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; Villeneuve 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 indicated. 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), tr-a-3(e1107); LGV, fog-2(q71i). In addition, we used two balancer chromosomes: \(not1\) (dpy-10(e128) unc-32(e444)), which suppresses recombination over the right half of chromosome II, and \(q1\) (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 suppressors of \(fem-3(gf)\) sterility as described (Barton and Kimble, 1990). At restrictive temperature, \(fem-3(gf)\) homoyogotes produce only sperm, whereas \(laf-1\) and \(fem-3(gf)\) animals produce both sperm and oocytes and consequently are self-fertile. \(laf-1\)(q267 and \(q80\) were isolated as suppressors of \(fem-3(q20gf)\), \(laf-1(q217)\) as a suppressor of \(fem-3(q96gt)\), and \(laf-1(q349)\) as a suppressor of \(fem-3(q95gt)\). All four alleles were backcrossed six times against wild-type animals. \(q267\) and \(q349\) failed to complement \(laf-1(q217)\), and all four mapped left of \(unc-93\) on linkage group III. Three factor mapping placed \(laf-1\) between \(dpy-1\) and \(daf-2\). From a parent of genotype \(dpy-1\(+/+\) \(daf-2\)), we isolated a map unit 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 \(laf-1\)\(daF-2\)\(+\)

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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 feedback 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 oocyte 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 \(laf-1q\) \(C\) \(1\) hermaphrodite at 20°C and then 24 hours later counting the number of unhatched embryos and arrested \(L\) \(1\) s. To determine when \(laf-1\) homoyogotes die, embryos were dissected from \(laf-1\) \(q\) \(C\) \(1\) 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 \(L\) \(1\) s from \(laf-1\) \(q\) \(C\) \(1\); \(him-8\) (which should include 37% \(XO\) animals) were scored for male-specific enlargement of the B blast cell. Approximately, 8% of the dying \(L\) \(1\) s were male, the expected number (\(n=53\)). Second, \(laf-1\) \(unc-32\) \(q\) \(C\) \(1\); \(him-8\) progeny were scored for survivors with an \(Unc-32\) phenotype. No Unc-32 progeny (male or hermaphrodi
te) 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, \(tra-2\) \((null)\) \(unc-4\) \(mn\) \(C\) \(1\) \ or \(tra-2\) \((null)\) \(unc-4\) \(hmn\) \(C\) \(1\); \(laf-1\) \(q217\) \(q\) \(C\) \(1\) animals were selfed, and Unc-4 non-Dpy animals scored \(unc-4\) as

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closely linked to tra-2 and serves as a marker; \(qCl\) homozygotes are Dpy. For \(laf-1/4\) and \(tra-3\) double mutants, \(dpy-1\) unc-32(\(qc1\) \(tra-3\), \(laf-1/4\) \(qc1\); \(tra-3\) and \(sadDf1/\(qc1\); \(tra-3\) hermaphrodites were selfed and non-Dpy progeny scored.

**Transgenic assay**

All transgene constructs were derived from the same parent vector, pCP16.41 (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 \(tra-2(+/+)\) 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 \(SalI\) and \(BglII\), respectively. The resulting PCR products were digested with \(SalI\) and \(BglII\) and subcloned into vector pK350 cut with the same enzymes. pK350 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 \(BglII\) and treated with mung bean nuclease. pBG1 was subsequently cloned with EcorI. The resulting fragment was cloned into pPD16.41 that had been cut with EcorI and \(StuI\).

Transgenic animals were generated using standard methods (Mello et al., 1991). The injection solution contained 25 ng/\(\mu\)l of test plasmid and 100 ng/\(\mu\)l 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 \(\beta\)-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.

**\(\beta\)-galactosidase assays**

\(\beta\)-galactosidase activity was assayed using a chlorophenol red-\(\beta\)-D-galactopyranoside substrate (Simon and Lis, 1987). Activity was calculated by dividing the change in O.D.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 \(tra-3(+/+)\) 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 Hodgkin, 1988). L1, L2, L3 and L4 were staged by scoring gonad and vulva development (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979).

**Oligo sequences**

\(\text{EBG-8} 5'-\text{TAATACGACTCACTATA-3'}\)
\(\text{EBG-9} 5'-\text{TGGACCAATTTGAGATGATAAGAAT-}\)
\(\text{TAAATACGACTCACTATA-3'}\)
\(\text{EBG-20} 5'-\text{GTTATTTGTTGCGACAATGTCTGGTTC-}\)
\(\text{EBG-21} 5'-\text{CTTTTTCACG-3'}\)
\(\text{EBG-25} 5'-\text{AAATTTATAGATCTTTATATCAGAGAAA-}\)
\(\text{AAAAA-3'}\)
\(\text{EBG-62} 5'-\text{AGTATCGGAGATTCCACAT-3'}\)
\(\text{EBG-70} 5'-\text{ACAAGACACTCCAAACACACCC-3'}\)
\(\text{EBG-71} 5'-\text{CGTTTTGTAGGGCGC-3'}\)

**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 \(fem-3(+/+)\) 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 \(j\)et and femini\(z\)ed. The \(laf-1\) alleles behave like a deficiency, \(sadDf1\), that removes the locus, indicating that all four alleles reduce \(laf-1\) function (see below). However, their phenotypes are more severe than \(sadDf1\), 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 heterozygous for \(laf-1\) or the deficiency, \(sadDf1\), can be transformed from hermaphrodites into females: \(laf-1(+/+)\) or \(sadDf1(+/+)\) 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 \(laf-1\) homozogotes 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 \(laf-1(+/+)\), \(laf-1(+/+)\) and \(laf-1(+/+)\).

\(laf-1\) may be a negative regulator of \(tra-2\)

Mutations in \(laf-1\) have an effect on sexual fate similar to that of \(tra-2(+/+)\) mutations that are defective in translational control (see Introduction). Like \(tra-2(+/+)\) mutations, \(laf-1(+/+)\) heterozygotes feminize both XX and XO animals and suppress \(fem-3(+/+)\) sterility (Table 1; Doniaich, 1986; Schedl and Kimble, 1987).
1988). Therefore, one simple hypothesis is that lava-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); lava-1(q217)/+ double mutants. The use of a heterozygote in double mutant analysis is not standard, but this was our only option since lava-1 homozygotes die. We found that tra-2(null); lava-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 lava-1 acts upstream of tra-2 in a genetic hierarchy and is consistent with lava-1 being a repressor of tra-2.

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

The similarity between the lava-1(+) and tra-2(gf) phenotypes suggested that lava-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(+); 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, respectively, had intestinal staining. Similar results 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 lava-1(lf) mutant background. A striking increase in the number of animals with β-gal expression was observed in lava-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. Motznj, personal communication). Again, sad1 and the lava-1 mutations did not alter the amount of reporter RNA (Table 2), consistent with the idea that lava-1 mutations affect translational control. lava-1 mutations also increased the expression of lacZ::tra-2(+) 3′UTR in XO animals (data not shown), suggesting that lava-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...
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(+/+) homozygotes. In contrast to our result with tra-2, we found that the presence of laf-1/+ or saDF1/+ could partially feminize tra-3(+/+) 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(+/+) double mutants. As predicted, tra-3(+/+) 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 1. laf-1 mutations feminize both XX and XO animals

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>Suppression of fem-3(gf)†</th>
<th>% animals with oocytes only</th>
<th>% animals with feminized germ line</th>
<th>% animals with feminized soma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>-</td>
<td>0% (n=55)</td>
<td>0%</td>
<td>0% (n=25)</td>
</tr>
<tr>
<td>laf-1(q267)/+</td>
<td>+</td>
<td>16% (n=150)</td>
<td>0%</td>
<td>12% (n=32)</td>
</tr>
<tr>
<td>laf-1(q80)/+</td>
<td>+</td>
<td>10% (n=139)</td>
<td>12%</td>
<td>23% (n=73)</td>
</tr>
<tr>
<td>laf-1(q217)/+</td>
<td>+</td>
<td>15% (n=145)</td>
<td>27%</td>
<td>27% (n=22)</td>
</tr>
<tr>
<td>laf-1(q349)/+</td>
<td>+</td>
<td>30% (n=200)</td>
<td>22%</td>
<td>27% (n=22)</td>
</tr>
<tr>
<td>saDF1/+</td>
<td>+</td>
<td>6% (n=123)</td>
<td>0%</td>
<td>3% (n=67)</td>
</tr>
</tbody>
</table>

*XX progeny were progeny from either dpy-lunc-32/tc1 (wild type), laf-1(+/)tc1, or saDF1/tc1; XO animals were progeny from either dpy-lunc-32/tc1;him-8 (wild type), laf-1(+/)tc1;him-8, or saDF1/tc1;him-8

†laf-1 alleles were obtained as fem-3(gf) suppressors (see Materials and methods). +, fem-3(q96gf) and saDF1/+; fem-3(q96gf) progeny were obtained from mothers raised at 15°C and then shifted to 25°C as adults. Approximately 40% of the saDF1/+; fem-3(q96gf) progeny were self-fertile, compared to 0% for fem-3/q96gf. saDF1/+ and laf-1(+/) are similar in their ability to suppress the fem-3(gf) self-sterile phenotype (data not shown).

‡XX progeny were scored at 20°C, 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°C. *n = number animals scored.

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

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>Transgene†</th>
<th>% animals with intestinal β-gal staining‡</th>
<th>β-gal Activity§</th>
<th>β-gal:let-2¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>lacZ::(wt)3'UTR</td>
<td>3% (n=112)</td>
<td>0.02 (0.01)</td>
<td>0.30</td>
</tr>
<tr>
<td>Wild type</td>
<td>lacZ::(-32)3'UTR</td>
<td>62% (n=132)</td>
<td>0.32 (0.03)</td>
<td>0.50</td>
</tr>
<tr>
<td>Wild type</td>
<td>lacZ::(-60)3'UTR</td>
<td>67% (n=117)</td>
<td>0.19 (0.01)</td>
<td>0.21</td>
</tr>
<tr>
<td>laf-1(q217)/+</td>
<td>lacZ::(wt)3'UTR</td>
<td>31% (n=83)</td>
<td>n.d.</td>
<td>0.32</td>
</tr>
<tr>
<td>saDF1/+</td>
<td>lacZ::(wt)3'UTR</td>
<td>35% (n=18)</td>
<td>n.d.</td>
<td>0.40</td>
</tr>
<tr>
<td>laf-1(q267)/+</td>
<td>lacZ::(-32)3'UTR</td>
<td>45% (n=67)</td>
<td>0.39 (0.01)</td>
<td>0.23</td>
</tr>
<tr>
<td>laf-1(q267)/+</td>
<td>lacZ::(-60)3'UTR</td>
<td>89% (n=25)</td>
<td>0.76 (0.07)</td>
<td>0.41</td>
</tr>
<tr>
<td>laf-1(q267)/+</td>
<td>lacZ::(-60)3'UTR</td>
<td>66% (n=34)</td>
<td>0.31 (0.01)</td>
<td>0.17</td>
</tr>
</tbody>
</table>

*Wild-type animals were N2 hermaphrodites; laf-1/+ animals were progeny from lacZ::(wt)tc1 and saDF1/+ were from saDF1/tc1 mothers. In all experiments, adult worms were heated 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). lacZ::tra-2(+/+) 3'UTR has the wild-type tra-2 3'UTR which has two DREs separated by a 4nt spacer; lacZ::(-32)3'UTR has a 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 630x magnification; genetic evidence suggests that control by the tra-2 3'UTR functions in intestinal cells (Donia, 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 OD730 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 transgenic 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.
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).

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.

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Removal of \textit{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 \textit{tra-3} (see above). A prediction of this model is that DRF activity might be observed in progeny of \textit{tra-3(lf)} mutants. To test this hypothesis, we made extracts from \textit{tra-3(lf)} homozygous embryos and tested the extracts for DRF activity. DRF was indeed present in the \textit{tra-3(lf)} embryonic extract (Fig. 7B). This finding is consistent with the idea that \textit{tra-3} promotes female development by removing the \textit{tra-2} translational repressor.

If \textit{tra-3} normally acts to repress the translational control of \textit{tra-2} in embryos, then removal of \textit{tra-3} might decrease expression of the \textit{lacZ::tra-2(wt) 3'UTR} transgene in embryos. Therefore, we examined the expression of \textit{lacZ::tra-2(wt) 3'UTR} in unlaid embryos of \textit{tra-3(lf)} hermaphrodites. As predicted, \textit{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 \textit{tra-3(+)} promoting female development by inhibiting the translational repression of \textit{tra-2}.

DISCUSSION

Translational control by \textit{laf-1} and \textit{tra-3}

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

The \textit{laf-1} gene is required for translational repression by the \textit{tra-2} 3'UTR and may encode part of the translational repressor. Three lines of evidence support this idea. First, a reduced level of \textit{laf-1} disrupts regulation of a reporter transgene by the \textit{tra-2} 3'UTR. In wild-type animals, a transgene bearing the intact \textit{tra-2} 3'UTR is translationaly repressed but, in \textit{laf-1(+)} mutant backgrounds, that transgene is partially released from repression. Second, the properties of \textit{laf-1} mutations are consistent with predictions for a \textit{tra-2} translational repressor. The phenotype of a loss-of-function mutation in the \textit{trans-acting} repressor is expected to mimic that of a gain-of-function mutation lacking the \textit{cis}-acting regulatory element. As predicted, both \textit{laf-1} and \textit{tra-2(sg)} mutations feminize XX and XO animals. Furthermore, the \textit{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 \textit{laf-1} acting upstream of \textit{tra-2}, the predicted position for a repressor of \textit{tra-2}.

The \textit{tra-3} gene (Hodgkin and Brenner, 1977) appears to act in the embryo to antagonize translational repression by the \textit{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 \textit{tra-3} mutant embryos, the 3'UTR is able to inhibit translation. Furthermore, DRF activity is present in \textit{tra-3} homozygous mutant embryos, but not in wild-type embryos.
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.

It should be noted that hermaphrodite spermatogenesis does not rely solely 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).

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

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>% animals with bilobed gonad†</th>
<th>% animals with vulva</th>
</tr>
</thead>
<tbody>
<tr>
<td>tra-3(e1107)</td>
<td>(n=82)</td>
<td>9%</td>
</tr>
<tr>
<td>laf-1(e267)/+;tra-3(e1107) (n=62)</td>
<td>21%</td>
<td></td>
</tr>
<tr>
<td>laf-1(e890)/+;tra-3(e1107) (n=53)</td>
<td>46%</td>
<td></td>
</tr>
<tr>
<td>laf-1(e217)/+;tra-3(e1107) (n=48)</td>
<td>40%</td>
<td></td>
</tr>
<tr>
<td>laf-1(e499)/+;tra-3(e1107) (n=45)</td>
<td>72%</td>
<td></td>
</tr>
<tr>
<td>saDF1/+;tra-3(e1107) (n=40)</td>
<td>25%</td>
<td></td>
</tr>
</tbody>
</table>

*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.

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

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>Transgene</th>
<th>% β-gal staining†</th>
<th>β-gal/UTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>laf-1(e217)/+;tra-3(e1107) lac-Z:/wt3UTR</td>
<td>(n=40)</td>
<td>15%</td>
<td></td>
</tr>
<tr>
<td>saDF1/+;tra-3(e1107) lac-Z:/wt3UTR</td>
<td>(n=50)</td>
<td>8%</td>
<td></td>
</tr>
<tr>
<td>N2 (embryos)</td>
<td>lac-Z:/wt3UTR</td>
<td>55% (n=325)</td>
<td>n.d.</td>
</tr>
<tr>
<td>tra-3(e1107) (embryos) lac-Z:/wt3UTR</td>
<td>(n=218)</td>
<td>27%</td>
<td></td>
</tr>
</tbody>
</table>

*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, unaided 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.
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 are 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 (Doniaich, 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 (Doniaich, 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 Schell 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 Mary Wickens, Eric Jan and Cindy Motzny for critical reading of the manuscript, Jim Kramer, Mary 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 the USDA and the Council for Tobacco Research. E. B. G. The generous support of the Howard Hughes Medical Institute to J. K. is also acknowledged.

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