Translational Regulation of tra-2 by Its 3’ Untranslated Region Controls Sexual Identity in C. elegans

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Summary

C. elegans hermaphrodites make sperm and then oocytes in an otherwise female animal. Gain-of-function mutations in the sex-determining gene tra-2 (tra-2(gf)) transform hermaphrodites into females (sterileless hermaphrodites). The tra-2(gf) mutations map to a perfect direct repeat in the 3’ untranslated region; each repeat is called a direct repeat element (DRE). Three experiments demonstrate that DRES repress tra-2 at the translational level. First, tra-2(gf) mRNAs are associated with larger polyisomes than are their wild-type counterparts. Second, translation of a reporter RNA is inhibited by DRES. Third, disruption of DRES does not increase tra-2 mRNA levels. An RNA binding activity specifically associates with the DRES. We propose that tra-2 translation is inhibited by association of an RNA binding-factor with the DRES and that this translational control is essential for development of C. elegans as a hermaphrodite/male species.

Introduction

The specification of cell fates during development is precisely regulated. Cells adopt particular fates according to their sex, position, and developmental stage. When regulators of cell fate are aberrantly expressed, dramatic and often deleterious effects are observed. A classic example is the generation of legs instead of antennae in the Drosophila mutant Antennapedia (for review see Aftolter, 1990). In this paper, we focus on regulation of the sex-determining gene tra-2 and specification of hermaphrodite rather than female development in the nematode Caenorhabditis elegans.

Sex determination in the nematode C. elegans is controlled by a cascade of regulatory genes that specify 1 of 2 sexual fates (Figure 1; for reviews see Hodgkin, 1990; Villeneuve and Meyer, 1990). Normally, XX animals are self-fertilizing hermaphrodites and XO animals are cross-fertile males. A hermaphrodite is essentially a somatic female that produces sperm and then oocytes. The tra-2 gene directs female cell fates within animals of the hermaphrodite sex and occupies a central position in the sex determination pathway (Hodgkin and Brenner, 1977; Hodgkin, 1990). 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 XX soma, tra-2 is active and specifies hermaphrodite development (Hodgkin and Brenner, 1977). Furthermore, the hermaphrodite level of tra-2 expression is controlled by positive feedback, which may ensure commitment to the hermaphrodite fate (Okkema and Kimble, 1991). In XO animals, tra-2 is inactivated and male development ensues (Hodgkin, 1980).

The development of XX animals as hermaphrodite rather than female depends on the negative regulation of tra-2 (Figure 1B). Dominant gain-of-function mutations of tra-2 (tra-2(gf)) transform XX animals into females (Doniach, 1986; Schedl and Kimble, 1988). Females and hermaphrodites are morphologically identical in somatic tissues (e.g., nerve, muscle, and gut), but differ in the germline; whereas a hermaphrodite makes both sperm and oocytes, a female produces only oocytes. The existence of tra-2(gf) mutants that develop as females indicates that tra-2 must be inhibited in hermaphrodites to permit the onset of spermatogenesis. In the absence of this germline regulation, tra-2 directs female development, and no sperm are produced.

In this paper, we investigate the molecular mechanism by which tra-2 is controlled to permit hermaphrodite rather than female development. First, we identify a regulatory element in the tra-2 3’ untranslated region (3’UTR) essential for negatively regulating tra-2 to allow the onset of spermatogenesis. Second, we demonstrate that this element regulates tra-2 at the translational level. Finally, we show that this regulatory element specifically binds a factor, and we propose a model by which that binding inhibits tra-2 translation. We discuss the implications of our findings for sex determination in C. elegans and for the evolution of cell fate control more generally.

Results

Molecular Characterization of tra-2(gf) Mutations

We previously showed that six tra-2(gf) mutations are associated with either a transposon insertion or small deletions within a BgIII-EcoRI restriction fragment located near the 3’ end of the tra-2 gene (Okkema and Kimble, 1991; see Experimental Procedures). In tra-2(+), this BgIII-EcoRI fragment contains 141 nt of protein-coding sequence, 220 nt of 3’UTR, and 648 nt 3’ to the polyadenylation site (Kuwabara et al., 1992). Located within the tra-2 3’UTR are two copies of a 28 nt sequence (elements A and B) arranged as a perfect direct repeat, separated by 4 nt (Figure 2; Kuwabara et al., 1992; for sequence of the direct repeat, see oligonucleotide EBG9 in Experimental Procedures). We call each 28 nt element a direct repeat element (DRE).

To learn the precise molecular defects associated with the tra-2(gf) mutations, we sequenced the BgIII-EcoRI restriction fragment from each mutant. In each mutant, we found an alteration in the direct repeat of the tra-2 3’UTR (Figure 2). Moreover, the extent to which the direct repeat is disrupted correlates with the strength of the allele (Fig-
Figure 1. Regulatory Pathways of Sex Determination in C. elegans

For simplicity, only genes specific for sex determination are shown. (A) In somatic tissues, seven genes specify sexual fate: her-1 (Hodgkin, 1980; Trent et al., 1988), tra-2 (Klass et al., 1976; Hodgkin and Brenner, 1977), tra-3 (Hodgkin and Brenner, 1977), fem-1 (Nelson et al., 1978; Doniach and Hodgkin, 1984), fem-2 (Kimble et al., 1984; Hodgkin, 1986), fem-3 (Hodgkin, 1986; Barton et al., 1987), and tra-1 (Hodgkin and Brenner, 1977; Hodgkin, 1987; Schedl et al., 1989). The primary determinant of sexual fate is the ratio of X chromosomes to autosomes: the X:A ratio (Madison and Herzenberg, 1979). In the XX soma, the genes regulating both somatic and germ line differentiation (e.g., the adh genes) inactivate her-1. tra-2 and tra-3 can then repress the fem genes so that tra-1 can promote female development. In the XO soma, her-1 is active and inhibits tra-2 and tra-3. Therefore, the fem genes negatively regulate tra-1, and male development occurs. (B) In the female germ line, nine genes regulate sexual cell fate: her-1; tra-2; tra-3; the fem genes; and four germ line-specific genes, fog-2 (Schedl and Kimble, 1988), fog-1 (Barton and Kimble, 1990), fog-3 (R. Ellis and J. K., unpublished data), and mog-1 (Graham and Kimble, 1993). In contrast with the soma, tra-1 is not the terminal regulator in the germ line. In the XX germ line, fog-2 represses tra-2, allowing the fem genes and fog-1 to promote spermatogenesis. After a brief period of spermatogenesis, mog-1 represses the fem genes and fog-1, resulting in oogenesis. In the XO germ line, her-1 inhibits tra-2 and tra-3, allowing the fem genes and fog-1 to promote continuous spermatogenesis.

Figure 2. The tra-2(gf) Mutations Disrupt the DREs in the 3'UTR

The hatched box represents the end of the open reading frame. The thin line represents the region corresponding to the 3'UTR with the translation stop (UAA) and polyadenylation site (AAUAAA) indicated. The DREs are depicted as two large arrows (A and B). Molecular lesions associated with tra-2(gf) mutations are diagrammed with the name of the allele listed on the right. The position of a transposon insertion is indicated by a triangle labeled Tc1. The position of deletion endpoints are indicated by brackets; the size of the deletion is shown in nucleotides.

Figure 3. tra-2(gf) Mutations Do Not Alter the Pattern of tra-2 mRNAs

Expressed during Development or Increase the Steady-State Levels of tra-2 mRNAs

(A) Northern blot of poly(A)+ RNA isolated from tra-2(e2020gf) animals. tra-2 produces a 4.7 kb (arrowhead), a 1.8 kb, and 1.9 kb (bracket) transcript. Animals were prepared from the embryos of synchronized animals (E); L1–L4 represent the first through fourth larval stages and A represents the adult (for a wild-type tra-2 RNA profile, see Okkema and Kimble, 1991).

(B) Northern blot of poly(A)+ RNA made from staged adults of either tra-2(q122gf), tra-2(e2020gf), or tra-2(+). The steady-state levels of the 4.7 kb and 1.8 kb tra-2 mRNAs were determined by measuring the ratio of tra-2 signal to myo-1 signal. For each experiment, the ratio of tra-2(+) to myo-1 signal was set to 1.0. The steady-state levels of the 4.7 kb transcript from tra-2(q122gf) and tra-2(e2020gf) were 0.8 and 0.9, respectively. The steady-state levels of the 1.8 kb transcript for tra-2(q122gf) and tra-2(e2020gf) were 0.5 and 0.2, respectively. spe-8 animals were used as a source of tra-2(+) RNA because, like the tra-2(gf) mutants, spe-8 is an obligate male/female strain. spe-8 causes a defect in hermaphrodite, not male, spermatogenesis and is therefore maintained as a stock of 50% male and 50% female animals (L'Hernault et al., 1988). The tra-2 RNA profile from spe-8 mutants is indistinguishable from that of the wild type (Okkema and Kimble, 1991). The 4.7 kb (arrowhead) and the 1.8 kb (arrow) mRNAs are indicated.

tra-2(gf) Mutations Do Not Alter the Expression Pattern of tra-2 mRNAs

To test whether the DREs in the tra-2 3'UTR regulate the production of tra-2 mRNAs, we compared tra-2 mRNAs in tra-2(+) and tra-2(gf) animals during development. Three tra-2 RNAs are detected during wild-type development: a 4.7 kb mRNA is found in all stages of both XX and XO animals; a 1.9 kb mRNA is found in XX hermaphrodite
larvae and in adult XO males; and a 1.8 kb mRNA is present only in the XX hermaphrodite adult germline and in embryos (Okkema and Kimble, 1991). In tra-2(e2020gf) XX animals, the developmental profile of tra-2 RNAs is similar to that of wild-type RNAs (Figure 3A). The 4.7 kb, 1.9 kb, and 1.8 kb messages are produced at normal times, and no unexpected RNAs are observed (see Okkema and Kimble, 1991). Therefore, it is unlikely that the tra-2(gf) mutations activate a cryptic promoter or alter splicing patterns, either of which might be predicted to affect the stage at which tra-2 mRNAs are expressed or to produce a novel transcript.

To address whether the tra-2(gf) mutations increase tra-2 activity by increasing RNA stability, we compared the steady-state levels of tra-2 mRNAs in tra-2(+) and tra-2(gf) animals (Figure 3B). The overall abundance of tra-2 mRNAs did not increase in the tra-2(gf) mutants. The amount of the 4.7 kb transcript was basically the same in animals of larval stage 3 (L3), L4, or adulthood, but the 1.8 kb transcript in both tra-2(q122gf) and tra-2(e2020gf) was reduced compared with the wild type (Figure 3B; see figure legend for values of steady-state RNA levels). It is not clear why the steady-state level of the 1.8 kb mRNA, but not the 4.7 kb mRNA, is decreased in the tra-2(gf) mutants. Perhaps disruption of the DREs removes an RNA stability element that is specific for the germline. It is unlikely that the decreased amount of the 1.8 kb mRNA results in the enhanced tra-2 activity observed in the tra-2(gf) mutants, since a decrease in the mRNA abundance would usually result in a decrease in expression. However, a decrease in the 1.8 kb mRNA could cause an increase in tra-2 activity if the 1.8 kb mRNA encoded a repressor of tra-2 activity. We think this unlikely since the 1.8 kb mRNA is present only in XX adult hermaphrodite germlines, yet the tra-2(e2020gf) mutation can feminize both the soma and germlines of XO males.

The DREs Are Translational Regulatory Elements tra-2(gf) Mutations Increase the Association of tra-2 mRNA with Polysomes

To test whether the DREs regulate the translation of tra-2 mRNA, the association of tra-2 mRNA with polysomes in tra-2(+) and tra-2(gf) adult animals was analyzed (Figure 4). If tra-2(gf) mRNA is more translationally active than tra-2(+) mRNA, then tra-2(gf) mRNA should be associated with polysomes more than tra-2(+) RNA is. Staged adult worms were collected and lysed into buffer containing either cycloheximide (which stabilizes the association of ribosomes with mRNA) or EDTA (which dissociates ribosomes from mRNA). The lysates were sedimented through a linear sucrose gradient (5%–45%). Then fractions from the gradient were analyzed by RNAase protection for the presence of the 4.7 kb tra-2 mRNA. The gradients were calibrated using yeast components; polysomes were found in the pellet and in fractions 1–5, while monosomes and free subunits were found at the top of the gradient in fractions 6–9 (Figure 4). Actin mRNA was used as an internal control for RNA recovery and for confirmation that different gradients could be compared.

The tra-2 RNA from tra-2(gf) animals was associated with larger polysomes than was tra-2 RNA from tra-2(+) animals (Figure 4; Table 1). Of the tra-2(+) mRNA, 51% was found in fractions containing monosomes and free subunits. However, only 31% of tra-2(q122gf) and 18% of tra-2(e2020gf) were found in these same fractions (Table 1). By contrast, in the presence of EDTA, both tra-2(gf) and tra-2(+) mRNAs were found at the top of the gradient. The sensitivity to EDTA suggests that the position of tra-2 mRNA on the gradient, in the presence of cycloheximide, reflected ribosome loading. Therefore, the tra-2(gf) mRNA is associated with larger polysomes than is tra-2(+) and hence is likely to be more translationally active. In principle, increased association with polysomes could reflect a reduction in the rate of translation elongation. However,
Table 1. Distribution of tra-2 mRNA from tra-2(+) and tra-2(gf) Animals on Polysome Gradients

<table>
<thead>
<tr>
<th>mRNA</th>
<th>tra-2</th>
<th>act-1</th>
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<tbody>
<tr>
<td></td>
<td>Cycloheximide (%)</td>
<td>EDTA (%)</td>
</tr>
<tr>
<td>tra-2(+) (n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pellet</td>
<td>10 (± 4)</td>
<td>2 (± 3)</td>
</tr>
<tr>
<td>1–5</td>
<td>39 (± 4)</td>
<td>21 (± 5)</td>
</tr>
<tr>
<td>6–9</td>
<td>51 (± 5)</td>
<td>77 (± 4)</td>
</tr>
<tr>
<td>tra-2(q122gf) (n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pellet</td>
<td>11 (± 4)</td>
<td>4 (± 4)</td>
</tr>
<tr>
<td>1–5</td>
<td>58 (± 5)</td>
<td>24 (± 6)</td>
</tr>
<tr>
<td>6–9</td>
<td>31 (± 7)</td>
<td>72 (± 4)</td>
</tr>
<tr>
<td>tra-2(e2020gf) (n = 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pellet</td>
<td>35 (± 4)</td>
<td>2 (± 1)</td>
</tr>
<tr>
<td>1–5</td>
<td>47 (± 6)</td>
<td>31 (± 6)</td>
</tr>
<tr>
<td>6–9</td>
<td>18 (± 4)</td>
<td>67 (± 9)</td>
</tr>
</tbody>
</table>

Yeast polysomes are found in the pellet and fractions 1–5; yeast monosomes and free subunits are found in fractions 6–9 (see legend of Figure 4; see Experimental Procedures). The values are the percentage of total tra-2 or act-1 RNA found in the indicated fractions. The standard deviation is indicated in parentheses. n is the number of experiments.

* spe-8 was used for tra-2(+) because, like tra-2(gf), it is an obligate male/female strain, approximately 50% are XO males and 50% are XX "females."

This explanation is unlikely; the increase in tra-2 activity seen in tra-2(gf) animals is inconsistent with the decrease in protein synthesis that would result from a decrease in elongation.

The size of polysomes containing tra-2 RNA correlated well with the strength of the allele. The tra-2(+) mRNA was primarily associated with free subunits, monosomes, and small polysomes, while tra-2(e2020gf) mRNA was found primarily in the pellet and larger polysome fractions (Figure 4; Table 1). Furthermore, the association of tra-2(q122gf) mRNA with polysomes was intermediate to that of tra-2(e2020gf) and tra-2(+) mRNAs (Figure 4; Table 1). The intermediate distribution of tra-2(q122gf) mRNA suggests that the presence of a single DRE results in partial regulation of the translation of tra-2 mRNA. Such partial regulation is consistent with genetic analysis that indicated partial control of tra-2 activity in tra-2(q122gf) mutants (Doniach, 1986; Schedl and Kimble, 1988).

The DREs Regulate the Translation of a Heterologous RNA

To test further whether the DREs regulate tra-2 expression at the translational level, we examined their ability to control the translation of a heterologous RNA. Specifically, RNA was synthesized in vitro from plasmids carrying the coding region for β-galactosidase (β-gal), which is followed by the entire tra-2(+) or tra-2(e2020gf) 3'UTR (called lacZ(+) and lacZ(gf), respectively). The reporter RNAs also encoded the SV40 nuclear localization signal and con-

Figure 5. The tra-2 3'UTR Can Translationally Regulate a Heterologous RNA

Reportor RNAs that encode β-gal fused with either the tra-2(+) (A) or tra-2(e2020gf) (B) 3'UTR were injected into the distal germline of N2 animals at 100 nM (see Experimental Procedures). The tra-2(+) 3'UTR is 206 nt long. (Top) Line drawings of injected gonads with the distal arm indicated: oocyte nuclei (arrow) and embryo nuclei (arrowhead). (Middle) Gonad injected with the reporter RNA. (Bottom) Diagram of injected RNAs: the hatched rectangle denotes the β-gal coding region, the thin line denotes tra-2 3'UTR, arrows denote the DREs, and brackets denote the endpoints of the tra-2(e2020gf) 108 nt deletion. The β-gal reporter protein includes an SV40 nuclear localization signal.
Table 2. Summary of Reporter RNA Experiments

<table>
<thead>
<tr>
<th>RNA*</th>
<th>β-Gal Staining in Distal Arms^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>lac-Z(+)</td>
<td>Unscored</td>
</tr>
<tr>
<td>lac-Z(gf)</td>
<td>48</td>
</tr>
</tbody>
</table>

* Reporter RNAs encoding β-gal carrying either the tra-2( + ) 3'UTR or the tra-2(e2020gf) 3'UTR were injected into the distal germelines of N2 animals at 100 nM as described in Experimental Procedures. In the tra-2( + ) 3'UTR, arrows denote the DREs. In the tra-2(e2020gf) 3'UTR, brackets denote the endpoints of the 108 nt deletion that removes the DREs.

b Diffuse staining that could only be detected as thin blue slashes at 630 x was defined as weak. Uniform staining with some nuclear localization that was visible at 50-250 x was defined as strong. Only the distal arms of germelines were scored. n is the total number of distal germelines scored from two separate experiments using two separate RNA preparations.

Referred to α-methyl GpppG cap and a poly(A) tail of 30 residues. lacZ( + ) and lacZ(gf) RNAs were injected into the distal arm of the germline of wild-type adult hermaphrodites. The injected animals were then incubated for 4 hr and subsequently fixed and stained for the presence of β-gal activity.

The lacZ(gf) RNA made more β-gal in the distal arm than did lacZ( + ) RNA. β-Gal activity was undetectable or weak in the distal arms injected with lacZ( + ) RNA (Figure 5A; Table 2). By contrast, β-gal activity was usually strong or weak in the distal arms injected with lacZ(gf) RNA (Figure 5B; Table 2). β-Gal activity was undetectable in only 4% of the distal arms injected with lacZ(gf) RNA. The lack of β-gal activity in distal arms injected with lacZ( + ) RNA must reflect translational repression rather than RNA turnover since strong staining was seen in the four to five most mature oocytes and in embryos (Figure 5A). Therefore, the lacZ( + ) RNA is translationally repressed by the tra-2 3'UTR.

Identification of a DRE-Binding Factor

How do the DREs regulate translation of tra-2 RNA? One possibility is that the DREs are a binding site for a negative regulator that inhibits tra-2 translation. In an attempt to identify such a binding activity, we assayed worm extracts by RNA gel retardation analysis. Incubation of a crude extract prepared from adult hermaphrodites with RNA containing the direct repeat (EBG-9; see Figure 6B) resulted in the appearance of several slower moving bands on a nondenaturing polyacrylamide gel (Figure 6A, lane 2). The formation of this complex demonstrates that a factor present in C. elegans binds to the direct repeats. We call the DRE binding activity a direct repeat factor (DRF).

The specificity of DRF binding to the DREs was determined by testing the ability of unlabeled RNA to compete with labeled RNA in the gel shift assay. In these experiments, excess unlabeled competitor RNA, which contained either both DREs (EBG-9), a single DRE (EBG-10), or a randomized RNA of the same base composition as one DRE (EBG-13), was included in the reaction. Loss of complex formation indicated that the competitor RNA could compete with the labeled RNA for binding to the factor. We found that increasing molar amounts of an RNA

![Figure 6. A Binding Factor Specifically Binds to the Direct Repeats](image)

(A) One femtomole of 32P-labeled EBG-9 RNA (see Figure 6B for schematic of EBG-9) was incubated with either no crude adult extract (lane 1) or 5 µg of crude adult extract (lanes 2–14) (see Experimental Procedures). Reactions were loaded on a 7% nondenaturing polyacrylamide gel. The gel was dried and autoradiographed. Slower migrating bands are due to complex formation (arrow); the faster band is uncomplexed probe. To determine binding specificity, increasing amounts (either 10 fmol, 50 fmol, or 100 fmol) of cold EBG-9 (lanes 2–5), EBG-10 (lanes 6–8), EBG-13 (lanes 9–11), or fem-3 3'UTR (lanes 12–14) were added to the binding reaction (see Figure 6B for schematic of competing RNAs).

(B) Summary of binding experiments in which different RNAs were tested for the ability to form a complex with DRF. The ability for an RNA to be bound by DRF was examined in two ways: 32P-labeled RNA was assayed directly for DRF binding, or unlabeled RNA was tested for the ability to compete with 32P-labeled EBG-9 RNA for binding. (Left) Names of the different RNAs used (see Experimental Procedures for sequences), (Middle) Schematics of the different RNAs. The open boxes represent tra-2 3'UTR sequences, arrows depict DREs, the position of deletion endpoints are indicated by brackets, and the sizes of the deletions are shown in nucleotides. The asterisks in EBG-13 denote a DRE sequence that has been randomized. The stippled box represents fem-3 3'UTR sequences. (Right) The different RNAs were scored for the ability (plus sign) or inability (minus sign) to form a complex.
containing either both DREs (EBG-9; Figure 6A, lanes 2–5) or one DRE (EBG-10; Figure 6A, lanes 6–8) competed with labeled probe. Qualitatively, EBG-10 appeared to be as good a competitor as EBG-9. However, increasing amounts of a randomized DRE (EBG-13; Figure 6A, lanes 9–11), used as a competitor, did not interfere with complex formation. To verify these results, the same RNAs were radioactively labeled and directly assayed for complex formation. RNAs that contained either both elements or a single element always formed a complex, whereas the randomized RNA did not (Figure 6B). In summary, the DREs are recognized specifically by an RNA-binding factor, the DRF. Moreover, a single DRE is necessary and sufficient for binding.

To assess further the specificity of DRF for RNAs containing the DREs, we examined the ability of DRF to bind the fem-3 3′UTR. The activity of fem-3, another sex determination gene, is also controlled by a regulatory site in its 3′UTR (Ahringer and Kimble, 1991; Barton et al., 1987). A 7 nt sequence (UUCUUUGU), located in the fem-3 3′UTR, is required for repression of fem-3 activity. The fem-3 sequence is similar to a sequence found within the tra-2 DREs (UUCUUAU). As can be seen in Figure 6A (lanes 12–14), the fem-3 3′UTR fails to compete with EBG-9 for complex formation. Thus, DRF is specific for the tra-2 DREs and, if DRF inhibits tra-2 translation, then tra-2 and fem-3 are likely to be controlled by different factors.

We also examined whether DRF is present specifically in one sex or tissue. We found that XO males contain DRF binding activity (data not shown), which is consistent with the observation that the tra-2(e2020g*) mutation causes inappropriate feminization in XO males (Doniach, 1986). Binding activity is also present in mutant animals that lack a germline, glp-1 and glp-4 (Figure 7, lanes 4 and 5; Austin and Kimble, 1987; Beanan and Strome, 1992), indicating that DRF activity is not germ-line specific. This is not surprising since tra-2(ge) mutations can feminize the soma (Doniach, 1986).

Discussion

tra-2 Is Translationally Controlled by a Regulatory Element in its 3′UTR

Dominant gain-of-function mutations of the sex-determining gene tra-2 activate this gene inappropriately and cause XX animals to develop as females rather than as hermaphrodites (Doniach, 1986; Schedel and Kimble, 1988). In this paper, we identify the site altered in six tra-2(ge) mutations and show that this site controls tra-2 activity at the translational level. Our conclusions are based on four lines of evidence. First, the tra-2(ge) mutations contain molecular defects in a perfect direct repeat located in the tra-2 3′UTR. Each repeat is called a DRE, for direct repeat element. Second, disruption of the DREs does not increase the steady-state level of tra-2 mRNA. Third, disruption of the DREs increases the association of the tra-2 mRNA with ribosomes. Fourth, the tra-2 3′UTR confers translational repression on a reporter RNA. In addition, we identify an RNA-binding factor that may be a translational inhibitor. We tentatively call this factor DRF, for direct repeat factor.

Figure 8 presents our working hypothesis for the translational regulation of tra-2 by its 3′UTR. Critical to this model is the notion that some trans-acting factor, perhaps DRF, binds to the DREs and inhibits translation. We postulate that binding to one DRE results in partial inhibition and that binding to both DREs results in full inhibition of translation. The ability of a single DRE to partially regulate tra-2 is
supported by several observations. \(tra-2(gf)\) alleles with one intact DRE retain partial regulation in vivo (Schedl and Kimble, 1988; this paper). Also, \(tra-2(gf)\) mRNA with one intact DRE is associated with polysomes at a level that is intermediate between that of mRNAs with two DREs and that of mRNAs with no DREs. Finally, a single DRE can bind DRF. In our model, we hypothesize that each DRE is a binding site for DRF. However, our experiments do not exclude the possibility that the direct repeat is a single binding site. The trans-acting factor may not bind to a classic RNA secondary structure, since no stable secondary structure was predicted for the DREs by computer analysis (data not shown).

How do the DREs inhibit translation? Elements that appear to regulate translation have been discovered in the 3'UTRs of numerous maternal mRNAs (for review see Standart, 1993). However, the mechanism by which these regulatory elements influence translation is not well understood. One possibility is that they may control the length of the poly(A) tail, which in turn may influence translation. Increases in polyadenylation usually correlate with increases in translation in species as diverse as clams, frogs, mice, and flies (Wickens, 1993). Similarly, the DREs may control translation of \(tra-2\) mRNA by controlling its polyadenylation. Alternatively, the DREs may sequester \(tra-2\) mRNA away from the translational machinery, or they may directly inhibit the interaction of initiation factors or of ribosomal subunits with the 5'UTR.

Identification of the regulator that binds and controls translation via the \(tra-2\) DREs will be essential to the analysis of this control. One approach is to identify the regulator genetically. A mutant lacking the regulator might be expected to have the same phenotype as that of the \(tra-2(gf)\) mutants. In addition, epistasis analysis should place the candidate gene upstream of \(tra-2\) in a genetic pathway. Although two genes, \(her-1\) and \(fog-2\), act upstream of \(tra-2\) as negative regulators (Figure 1B), their loss-of-function phenotypes differ significantly from those of \(tra-2(gf)\) mutants. The \(tra-2(gf)\) phenotype is transformation of XX animals from hermaphrodite to female, and the most severe \(tra-2(gf)\) also feminizes the germline and intestine of older XO adults. By contrast, loss-of-function mutations of \(her-1\) transform XO animals into hermaphrodites, and loss-of-function mutations of \(fog-2\) do not affect XO males at all. Therefore, no gene in the existing sex determination pathway is a good candidate for the translational regulator of \(tra-2\).

\textbf{tra-2 Is Controlled at Two Levels to Achieve Hermaphrodite Spermatogenesis}

Another class of \(tra-2\) dominant mutations, known as \(tra-2(mx)\) (for mixed character), also disrupts the onset of hermaphrodite spermatogenesis. Like \(tra-2(gf)\), the \(tra-2(mx)\) alleles transform XX animals into females. However, unlike \(tra-2(gf)\), \(tra-2(mx)\) alleles do not affect XO animals (Doniach, 1986; Schedl and Kimble, 1988), and the \(tra-2(mx)\) alleles are not associated with a molecular change in the \(tra-2\) 3'UTR (P. Kuwabara, P. G. O., and J. K., unpublished data). Therefore, the \(tra-2(gf)\) and \(tra-2(mx)\) mutations have different phenotypes and distinct molecular lesions and are likely to represent defects in two separate controls of \(tra-2\). Both controls are clearly essential for the onset of hermaphrodite spermatogenesis, since a defect in either one feminizes the germline. We suggest that translational regulation of \(tra-2\) via the DREs acts in concert with this second \(tra-2\) control to ensure complete repression of \(tra-2\) activity in the hermaphrodite germline. One reason that \(tra-2\) may be so tightly controlled is that the \(tra-2\) gene is subject to positive feedback regulation within the sex determination pathway (Okkema and Kimble, 1991). Since a little \(tra-2\) activity is predicted to generate much more \(tra-2\) activity, which would reinforce the commitment in female cell fates, it is not surprising that this key sex-determining gene is carefully controlled.

\textbf{Translational Control and Specification of Cell Fate in the Hermaphrodite Germline}

Determination of a germ cell as sperm or oocyte requires regulation of the genes that specify sexual fates in the germline (Figure 1B; see legend for a description of the germline sex determination pathway; for review see Hodgkin, 1990; Villeneuve and Meyer, 1990). In C. elegans, both the onset of hermaphrodite spermatogenesis and the switch from spermatogenesis to oogenesis depend on translational controls (Figure 9). The translational regulation of \(tra-2\) releases the \(fem\) genes and \(fog-1\) to direct spermatogenesis (this paper); subsequently, inhibition of \(fem-3\) permits the switch to oogenesis (Barton et al., 1987; Ahringer and Kimble, 1991). It is unclear whether \(tra-2\) must be activated for oogenesis to occur. The RNA reporter experiments suggest that the DREs can inhibit the translation of \(tra-2\) during oogenesis (Figure 5A). However, \(tra-2\) could be activated by the removal of another regulation, such as removal of the \(tra-2(mx)\) or \(fog-2\) controls. Alternatively, \(tra-2\) activity may remain low, and the switch
to oogenesis may be mediated by the inhibition of fem-3 by other genes. Gain-of-function mutations of fem-3 interfere with the switch from spermatogenesis to oogenesis and cause continuous spermatogenesis in an XX female soma. The fem-3(ge) mutations possess single nucleotide changes in the middle of the fem-3 3'UTR and cause increased polyadenylation of the fem-3 mRNA (Ahriniger and Kimble, 1991). The regulatory elements located in the tra-2 and fem-3 3'UTRs appear to bind distinct factors; the fem-3 3'UTR fails to compete for binding to the tra-2 regulatory element. Therefore, two distinct translational controls appear to be essential for production of a limited number of sperm in hermaphrodites.

The evolution of a species with males and self-fertilizing hermaphrodites from one with males and females may have been achieved, at least in part, by the modification of preexisting translational controls. In many organisms, including worms, flies, clams, sea urchins, frogs, and mice, maternal mRNAs are controlled at the translational level by regulatory elements in their 3'UTRs (for review see Wickens, 1993). Since both tra-2 and fem-3 encode maternal RNAs, it is conceivable that translational controls of maternal mRNAs may have been co-opted in the germ line to regulate the sex-determining machinery and to control the transient production of sperm typical of self-fertilizing hermaphrodites. For example, translational controls for maternal tra-2 mRNA may have been altered so that tra-2 would be repressed prior to gametogenesis. Such a change would free the fem genes and fog-1 to drive spermatogenesis.

The tra-2 3'UTR May Be Involved in Masking Maternal tra-2 mRNA

Maternal mRNAs from a variety of different organisms are translationally inhibited in oocytes, only to become activated upon fertilization. It has been proposed that proteins associated with maternal mRNAs may act as translational inhibitors and that these masking proteins are destroyed or modified to liberate the mRNAs for translation (Spirin, 1966). In clam oocytes, the 3'UTR is clearly involved in the masking of maternal mRNAs (Standart et al., 1990). Although tra-2 has not been shown genetically to have a maternal effect, our results indicate that the tra-2 3'UTR may similarly have a site required for masking maternal tra-2 mRNA. When reporter RNA carrying the wild-type tra-2 3'UTR (lacZ+) was assayed, the RNA was not detectably translated in the distal arm or in immature oocytes, but the RNA was translated in mature oocytes (see Figure 5A). We postulate that endogenous tra-2 mRNA may be translated in a pattern similar to that of the lacZ+ reporter RNA. If this is true, maternal tra-2 mRNA may be masked in immature oocytes by a protein binding to the tra-2 3'UTR (perhaps by DRF binding to the DREs) and then unmasked in mature oocytes before fertilization. Consequently, Tra-2 protein would be maternally contributed to the embryo, which could be critical if sexual fate decisions are made soon after fertilization.

Translational Control and Developmental Regulation

The control of developmental regulatory genes at the translational level is not unique to tra-2 and germline sex determination. In C. elegans, lin-14 and the proper progression through larval development appear to be controlled by elements in the lin-14 3'UTR (Wightman et al., 1991). In Drosophila, nanos is thought to inhibit the translation of hunchback mRNA by binding elements in the hunchback 3'UTR (Wharton and Struhl, 1991). The use of translational controls for maternal RNAs has been rationalized by the fact that their genes are limited to posttranscriptional mechanisms of gene expression. Yet translational controls are also used for somatic cell fate decisions. In the case of lin-14, translational control of developmental fates is clearly somatic; lin-14 is regulated in the hypodermis to control stage-specific lineages (Araus et al., 1991). In addition, the strongest tra-2(ge) allele alters the sexual phenotype of the X0 soma. Consistent with the somatic phenotype of tra-2(ge), the RNA binding activity specific for the tra-2 DREs is present in males and in somatic tissues. Decreased translation of tra-2 in the soma may be necessary to ensure full inhibition of tra-2 activity by her-1 (see Figure 1A). Therefore, controls at the translational level are critical for cell fate regulation in both germline and somatic tissues and are not limited to maternal mRNAs. The importance of translational regulation to maternal mRNAs has been known for years, but the more general significance of translational regulation to the control of cell fate throughout development is just beginning to emerge.

Experimental Procedures

Sequencing of tra-2(+) and tra-2(ge) 3'UTRs

The standard methods for manipulating nucleic acids were used (Maniatis et al., 1982). Sequenase T7 DNA polymerase (U. S. Biochemicals) was used in sequencing following the method of Tabor and Richardson (1987). The tra-2(ge) alleles were sequenced on one strand using primers designed from the wild-type sequence (Kuwabara et al., 1992). For the tra-2(ge) alleles q122ge, q244ge, e2048ge, and e2020ge, a BglII–EcoRI fragment located at the 3' end of tra-2 was cloned following polymerase chain reaction amplification (Saiki et al., 1988) of genomic DNA isolated from a population of tra-2(ge) homozygous animals. tra-2-specific primers were used that were located outside the BglII–EcoRI fragment (PO6, 5'-ATCAAGAAAGAAGCACC-3', and PO7, 5'-TCAACTTACCAGCCGTA-3'). Amplified DNA was digested with BglII and EcoRI and cloned into pBlB76 (International Biotechnologies) for sequencing. For q1039f, the fragment spanning the 3' junction of Tc1 and tra-2 was amplified using a primer within Tc1 (5'ATCAGACCTGATG) provided by A. Rushforth and P. Anderson, University of Wisconsin-Madison and a tra-2-specific primer (PK21, 5'-TTGAGGTCCAGGTGACGAT-3'). Polymerase chain reaction amplifications were done using 10 ng of C. elegans genomic DNA and 100 μM of each primer. DNA was denatured at 95°C; primers were annealed at 42°C and extended at 72°C. For q101gf, the 4.5 kb BamHI–HindIII fragment containing the tra-2 3' end was cloned from a Charon 27 λ phage library made from homozygous tra-2(q101gf) animals and was subsequently cloned into pBlB76 for sequencing.

Northern Blot Analysis

Isolation of C. elegans RNA followed standard protocols (Emmons and Yesner, 1984; Rosenquist and Kimble, 1988). Northern blots were performed as described by Okkama and Kimble (1991). 32P-labeled RNA probes were made using SP6 or T7 RNA polymerase following reaction conditions similar to those described by Melton et al. (1984). For quantitation, a blot was rehybridized using a probe (pJK191) specific for the phyrgenase-specific myo-1 gene (Miller et al., 1986). Quantification of transcript bands on autoradiographs was performed using a Zeineh model SL-504-XL scanning laser densitometer. Each lane was scanned in two or three locations, and the mean peak area was calculated.
Polysomes Analysis
Polysomes were isolated from developmentally staged adult worms (see below) grown at 20°C. The worms were collected and washed three times in M9 (22 mM potassium phosphate monobasic, 84 mM sodium phosphate dibasic, 90 mM sodium chloride, and 1 mM magnesium sulfate) and then one time in either room temperature (~22°C) cycloheximide lysis buffer (300 mM NaCl, 50 mM HEPES [pH 7.3], 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM EDTA, 2 mg/mL heparin, 400 U of RNasin [Promega], 2 mM vanadyl ribonucleoside complex [Sigma], 2.5 mM phenylmethylsulfonyl fluoride, and 0.2 mg/mL cycloheximide) or in room temperature EDTA lysis buffer (300 mM NaCl, 50 mM HEPES [pH 7.3], 1 mM dithiothreitol, 1 mM EDTA, 0.2 mg/mL heparin, 400 U of RNasin, 2 mM vanadyl ribonucleoside complex, 2.5 mM phenylmethylsulfonyl fluoride, and 30 mM EDTA). All solutions contained in the cycloheximide lysis and EDTA lysis buffers, except for the RNasin, vanadyl ribonucleoside complex, and phenylmethylsulfonyl fluoride, were treated with diethyl pyrocarbonate. After the final wash, the volume of worms was measured and brought up to 0.75 ml with either cycloheximide lysis buffer or EDTA lysis buffer. Immediately before use, the suspension was French pressed at 4°C using 8000 psi. The lysate was then centrifuged at 12,000 x g for 10 min at 4°C, and the resulting supernatant was loaded onto a 10 ml 5%-45% linear sucrose gradient made in either cycloheximide lysis buffer or EDTA lysis buffer. Polysomes were centrifuged in a SW41 Ti Beckman swinging bucket rotor at 21,000 x g for 2 h at 4°C. After centrifugation, 1,25 ml fractions were collected, and ethanol was precipitated. The pellets at the bottom of the gradient were also kept for further analysis. The ethanol pellets and the gradient pellet were extracted with 0.25 ml of 4 M guanidine thiocyanate, 50 mM Tris-HCl (pH 7.6), 5 mM EDTA, and 0.5% Na-lauroylsarcosine (sodium salt), followed by three phenol–chloroform and two chloroform extractions. The RNA was precipitated by the addition of 0.025 vol of 1 M acetic acid and 0.75 vol of 100% ethanol and stored at −20°C. After centrifugation, the purified RNAs were resuspended in equal volumes (0.1 ml) of diethyl pyrocarbonate-treated dH₂O, and RNA yields were determined spectrophotometrically. Samples (0.05 ml) were analyzed by RNAase protection assay.

RNAase protections were performed using the Ambion RPAII kit, a modification of the method of Lee and Costow (1987). The tra-2ΔP-RNA probe was made from pJK450 linearized with ClaI using T7 RNA polymerase (Melton et al., 1984). pJK500 contains the Pati-BamHI fragment of the tra-2 CDNA (nucleotides 2519–3015; see Kwekbara et al., 1992). act-1 RNA probe was synthesized from an act-1 specific clone (kindly provided by M. Krause) linearized with EcoRI using T3 RNA polymerase. The reactions were run on a 5% denaturing polyacrylamide–urea gel. The signals were quantitated using a β-scanner (Betalgen, Waltham, Massachusetts). The act-7 signal was used to normalize the loading difference between a particular tra-2(+)/tra-2(−) and the same tra-2(Δp). The ratio of the act-1 counts per minute in tra-2(+)/tra-2(−) compared with the act-1 counts per minute in the tra-2(Δp) fraction was determined, and the resulting number was multiplied by the counts of the tra-2(Δp) 4.7 kb message.

Reporter RNA Synthesis and Microinjections
Reporter RNAs were synthesized in vitro essentially as described in Melton et al. (1984). cDNA templates were made by PCR using pJK350. All RNAs were synthesized to contain the 7-methyl GpppG cap at the 5′ end and a 3′ poly(A) tail of 30 residues. Full-length transcripts were purified on oligo(dT)–cellulose. RNA synthesis protocols and the pJK350 vector will be described in detail elsewhere (T. Evans, S. Crittenden, and J. K., unpublished data). The reporter RNAs encode β-gal that contains the SV40 nuclear localization signal at the N-terminus (T. Evans, et al., 1990). The tra-2(+)/+ and tra-2(Δp) 3′UTR clones were cloned following polymerase chain reaction amplification using tra-2 3′UTR specific primers EBG-20 [5′-ATTTTATTGGTGGCAATGCATGTTCCCTTTTACG-3′] and EBG-21 [5′-AAATATTTGATCTATGTGACATTTAACAAGAAAACCAAA-3′] from genomic DNA isolated from either tra-2(+) or tra-2(Δp) animals, respectively. The polymerase chain reaction products were digested with Sall and BglII and were cloned into the 3′ promoter of pJK350.

Reporter RNAs were microinjected at 100 nM into the distal arm of N2 hermaphrodite germlines, as described elsewhere (Fire, 1986; T. Evans, S. Crittenden, and J. K., unpublished data). Injected hermaphrodites were incubated in recovery buffer (Fire, 1986) for 30 min and were placed on seeded plates for 4 hr at 20°C. Worms were washed in M9 and then fixed and stained with X-Gal as described (Fire et al., 1990).

Analysis of RNA–Protein Interactions
RNA gel shifts were performed using a modification of the method of Müller et al. (1989). Worms were washed off petri plates with M9 salts and were washed one time in lysis buffer (10 mM HEPES [pH 7.6], 10 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol, 7 µg/ml pepstatin, 5 µg/ml chymostatin, 25 µg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride). The worms were homogenized using a French press at 4°C using 8000 psi. The homogenate was spun at 12,000 x g for 15 min. Protein concentration of the supernatant was determined using the Coomassie protein assay reagent (Pierce). For the binding reaction, 5 µg of extract was incubated with 1 fmol of EB9-9 in binding buffer (10 mM HEPES [pH 7.3], 25 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mg/ml TRNA, and 5% glycerol) in a final reaction volume of 20 µl at 25°C. After 15 min, heparin was added to a final concentration of 5 mg/ml, and the reaction was placed at 4°C for 5 min. The samples were then loaded on a 7% nondenaturating polyacrylamide gel (ratio of acrylamide to bis was 29:1.2), which had been pre-equilibrated to 4°C. The gel was run at 4°C for 1 hr using 10 W; then it was dried and autoradiographed at −80°C. In some experiments, different and dried autoradiographs of cold competitor RNA were added to the reactions. The final reaction conditions were kept constant by adjusting the volume with dH₂O. It made no difference to the results whether the competitor RNA was added before or after the 15 min incubation.

For the 32P-RNA probes containing the 3′UTRs of tra-2(Δp), tra-2(Δp)2(Δ212gff), and tra-2(Δp)2(Δ202gff), the vectors pJK66, pJK825, and pJK265 were cut with Mael. For the fem-3 3′UTR, the vector pJK600 was linearized with DraI. The probes were made using T7 RNA polymerase as described above. All other 32P-RNA probes (EB9-9, EB9-10, EB9-11, and EB9-13) were made by the method of Milligan and Uhlenbeck (1989). For unlabeled probes, a trace amount of [32P]UTP was added to the reaction, and Cheneryov counting was used for quantitation. The CRE sequence of EB-13 was randomized by placing paper containing individual nucleotides into a container and withdrawing them one at a time. The oligo names and their sequences are listed below. The CRE sequence is in bold; the –17 to –1 of T7 RNA polymerase promoter plus an additional 6 nt, which were included to optimize transcription, are underlined. The scrambled CRE of EB-13 is italicized.

EB9-9: 5′-TGGAGCATAATAGATGAGTTGTAATGATAAGAAATATTAATGATGATGATGATTAGCTTACCTGCGTGTATTA3′

EB9-10: 5′-TGGAGCATTGATGATGAGTTGTAATGATAAGAAATATTAATGATGATGATGATTAGCTTACCTGCGTGTATTA3′

EB9-11: 5′-TGGACAGATGAAATGGAAAATTGTGACAAATATGAAGAAACGAAATGATGAAAGAATTTGTGGAACCCATTACGCTCGTGTATTA3′

EB9-13: 5′-TGGACAGATGAAATGGAAAATTGTGACAAATATGAAGAAACGAAATGATGAAAGAATTTGTGGAACCCATTACGCTCGTGTATTA3′

Characterization of DRF Binding Activity

Binding of DRF to the DREs was inhibited by Mg²⁺ and Ca²⁺ and was sensitive to protease treatment (data not shown). No complex was formed with antisense tra-2 3′UTR RNA (data not shown). Also, no complexes were formed with double-stranded or single-stranded DNA that encoded the tra-2 3′UTR, demonstrating that the binding activity is specific for RNA (data not shown). Consistently, at least two complexes were detected in each RNA retardation experiment. It is presently unclear why multiple complexes were seen. One possibility is that distinct complexes result from the proteolytic degradation of a single binding factor. This hypothesis is supported by the observation that incubations of extract for 10 min or longer increased the intensity of lower bands with a simultaneous decrease in intensity of the upper bands (data not shown). Another possibility is that the multiple bands are due to different factors binding to the DREs or posttranslational modification of the binding activity.
Synchronizing Animals
Developmentally staged animals were grown from embryos isolated by hypochloric treatment of gravid hermaphrodites (Sulston and Hodgkin, 1988). More tightly synchronized animals were obtained for Northern analysis, by isolating embryos as described above and allowing them to develop for approximately 6 hr at room temperature (≈22°C), followed by a second hypochloric treatment. L1, L2, L3, and L4 were staged by scoring gonad and vulva development (Kimble and Hirsh, 1979; Sulston and Horvitz, 1977).

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