both sperm and oocytes and consequently are self-fertile. *laf-1(q267* and *q80)* were isolated as suppressors of *fem-3(q20*gf*)*, *laf-1(q217)* as a suppressor of *fem-3(q96*gf*)*, and *laf-1(q349)* as a suppressor of *fem-3(q95*gf*)*. All four alleles were backcrossed six times against wild-type animals. *q267, q80* and *q349* failed to complement *laf-1(q217)*, and all four mapped left of *unc-93* on linkage group III. Three factor mapping placed *laf-1(q217)* between *dpy-1* and *daf-2*: From a parent of genotype *dpy-1+daf-2/+laf-1(q217)+*, 82/99 Dumpy nonDaf and 13/95 Daf nonDpy recombinants carried *laf-1(q217)*, placing *laf-1* approximately 1.5-1.8 map units left of *daf-2*. From a parent of genotype *laf-1(q217)daf-2/++*, progeny were produced at 15°C, and then shifted to 25°C to score for the Daf phenotype. Since *laf-1* is lethal, the only Daf surviving progeny were recombinants of genotype *laf-1daf-2/+daf-2*.

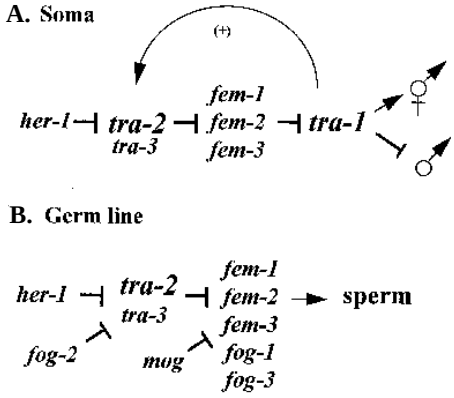


**Fig. 1.** Genetic control of sex determination in *C. elegans*. For simplicity, genes that act early to control both sex determination and dosage compensation are omitted. (For review and detailed references, see Villeneuve and Meyer, 1990). (A) Sex determination in somatic tissues. Seven genes are critical determinants of somatic sexual fates: *her-1*, three *tra* genes and three *fem* genes. In XO animals, *her-1* inhibits *tra-2* and *tra-3*, the *fem* genes inhibit *tra-1*, and male development ensues. In XX animals, *her-1* is not active; therefore, *tra-2* and *tra-3* repress the *fem* genes and *tra-1* promotes female development. In addition, *tra-1* may positively feed back on to *tra-2* to amplify commitment to female development (Okkema and Kimble, 1991). (B) Sex determination in the germ line. Six genes that regulate somatic sexual fates also play a major role in regulation of germ-line sexual fates: *her-1*; *tra-2*; *tra-3* and the three *fem* genes. In addition, three *fog* genes (Schedl and Kimble, 1988; Barton and Kimble, 1990; Ellis and Kimble, 1994), and six *mog* genes (Graham and Kimble, 1993; Graham et al., 1993) affect germ line but not somatic sexual fates. In XO animals, *her-1* inhibits *tra-2* and *tra-3* permitting *fog-1, fog-3* and the *fem* genes to direct spermatogenesis. The XX germ line is more complex because first sperm and then oocytes are made. The *fog-2* gene is thought to repress *tra-2* and *tra-3* to promote spermatogenesis; then after a brief period of spermatogenesis, the *mog* genes repress male-determining genes so that oogenesis can proceed. In contrast to the soma, *tra-1* is not a terminal regulator in germ-line sex determination. Although *tra-1* influences germ-line sex determination in both XX and XO animals, its role is not yet clear (Hodgkin, 1987, Schedl et al., 1989; De Bono and Hodgkin, 1995). Consideration of the relationship of *laf-1* to *tra-1* is beyond the scope of this paper.

6/616 surviving embryos were Daf, placing *laf-1* approximately 1.5 map units to the left of *daf-2*.

Lethality was scored by counting the total number of eggs laid by a *laf-1/qC1* hermaphrodite at 20°C and then 24 hours later counting the number of unhatched embryos and arrested L1s. To determine when *laf-1* homozygotes die, embryos were dissected from *laf-1/qC1* mothers, incubated at 20°C, and scored over the next 24 hours using Nomarski DIC optics. Approximately 22% of the embryos arrested. Sex-specific lethality was determined as follows. First, dying L1s from *laf-1/qC1; him-8* (which should include 37% XO animals) were scored for male-specific enlargement of the B blast cell. Approximately, 8% of the dying L1s were male, the expected number (*n*=53). Second, *laf-1 unc-32/qC1; him-8* progeny were scored for survivors with an Unc-32 phenotype. No Unc-32 progeny (male or hermaphrodite) survived.

### Double mutant analysis

Using DIC Nomarski optics or dissecting scope, several tissues (tail, intestine, gonad, vulva formation and germ line) were examined for feminization:. For *tra-2(null); laf-1(x)/+* double mutants, *tra-2(null)unc-4/mnC1* or *tra-2(null)unc-4/mnC1; laf-1(q217)/qC1* animals were selfed, and Unc-4 non-Dpy animals scored [*unc-4* is

closely linked to *tra-2* and serves as a marker; *qC1* homozygotes are Dpy]. For *laf-1/+; tra-3* double mutants, *dpy-1 unc-32/qC1 tra-3, laf-1/qC1*; *tra-3* and *saDf1/qC1; tra-3* hermaphrodites were selfed and non-Dpy progeny scored.

### Transgenic assay

All transgene constructs were derived from the same parent vector, pPC16.41 (kind gift of Dr Peter Candido). This vector contains the inducible heat-shock promoter, 16.41, the *lacZ*-coding region, and a polylinker (Stringham et al., 1992). To construct 3′UTR reporter transgenes, the desired 3′UTR was PCR amplified from either *tra-2(+)* or *tra-2(gf)* genomic DNA (Goodwin et al., 1993) using primers EBG-20 and EBG-21 (see below for sequences). EBG-20 and EBG-21 introduce restriction sites *Sal*I and *Bgl*II, respectively. The resulting PCR products were digested with *Sal*I and *Bgl*II and subcloned into vector pJK350 cut with the same enzymes. pJK350 encodes the *lacZ*-coding region (including the SV40 nuclear localization signal), a 3′ polylinker, and a poly(A) tract of 30 residues (Evans et al., 1994). The resulting vector pBG1 was cut with *Bgl*II and treated with mung bean nuclease. pBG1 was subsequently cut with *Eco*RI. The resulting fragment was cloned into pPD16.41 that had been cut with *Eco*RI and *Stu*I.

Transgenic animals were generated using standard methods (Mello et al., 1991). The injection solution contained 25 ng/μl of test plasmid and 100 ng/μl of plasmid RF46 (RF46 carries the dominant Rol-6 marker). Transgenes were integrated into the genome as described (Mello and Fire, 1995), and expression of β-gal was assayed as described (Fire, 1992). RT-PCR was used to measure the level of transgenic RNA produced from each transgene. RNA was isolated by the method of Chomczynski and Sacchi (1987), reverse transcribed using an oligo(dT) primer and amplified using EBG-62 and EBG-21 primers. As an internal control, oligos EBG-70 and EBG-71 were used to amplify *let-2* sequences. *let-2* codes for a type IV collagen (Sibley et al., 1993). The linear range of the reaction was determined by using increasing concentrations of test cDNA, which were amplified for 25, 30 or 35 cycles. For each cycle, the concentration of cDNA versus the amount of product made were plotted. The ratio of the transgenic to collagen RNA was determined by comparing the amount of transgenic to collagen PCR products. Only values that fell within linear range of the PCR reaction for both the transgenic and the collagen products were used. To relate the amount of transgenic RNA produced by different constructs, the ratio of the *let-2* and transgene PCR products was compared between lines.

### β-galactosidase assays

β-galactosidase activity was assayed using a chlorophenol red-β-D-galactopyranoside stubstrate (Simon and Lis, 1987). Activity was calculated by dividing the change in $OD_{574}$ over time by the amount of total protein in each extract.

### RNA gel shift analysis

RNA gel shifts were performed as described (Goodwin et. al,1993). A [$^{32}$P]RNA probe containing both DREs was made by the method of Milligan and Uhlenbeck (1989) using T7 polymerase. Labelled RNA was synthesized from two oligos: EBG-8, which contains the T7 promoter, and EBG-9, which contains both DREs. The oligo names and sequences are listed below. DRE sequences are in bold; –17 to –1 of T7 RNA polymerase promoter plus an additional 6 nt are underlined. Extracts were made as described (Goodwin et al., 1993). For embryonic extracts, gravid hermaphrodites were treated with hypochlorite to dissolve the body and release intact embryos. To ensure that similar populations of wild-type and *tra-3(lf)* embryos were compared, small aliquots of each sample were removed and the age of the embryos scored using Nomarski DIC optics. In all experiments, embryos were premorphogenesis, ranging from one cell to several hundred.

### Synchronizing animals

Developmentally staged animals were grown from embryos isolated by hypochlorite treatment of gravid hermaphrodites (Sulston and Hodkgin, 1988). L1, L2, L3 and L4 were staged by scoring gonad and vulva development (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979).

### Oligo sequences

EBG-8 5′-TAATACGACTCACTATA-3′
EBG-9 5′-TGGACGATT**AGATATGAGATGATAAGAAAT-TAAATA**TGAG**TAGATATGAGTAGATAAGAAATTAAAT**AAT-GAAATGGAAATTGTCGCCCTATAGTGAGTCGTATTA-3′
EBG-20 5′-ATTTTTATTGTCGACAATGTCTGTTTC-CTTTTTCAG-3′
EBG-21 5′-AAATTTTATAGATCTTTTATTAACAAGAAAA-CAAAA-3′
EBG-62 5′-AGTATCGGCGGAATTCCAACT-3′
EBG-70 5′-TCAAGCACTCCCAAACCACC-3′
EBG-71 5′-CGTTTCTGGAGGCGTAGTTG-3′

## RESULTS

### The *laf-1* gene is required for spermatogenesis in XX hermaphrodites and for male development in XO males

Four *laf-1* alleles were isolated by genetic selections for dominant suppressors of *fem-3(gf)* sterility (see Materials and Methods). All four *laf-1* alleles have two phenotypes: *laf-1*/+ heterozygotes are feminized, while *laf-1* homozygotes die (see below). This dual phenotype inspired the name *laf*, for lethal and feminized. The *laf-1* alleles behave like a deficiency, *saDf1*, that removes the locus, indicating that all four alleles reduce *laf-1* function (see below). However, their phenotypes are more severe than *saDf1* suggesting that the *laf-1* alleles have dominant negative activity (Table 1, see below).

*laf-1* mutations feminize both XX and XO animals (Fig. 2, Table 1). XX animals heterozgyous for *laf-1* or the deficiency, *saDf1*, can be transformed from hermaphrodites into females: *laf-1/ +* or *saDf1/+* animals often fail to make sperm, but instead make oocytes (Fig. 2B; Table 1). Similarly, *laf-1/+* XO heterozygotes sometimes make gametes with an oocyte-like morphology (Fig. 2D, Table 1). The *laf-1* alleles partially feminize the XO soma as well: the sensory rays and fan of the male tail are truncated or missing, and occasionally a vulva is made (Table 1; Fig. 2D). In conclusion, *laf-1* mutations feminize both XX and XO animals, which suggests that wild-type *laf-1* represses female development.

Both XX and XO *laf-1* homozgotes die, either as embryos or first stage larva (see Materials and Methods). Dying embryos contain several hundred cells, which differentiate; pharynx and intestine are made, gut granules are visible and muscle cells are present as indicated by contractions. However, morphogenesis is defective, resulting in severely abnormal embryos (Fig. 3B). This embryonic phenotype is seen with little variation among progeny of *laf-1(q267), laf-1(q217)* and *laf-1(q349).*

### *laf-1* may be a negative regulator of *tra-2*

Mutations in *laf-1* have an effect on sexual fate similar to that of *tra-2(gf)* mutations that are defective in translational control (see Introduction). Like *tra-2(gf)* mutations, *laf-1/+* heterozygotes feminize both XX and XO animals and suppress *fem-3(gf)* sterility (Table 1; Doniach, 1986; Schedl and Kimble,

1988). Therefore, one simple hypothesis is that *laf-1(+)* may inhibit female development by repressing *tra-2*.

To test this prediction, we compared the phenotypes of *tra-2(null)* single mutants and *tra-2(null); laf-1(q217)/+* double mutants. The use of a heterozygote in double mutant analysis is not standard, but this was our only option since *laf-1* homozygotes die. We found that *tra-2(null); laf-1(q217)/+* double mutants develop as non-mating males (*n*=168), a phenotype indistinguishable from that of *tra-2(null)* single mutants (Fig. 4B). This result suggests that *laf-1* acts upstream of *tra-2* in a genetic heirarchy and is consistent with *laf-1* being a repressor of *tra-2*.

### *laf-1* affects translational regulation by the *tra-2* 3′UTR

The similarity between the *laf-1/+* and *tra-2(gf)* phenotypes suggested that *laf-1* may be involved in regulating *tra-2* at the translational level. To test this idea, we developed a transgenic reporter assay to examine translational regulation dependent on the *tra-2* 3′UTR. Specifically, we generated transgenic animals carrying the *lacZ*-coding region fused to a *tra-2* 3′UTR and placed under control of a heat-shock promoter (Fig. 5; Table 2). Three different reporter transgenes were made: *lacZ::tra-2(+) 3′UTR* carries the full-length (206 nt) wild-type *tra-2* 3′UTR; *lacZ::tra-2(-32) 3′UTR* is identical except that it lacks one DRE; and *lacZ::tra-2(-60) 3′UTR* is again identical except that it lacks both DREs.

The activities of the three reporter transgenes differ dramatically when examined in a wild-type background. For *lacZ::tra-2(+) 3′UTR*, only 3% of XX adult animals displayed β-gal staining in intestinal cells (Fig. 5A; Table 2). However, for *lacZ::tra-2(-32)* and *lacZ::tra-2(-60) 3′UTR*, 62% and 67% of the adult animals, respectivly, had intestinal staining. Similar result were obtained when total β-gal activity was measured using an in vitro assay (Table 2; Simon and Lis, 1987). We also examined the expression of the transgenes in XO animals. Similar to XX animals, only a few animals carrying *lacZ::tra-2(+) 3′UTR* showed intestinal β-gal staining, however, greater than 40% of XO animals with *lacZ::tra-2(-32)* or *lacZ::tra-2(-60) 3′UTR* had β-gal activity (data not shown). The different transgenes produce similar amounts of RNA (see Materials and Methods; Table 2). Therefore, the difference in β-gal expression is not likely due to differences in production or stability of the RNA, but instead, to differences in translational control.

We next examined β-gal expression from the three reporter transgenes in a *laf-1/+* mutant background. A striking increase in the number of animals with β-gal expression was observed in *laf-1/+* mutants carrying the *lacZ::tra-2(+) 3′UTR* reporter (Fig. 5B; Table 2). It is unlikely that the enhancement of β-gal activity resulted from the heat shock, since a reporter transgene under the control of a non-inducible promoter gave similar results (C. Motzny, personal communication). Again, *saDf1* and the *laf-1* mutations did not alter the amount of reporter RNA (Table 2), consistent with the idea that *laf-1* mutations affect translational control. *laf-1* mutations also increased the expression of *lacZ::tra-2(+) 3′UTR* in XO animals (data not shown), suggesting that *laf-1* regulates *tra-2* translation in males as well. Two other feminizing mutations, *fem-3(lf)* and *fog-2(lf)*, do not increase β-gal

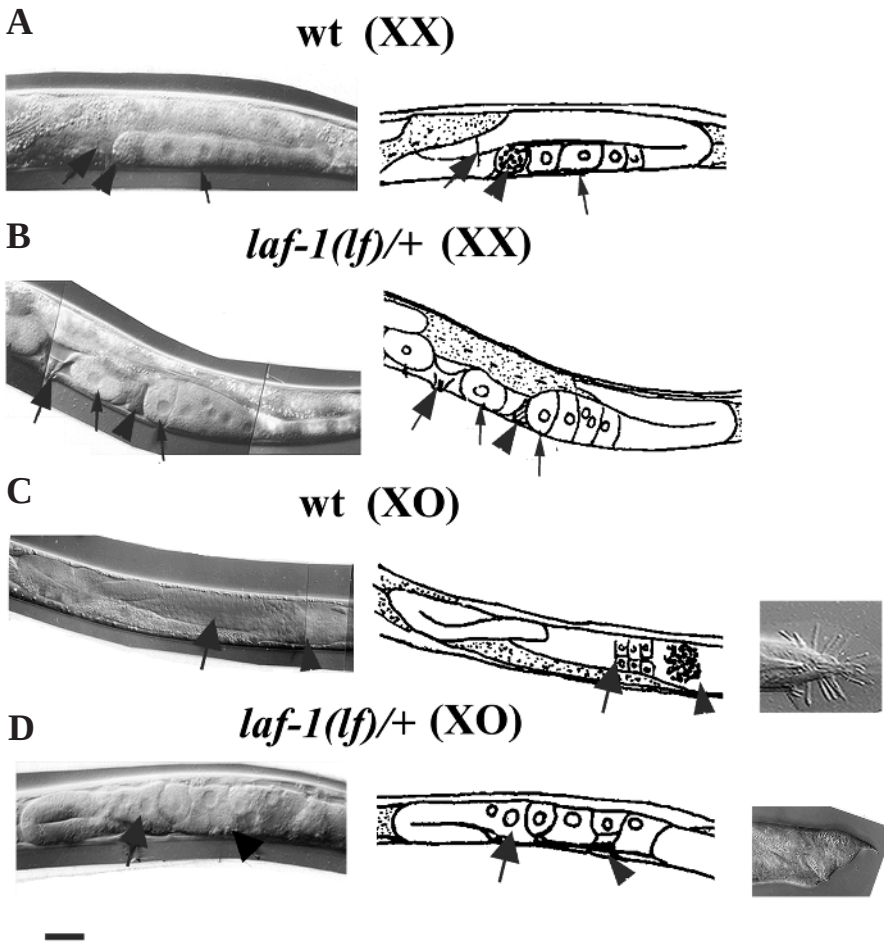


**Fig. 2.** Sexual phenotypes of *laf-1* /+ XX and XO animals. Posterior mid-sections of young adults; anterior to left. Line drawings (right) illustrate gamete type and gonad shape; intestine is stippled. (A) Wild-type XX hermaphrodite, ventral view. Note typical bilobed gonad, vulva (large arrow), sperm (arrowhead) and oocytes (small arrow). (B) *laf-1(q217)/+* XX animal, lateral view. The somatic gonad is bilobed and the vulva appears normal (large arrow). However, the germ line is feminized. Only oocytes are produced (small arrow); sperm are not observed in the spermatheca (arrowhead) and cross-progeny are not made. An unfertilized oocyte (small arrow) is present in the uterus. (C) Wild-type XO male, ventral view. The somatic gonad is a single U-shaped gonad with sperm (arrowhead) and primary spermatocytes (large arrow). The tail has typical male sensory rays and fan structure (inset). (D) *laf-1(q217)/+* XO animal, lateral view. The somatic gonad is a single U-shaped gonad and has some sperm (arrowhead). However, the germ line is feminized as indicated by oocyte-like cells (arrow). The tail is also feminized: rays are truncated and the fan is absent. Magnification bar equals 100 μm, except for the tail insets where it equals 25 μm.

**Table 1. *laf-1* mutations feminize both XX and XO animals**

| Genotype* | Suppression of *fem-3(gf)*† | XX‡ % animals with oocytes only | XO§ % animals with feminized germ line | XO§ % animals with feminized soma |
|---|---|---|---|---|
| Wild type | − | 0% (*n*=55) | 0% | 0% (*n*=25) |
| *laf-1(q267)/+* | + | 16% (*n*=150) | 0% | 12% (*n*=52) |
| *laf-1(q80)/+* | + | 10% (*n*=139) | 12% | 23% (*n*=73) |
| *laf-1(q217)/+* | + | 20% (*n*=145) | 27% | 36% (*n*=146) |
| *laf-1(q349)/+* | + | 30% (*n*=200) | 22% | 27% (*n*=22) |
| *saDf1/+* | + | 6% (*n*=123) | 0% | 3% (*n*=67) |

*XX animals were progeny from either *dpy-1unc-32/qC1* (wild type), *laf-1(lf)/qC1*, or *saDf1/qC1;* XO animals were progeny from either *dpy-1unc-32/qC1;him-8* (wild type), *laf-1(lf)/qC1;him-8*, or *saDf1/qC1;him-8*

†*laf-1* alleles were obtained as *fem-3(gf)* suppressors (see Materials and methods). +; *fem-3(q96*gf*)* and *saDf1/+; fem-3(q96*gf*)* progeny were obtained from mothers raised at 15°C and then shifted to 25°C as adults. Approximately 40% of the *saDf1/+; fem-3(q96*gf*)* progeny were self-fertile, compared to 0% for *fem-3(q96*gf*)*. *saDf1/+* and *laf-1(lf)/+* are similar in their ability to suppress the *fem-3(gf)* self-sterile phenotype (data not shown).

‡XX progeny were scored at 20ºC. *n* = number animals scored.

§XO progeny were scored at either 20°C or 25°C. Similar phenotypes were detected at each temperature, although feminization was less frequent at 20°C than at 25°C. The above values were obtained from progeny of mothers raised at 20°C and then shifted as young adults to 25°. *n* = number animals scored.

activity in the same assay (data not shown), indicating that the effect on reporter expression is not simply due to feminization.

To investigate whether *laf-1* acts through the DREs to repress the activity of the transgene, we asked whether *laf-1* mutations could affect the activity of *lacZ::tra-2(-32)* but not *lacZ::tra-2(-60) 3′UTR*. Both in vivo and in vitro assays demonstrated that *laf-1(q267)/+* increased β-gal expression of *lacZ::tra-2(-32)*, but did not affect expression of *lacZ::tra-2(-60) 3′UTR* (Table 2). The *laf-1* mutation did not increase the steady state levels of reporter RNA (Table 2). The simplest interpretation is that a decrease in *laf-1* activity relieves translational repression of *tra-2* by the 3′UTR. Therefore, *laf-1* mutations are likely to feminize animals by reducing the translational repression of *tra-2* by the DREs.

### *tra-3* may inhibit translational repression of *tra-2*

Previous genetic analyses have not separated the activities of *tra-2* and *tra-3* in the genetic hierarchy (Hodgkin, 1980). Since *laf-1* appears to act upstream of *tra-2*, we next asked whether *laf-1* also acts upstream of *tra-3*. To this end, we examined the phenotype of a *laf-1/+; tra-3(null)* double mutant. In this experiment, we used all four *laf-1* alleles as well as the deficiency, *saDf1*. Furthermore, because *tra-3* has a strong maternal effect, we scored progeny from *laf-1/+; tra-3(lf)* homozygotes. In contrast to our result with *tra-2*, we found that the presence of *laf-1/+* or *saDf1/+* could partially feminize *tra-3(lf)* mutants (Fig. 4D; Table 3). One interpretation is that *tra-3* acts upstream of *laf-1* and that *tra-3* represses *laf-1* activity. Alternatively, *laf-1* and *tra-3* may act in parallel pathways.

If *tra-3(+)* normally inhibits *laf-1(+)*, then removal of *tra-3* might suppress the effect of reducing *laf-1* activity. To test this, we examined expression of *lacZ::tra-2(+) 3′UTR* in progeny of *laf-1/+; tra-3(lf)* double mutants. As predicted, *tra-3(lf)* significantly decreased the number of animals with intestinal β-gal staining (Tables 2 and 4). These results are consistent with *tra-3(+)* promoting female development by blocking the translational repression of *tra-2*.

**Table 2. *laf-1* Mutations disrupt DRE-mediated regulation of a reporter transgene**

| Genotype* | Transgene† | % animals with intestinal β-gal staining‡ | β-gal Activity§ | β-gal:*let-2*¶ |
|---|---|---|---|---|
| wild-type | *lac-Z::(wt)3′UTR* | 3% (*n*=112) | 0.02 (0.01) | 0.30 |
| wild-type | *lac-Z::(-32)3′UTR* | 62% (*n*=132) | 0.32 (0.03) | 0.50 |
| wild-type | *lac-Z::(-60)3′UTR* | 67% (*n*=117) | 0.19 (0.01) | 0.21 |
| *laf-1(q217)/+* | *lac-Z::(wt)3′UTR* | 31% (*n*=83) | n.d. | 0.32 |
| *saDf1/+* | *lac-Z::(wt)3′UTR* | 35% (*n*=18) | n.d. | 0.40 |
| *laf-1(q267)/+* | *lac-Z::(wt)3′UTR* | 45% (*n*=67) | 0.39 (0.01) | 0.23 |
| *laf-1(q267)+* | *lac-Z::(-32)3′UTR* | 89% (*n*=25) | 0.76 (0.07) | 0.41 |
| *laf-1(q267)/+* | *lac-Z::(-60)3′UTR* | 66% (*n*=34) | 0.31 (0.01) | 0.17 |

*Wild-type animals were N2 hermaphrodites; *laf-1/+* animals were progeny from *laf-1(lf)/qC1* and *saDf1/+* were from *saDf1/qC* mothers. In all experiments, adult worms were heat shocked for 2 hours at 33°C and allowed to recover for an additional 2 hours at 20°C before being fixed and stained for β-gal activity.

†Three different transgenes were integrated into the *C. elegans* genome. All three transgenes carry the *lacZ* coding region under control of the inducible heat shock promoter (16.41). *lac-Z::tra-2(+)3′UTR* has the wild-type *tra*-2 3′UTR which has two DREs separated by a 4nt spacer; *lac-Z::(-32)3′UTR* has a mutant *tra-2* 3′UTR which is deleted for one DRE plus the spacer; and *lac-Z::(-60)3′UTR* has a different mutant *tra-2* 3′UTR which is deleted for both DREs plus spacer.

‡Animals were scored as positive if blue precipitate was detectable in intestinal cells at 630× magnification; genetic evidence suggests that control by the *tra-2* 3′UTR functions in intestinal cells (Doniach, 1986). *n*= total number animals scored from at least five different experiments.

§Numbers represent β-gal activity present in crude lysates of adult worms. Units are change of $OD_{574}$ from CPRG hydrolysis per min per mg protein, and are mean values of at least four different experiments. Standard deviations are in parentheses.

¶RT-PCR was used to measure the amount of trangenic RNA made from the different transgenes after a 2 hour heat shock (see Materials and methods for details). As an internal control, mRNA from the *let-2* gene was amplified. Shown is the ratio of PCR product from the transgene to PCR product from *let-2*.

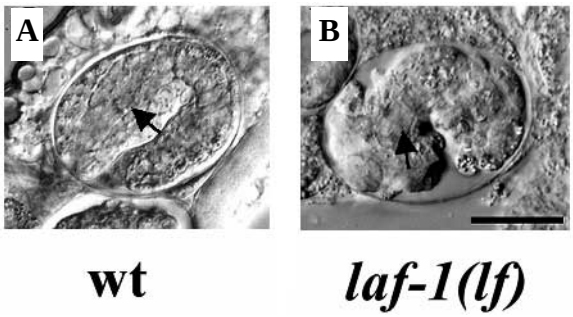


**Fig. 3.** Embryos homozygous for *laf-1* mutations arrest with defects in morphogenesis. (A) Wild-type embryo at two-fold stage. (B) *laf-1(q267)* homozygous embryo at similar age. Morphogenesis is defective, but major tissue types are made: pharyngeal tissue (arrow) and intestine (not visible in this focal plane) are present. Magnification bar equals 10 μm.

### Translational repression by the *tra-2* 3′UTR is influenced by developmental stage

We next used the *lacZ::tra-2 3′UTR* reporter transgenes to examine translational repression during development. In adults, β-gal activity from *lacZ::tra-2(+) 3′UTR* is generally not detected in intestinal cells, but is usually present in unlaid embryos (Fig. 5A), suggesting that the translational repression by the *tra-2* 3′UTR is reduced in embryos.

To further pursue the developmental stages at which *tra-2* translational repression occurs, we examined expression of reporter transgenes during larval development. Specifically, we measured expression of the *lacZ::tra-2(-32) 3′UTR* reporter transgene in the four larval stages (L1, L2s, L3s and L4s). This transgene was used because one DRE can partially regulate *tra-2* translation (Goodwin et al., 1993) and therefore might provide a sensitive assay. We found that the first three larval stages had less β-gal activity as compared to L4s and adults (Fig. 6). L2s and L3s had particularly low β-gal expression; only 8% and 3% of the animals, respectively, had intestinal staining. The decrease in expression from *lacZ::tra-2(-32) 3′UTR* depends on DRE regulation, since greater than 60% of larval animals with *lacZ::tra-2(-60) 3′UTR* had β-gal activity in intestinal cells (Fig. 6). Again, the different transgenes made similar amounts of RNA (data not shown). We conclude that the translational repression of *tra-2* by its 3′UTR is strongest in L2s and L3s.

### The presence of DRF-binding activity correlates with the strength of translational repression during development

Previously, we identified a binding activity specific to the *tra-2* DREs, called DRF, and proposed that DRF represses translation (Goodwin et al., 1993; see Introduction). To test whether DRF-binding activity correlates with strength of repression, we examined DRF at specific stages of development. Extracts were made from embryos, the four larval stages and adults, and then assayed for DRF activity. We found no DRF in extracts made from embryos, but did see activity in the four larval stages and adults, with a peak of activity in L2s and L3s (Fig. 7A). This developmental profile of DRF activity correlates remarkably well with the strength of translation repression by the *tra-2* 3′UTR during development (see above).

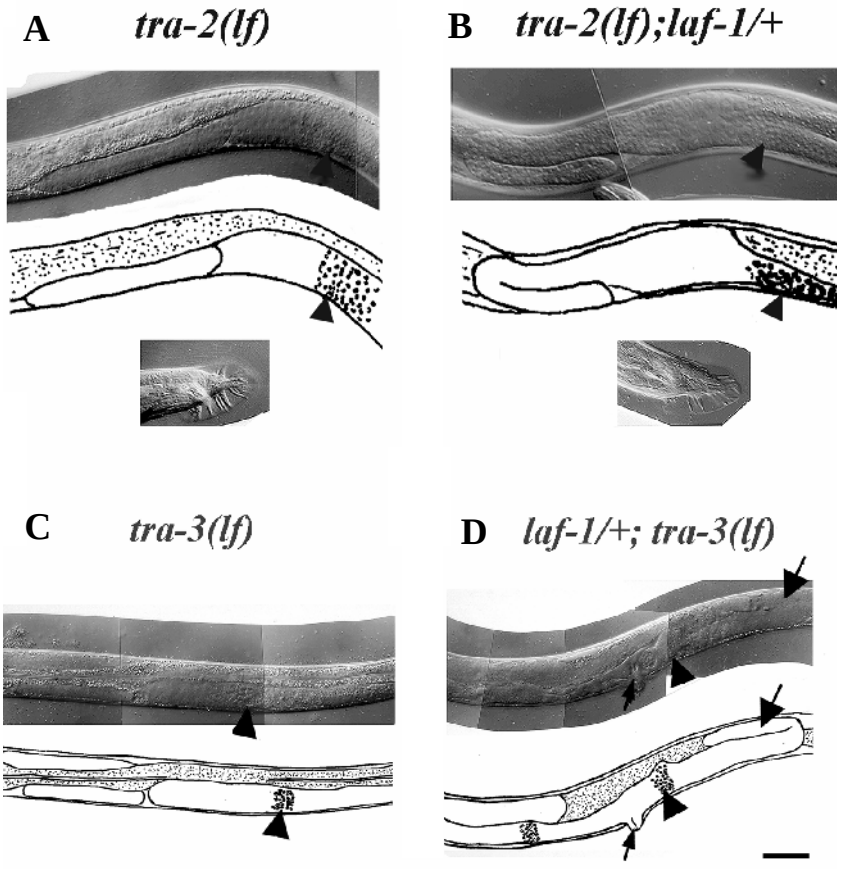


**Fig. 4.** *laf-1* may act upstream of *tra-2* in the hierarchy of sex-determining genes. Mid-regions shown in ventral view with anterior to left; tails shown below. (A) XX *tra-2(e1095)* homozygote. The phenotype of the *tra-2(e1095)* null allele was described previously (Hodgkin and Brenner, 1977; Kuwabara et al., 1992). Animals have a masculinized soma with slightly truncated fan and sensory rays, fail to display mating behaviour and make only sperm (arrowhead). (B) XX *tra-2(1095); laf-1(q217)/+* double mutant. The double mutant is indistinguishable from a *tra-2(e1095)* single mutant: it develops as a non-mating male with truncated tail structures and the germ line produces sperm (arrowhead). Magnification bar equals 100 μm except for tails where it equals 25 μm. *tra-3* may act upstream of *laf-1* in the hierarchy of sex-determining genes. (C,D) Posterior mid-sections of young adults; lateral views with anterior to left. Tails are not shown since *laf-1* did not further feminize this structure. (C) XX *tra-3(e1107)* single mutant. The phenotype of the *tra-3(e1107)* putative null allele was described previously (Hodgkin and Brenner, 1977). Both soma and germ line are masculinized; sperm are indicated (arrowhead). (D) XX *laf-1(q217)/+; tra-3(e1107)* double mutant. Feminization is observed by presence of a vulva (small arrow) and a reflexed gonad (large arrow) In this animal, the germ line is not feminized; it produced only sperm (arrowhead). Magnification bars equal 100 μm.

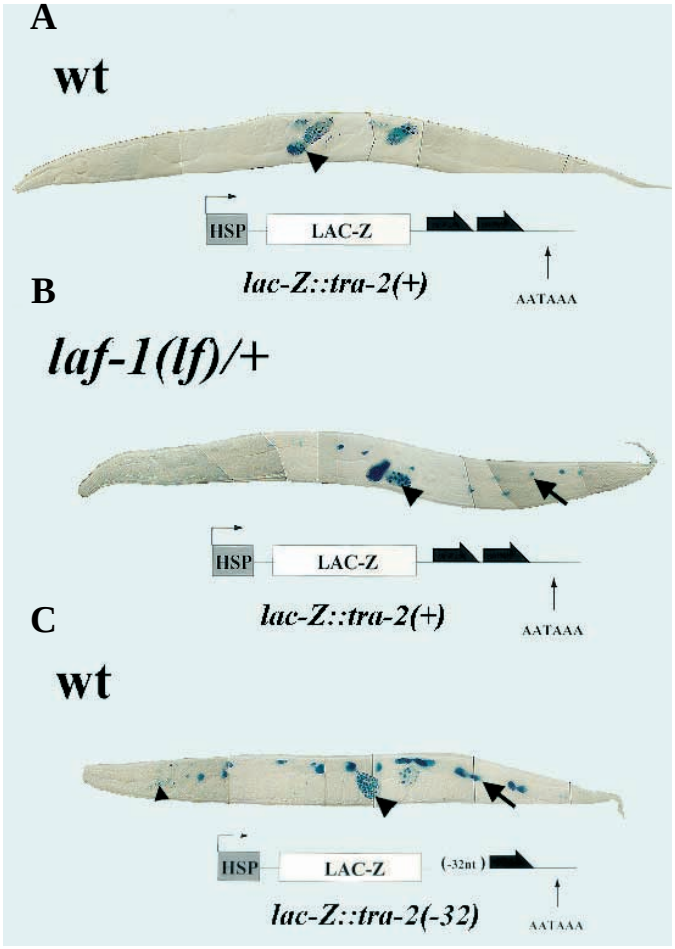


**Fig. 5.** Translational regulation of a reporter transgene by the *tra-2* 3′UTR is disrupted in *laf-1*/+ animals. Lateral views with anterior to left; cartoons and names of particular transgenes indicated below each animal. The β-gal reporter is fused to a nuclear localization signal, so that staining is primarily nuclear. (A) Wild-type animal carrying *lacZ:: tra-2(+)*3′UTR transgene. No β-gal activity is detected in somatic tissues, but embryos show strong β-gal staining (arrowhead). (B). *laf-1(q267)/+* carrying *lacZ:: tra-2(+)*3′UTR transgene. β-gal activity is not only found in embryos (arrowhead), but also observed in intestinal cells (arrow; 12 large intestinal nuclei are easily visible). (C) Wild-type animal carrying *lacZ:: tra-2(-32)*3′UTR transgene. β-gal activity is observed in embryos (arrowhead), intestinal cells (arrow; 13 intestinal nuclei can be seen) and several unidentified somatic cells (small arrowhead).

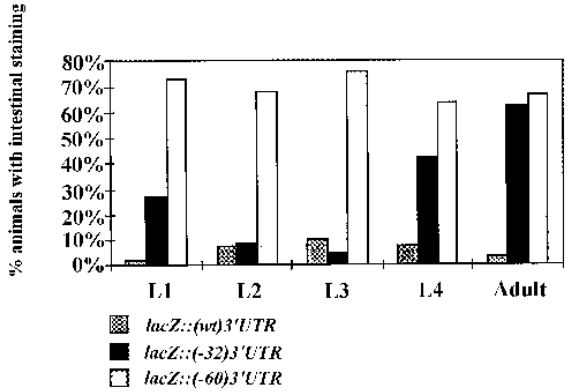


**Fig. 6.** Developmental regulation of the translational control. Wild-type animals carrying *lacZ:: tra-2(wt)*3′UTR, *lacZ:: tra-2(-32)*3′UTR, or *lacZ:: tra-2(-60)*3′UTR transgenes were heat shocked and stained for β-gal activity. The number of animals showing β-gal staining in intestinal cells were scored. Each data point represents at least 50 animals from three different experiments.

### Removal of *tra-3* increases DRF activity in embryos

The lack of DRF activity in early embryos might be explained by the presence of an activity that inhibits DRF. The best candidate for such an inhibitor is *tra-3* (see above). A prediction of this model is that DRF activity might be observed in progeny of *tra-3(lf)* mutants. To test this hypothesis, we made extracts from *tra-3(lf)* homozygous embryos and tested the extracts for DRF activity. DRF was indeed present in the *tra-3(lf)* embryonic extract (Fig. 7B). This finding is consistent with the idea that *tra-3* promotes female development by removing the *tra-2* translational repressor.

If *tra-3* normally acts to repress the translational control of *tra-2* in embryos, then removal of *tra-3* might decrease expression of the *lacZ::tra-2(wt) 3′UTR* transgene in embryos. Therefore, we examined the expression of *lacZ::tra-2(wt) 3′UTR* in unlaid embryos of *tra-3(lf)* hermaphrodites. As predicted, *tra-3(lf)* decreased the number of embryos with β-gal staining (Table 4). In conclusion, the gel shift and transgene results are consistent with *tra-3(+)* promoting female development by inhibiting the translational repression of *tra-2*.

## DISCUSSION

### Translational control by *laf-1* and *tra-3*

In this paper, we identify two genes, *laf-1* and *tra-3*, that regulate the translation of *tra-2* by the 3′UTR, and we propose a pathway by which these two genes may control sex determination.

The *laf-1* gene is required for translational repression by the *tra-2* 3′UTR and may encode part of the translational repressor. Three lines of evidence support this idea. First, a reduced level of *laf-1* disrupts regulation of a reporter transgene by the *tra-2* 3′UTR. In wild-type animals, a transgene bearing the intact *tra-2* 3′UTR is translationally repressed but, in *laf-1/+* mutant backgrounds, that transgene is partially released from repression. Second, the properties of *laf-1* mutations are consistent with predictions for a *tra-2* translational repressor. The phenotype of a loss-of-function mutation in the *trans*-acting repressor is expected to mimic that of a gain-of-function mutation lacking the *cis*-acting regulatory element. As predicted, both *laf-1* and *tra-2(gf)* mutations feminize XX and XO animals. Furthermore, the *laf-1* locus is dosage sensitive, consistent with the observation that the number of DREs influences the extent of the control (Goodwin et al., 1993). Third, double mutant analysis is compatible with *laf-1* acting upstream of *tra-2*, the predicted position for a repressor of *tra-2*.

The *tra-3* gene (Hodgkin and Brenner, 1977) appears to act in the embryo to antagonize translational repression by the *tra-2* 3′UTR, perhaps by inactivating DRF. In wild-type embryos, the 3′UTR does not repress translation of a reporter transgene but, in *tra-3* mutant embryos, the 3′UTR is able to inhibit translation. Furthermore, DRF activity is present in *tra-3* homozygous mutant embryos, but not in wild-type embryos. In

**Table 3. *laf-1* mutations can feminize *tra-3(7lf)* animals***

| Genotype* | | % animals with bilobed gonad† | % animals with vulva |
|---|---|---|---|
| *tra-3(e1107)* | (*n*=82) | 0% | 6% |
| *laf-1(q267)/+;tra-3(e1107)* | (*n*=62) | 21% | 10% |
| *laf-1(q80)/+;tra-3(e1107)* | (*n*=53) | 46% | 16% |
| *laf-1(q217)/+;tra-3(e1107)* | (*n*=48) | 40% | 17% |
| *laf-1(q349)/+;tra-3(e1107)* | (*n*=45) | 72% | 56% |
| *saDf1/+;tra-3(e1107)* | (*n*=40) | 25% | 27% |

*Animals were obtained as progeny from either *dpy-1 unc-32/qC1; tra-3(e1107)* or *laf-1(lf)/qC1; tra-3(e1107)* hermaphrodites and scored at 20°C. *n* = number animals scored. Results were obtained from at least two different experiments.

†A bilobed gonad was scored if two distinct arms were seen. Many times one arm did not reflex properly, and often one arm was smaller than the other. Occasionally abnormal gonads were observed and were not scored as either bilobed or single lobed.

addition, derepression of a reporter transgene, bearing the intact *tra-2* 3′UTR, in a *laf-1*/+ mutant background is suppressed by *tra-3(lf)*. Finally, double mutant analysis suggests that *tra-3* acts upstream of *laf-1* in a genetic hierarchy, as would be predicted if *tra-3* were a regulator of *laf-1*.

Our working model is that *tra-3* negatively regulates *laf-1* and that *laf-1*, in turn, negatively regulates translation of *tra-2* (Fig. 8A). This model is based on experiments that utilized both the deficiency *saDf1* and the *laf-1* alleles, and is the simplest interpretation of our results.

## Roles of *laf-1* and *tra-3* in sex determination

The control of *tra-2* translation by *laf-1* and *tra-3* is necessary for sexual development in both the somatic and germ-line tissues of XX and XO animals. We postulate that *tra-3* acts in the early embryo to release *tra-2* from negative translational regulation by *laf-1* (Fig. 8B). In XX embryos, translation of *tra-2* leads to inhibition of the *fem* genes and hermaphrodite development. In XO embryos, translation of *tra-2* has little effect due to the presence of *her-1*, which is thought to repress *tra-2* post-translationally and thereby direct the embryo down the male pathway of differentiation (Hunter and Wood, 1992; Kuwabara et al., 1992; Perry et al., 1993).

Once the embryo hatches, we suggest that *laf-1* represses *tra-2* translation in L2 and L3 hermaphrodites to permit spermatogenesis (Fig. 8C). The effect of maternal *tra-3* may have dissipated by this stage, or *tra-3* may be regulated in a tissue-specific manner so that *laf-1* is free to repress *tra-2* translation. In addition, *laf-1* is required in XO animals to inhibit *tra-2* translation for normal male development.

**Table 4. Loss of *tra-3* increases translational repression of *tra-2***

| Genotype* | Transgene | % β-gal staining† | β-gal/ let-2‡ |
|---|---|---|---|
| *laf-1(q217)/+;tra-3(e1107)* | *lac-Z::(wt)3′UTR* | 15% (*n*=40) | 0.37 |
| *saDf1/+;tra-3(e1107)* | *lac-Z::(wt)3′UTR* | 8% (*n*=50) | 0.33 |
| N2 (embryos) | *lac-Z::(wt)3′UTR* | 55% (*n*=325) | n.d. |
| *tra-3(e1107)* (embryos) | *lac-Z::(wt)3′UTR* | 27% (*n*=218) | n.d. |

*Animals were obtained as progeny from either wild-type (N2), *tra-3(e1107)* or *laf-1(lf)/qC1; tra-3(e1107)* hermaphrodites In all experiments, adult worms were heat shocked for 2 hours at 33°C, and then allowed to recover for an additional 2 hours at 20°C before being fixed and stained for β-gal activity. To determine whether *tra-3(lf)* mutations increased *tra-2* 3′UTR-mediated regulation in embryos, unlaid embryos of mothers of the indicated genotype were scored for β-gal activity.

†For adults, *n*=number animals scored for β-gal staining in intestinal cells. For embryos, all tissues were scored for β-gal activity.

‡See Table 2 legend.

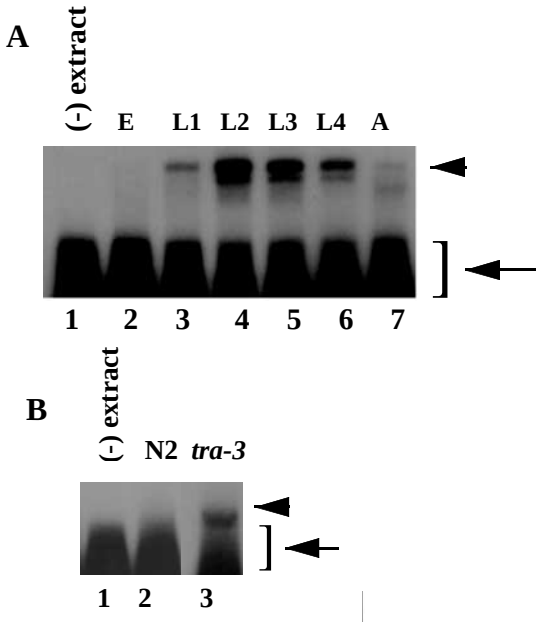


**Fig. 7.** Developmental regulation of DRF activity. (A) DRF activity during development as assayed by RNA gel shifts. One femtomole of $^{32}$P-labeled EBG-9 RNA was incubated with either no extract (lane 1), or 5 μg crude extract made from embryos (lane 2), L1s (lane 3), L2s (lane 4), L3s (lane 5) L4s (lane 6) and adults (lane 7). Reactions were loaded on a 7% nondenaturing polyacrylamide gel. The gel was dried and autoradiographed. Slower migrating bands are due to complex formation (arrowhead); the faster band is uncomplexed probe (arrow). (B) To examine whether DRF activity is influenced by the state of *tra-3* in embryos, 5 μg crude extract made from either wild-type embryos (lane 2) or *tra-3(e1107)* homozygous embryos (lane 3) were incubated with one femtomole of EBG-9 RNA and then run on a 7% nondenaturing polyacrylamide gel. Complex (arrowhead) and free probe (arrow) are indicated.

It should be noted that hermaphrodite spermatogenesis does not rely soley on control of *tra-2* at the translational level (Doniach, 1986; Schedl and Kimble, 1988; Goodwin et al., 1993). The onset of hermaphrodite spermatogenesis also requires an apparently post-translational regulation of *tra-2* (P. Kuwabara, P. Okkema, and J. Kimble, unpublished data) as well as regulation by *fog-2* (Schedl and Kimble, 1988). The mechanism by which *fog-2* controls sex determination is not understood. However, it does not appear to be at the level of *tra-2* translation since *fog-2* mutants have no effect on the expression of a reporter transgene under control of the *tra-2* 3′UTR (E. B. Goodwin, unpublished results).

## *laf-1* and *tra-3* may control other genes in addition to *tra-2*

*laf-1* and *tra-3* may have other regulatory activities in addition to their control of translation by the *tra-2* 3′UTR. Neither loss-of-function nor gain-of-function *tra-2* mutations affect viability (Hodgkin and Brenner, 1977; Doniach, 1986), but both XX and XO *laf-1* homozygous embryos die. The most likely explanation

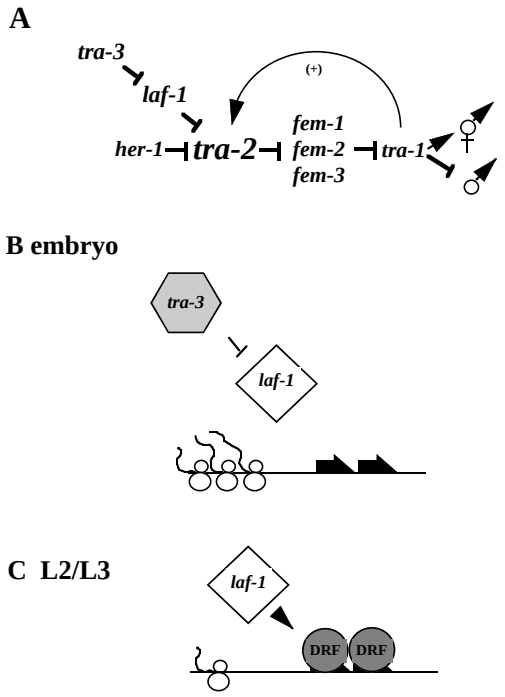


**Fig. 8.** Model for translational regulation of *tra-2* by *laf-1* and *tra-3*. (A) Genetic pathway for translational control of *tra-2*. In the embryo, *tra-3* inhibits *laf-1* and, in turn, *tra*-2 can promote female development. In XO embryos, the presence of *her-1* inhibts *tra-2* post-transcriptionally, allowing for male development. During the L2 and L3 stages of XX development, *tra-3* is absent. Therefore, *laf-1* inhibits *tra-2* allowing for spermatogenesis. During XO male development, *her-1* and *laf-1* are required to maintain low *tra-2* activity. (B,C) Molecular model for how *tra-3* and *laf-1* modulate the translation of *tra-2*. (B) In embryos, *tra-3* removes *laf-1*. Hence, DRF does not bind to the DREs and *tra-2* translation is not inhibited. (C) In L2s and L3s, *tra-3* is inactive, therefore, *laf-1* promotes DRF binding to the DREs and *tra-2* translation is repressed.

is that *laf-1* regulates other mRNAs in addition to *tra-2* and that misexpression of these mRNAs results in death. Similarly, *tra-3* may regulate other genes in addition to *tra-2*. If the effects of *tra-3* were exerted only through the *tra-2* 3′UTR, then a strong *tra-2(gf)* mutation, which deletes both DREs, would be predicted to completely feminize a *tra-3* mutant. However, this is not the case (Doniach, 1986). Since *tra-3* mutants only appear to affect sexual fates, *tra-3* may normally regulate other genes in the sex determination pathway in addition to *tra-2*. Alternatively, *laf-1* may normally repress other female-determining genes or additional *cis*-acting regulatory elements in the *tra-2* 3′UTR may exist.

## A molecular model for translational regulation by the *tra-2* 3′UTR

We previously proposed that DRF binds the *tra-2* DREs to negatively regulate translation (Goodwin et al., 1993). The results reported here support this hypothesis. The presence of DRF correlates well with the strength of repression. In embryos, neither DRF activity nor translational repression is observed. However, in L2s and L3s, DRF activity peaks as does translation repression. Our results also link the activities of *laf-1* and *tra-3* to the DRE-mediated regulation. A loss of *tra-3* activity increases both translational repression and DRF activity. Similarly, the effect of *laf-1* is likely to occur via the DREs. Loss of *laf-1* activity affects the control of 3′UTRs bearing either one or two DREs, but has little or no affect on a 3′UTR with no DREs.

The molecular nature of the *laf-1* gene product remains unknown. However, we speculate that *laf-1* may encode either a component of DRF itself or a factor that promotes DRF activity. RNA gel shift analysis was performed to determine if *laf-1* mutations may alter DRF binding. Since, *laf-1* mutations are homozygous lethal, it was necessary to use extracts made from *laf-1*/+ heterozygote animals. We found that DRF activity in *laf-1*/+ extracts was similar to wild-type extracts (data not shown). However, the presence of one wild-type copy of the gene makes this result difficult to interpret. The mechanism by which DRF represses *tra-2* translation is not understood. DRF might control length of the poly(A) tail, it might sequester *tra-2* mRNA from the translational apparatus or it might inhibit binding of translational initiation factors or ribosomal subunits to the 5′UTR.

The *tra-3* gene encodes a calpain-like protease (Barnes and Hodgkin, 1996). The molecular identity of *tra-3* suggests a mechanism by which *tra-3* could regulate *laf-1*/DRF. TRA-3 may destroy DRF activity by proteolytic cleavage of one or more its components.

The simplest molecular model for the roles of *laf-1* and *tra-3* is depicted in Fig. 8. The most direct role for *laf-1* in 3′UTR regulation is as a component of DRF; the most direct role for *tra-3* is a proteolytic negative regulator of *laf-1*. However, it is also possible that either gene might act indirectly on DRF to enhance (*laf-1*) or suppress (*tra-3*) its activity. Furthermore, although some links to the DREs have been made, it remains formally possible that either might work through some other element in the *tra-2* 3′UTR.

## Why control *tra-2* at the translational level?

We suggest that this control may have been selected for several reasons. First, sex determination occurs in the early embryo, where translational control is prominent in many organisms. Although *tra-3* can be supplied either maternally or zygotically (Hodgkin and Brenner, 1977), the *tra-3/laf-1* control may have evolved from a time when the embryo was under more strict translational control. Alternatively, since *tra-3* and *laf-1* are likely to act on other genes as well (see above) perhaps this control impinges on other mRNAs supplied exclusively from the maternal dowry.

Second, the interaction of TRA-2 with other regulatory proteins in the pathway may demand a precise control over the level of TRA-2 protein. If too much TRA-2 is made, feminization occurs abnormally in both XX and XO animals (Doniach, 1986). In XO animals, negative control by *her-1* is not sufficient to down-regulate *tra-2* and, in XX animals, negative control by *fog-2* is similarly not sufficient. Because a positive feedback upon *tra-2* may amplify commitment to female development (Okkema and Kimble, 1991), inappropriate *tra-2* expression could be augmented and result in inappropriate female development. Therefore, *tra-2* protein must be maintained at a level that can be controlled by various other regulators, such as *her-1* and *fog-2*.

Third, *tra-2* is transcribed as part of an operon and shares a promoter with a second transcript. Perhaps, the translational control of *tra-2* evolved to accommodate the constraints put on the *tra-2* promoter by this genome structure.

We are grateful to Kathy Barton and Tim Schedl for isolation of *fem-3(gf)* suppressors, Peter Candido for the pPD16.41 vector and Kenneth Ebie for invaluable help with the transgene experiments. We thank Marv Wickens, Eric Jan and Cindy Motzny for critical reading of the manuscript, Jim Kramer, Marv Wickens, Eric Jan and Cindy Motzny for valuable discussions, and Ken Pobloske for help with illustrations. This work was supported in part by grants from the NIH, NSF, Council for Tobacco Research and ACS to J. K. and by grants from NIH and Council for Tobacco Research to E. B. G. The generous support of the Howard Hughes Medical Institute to J. K. is also acknowledged.

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