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The STAR protein, GLD-1, is a translational regulator of sexual identity in *Caenorhabditis elegans*

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The *Caenorhabditis elegans* sex determination gene, *tra-2*, is translationally regulated by elements in the 3′-untranslated region called TGEs. TGEs govern the translation of mRNAs in both invertebrates and vertebrates, indicating that this is a highly conserved mechanism for controlling gene activity. A factor called DRF, found in worm extracts binds the TGEs and may be a repressor of translation. Using the yeast three-hybrid screen and RNA gel shift analysis, we have found that the protein GLD-1, a germline-specific protein and a member of the STAR family of RNA-binding proteins, specifically binds to the TGEs. GLD-1 is essential for oogenesis, and is also necessary for spermatogenesis and inhibition of germ cell proliferation. Several lines of evidence demonstrate that GLD-1 is a translational repressor acting through the TGEs to repress *tra-2* translation. GLD-1 can repress the translation of reporter RNAs via the TGEs both *in vitro* and *in vivo*, and is required to maintain low TRA-2A protein levels in the germline. Genetic analysis indicates that GLD-1 acts upstream of the TGE control. Finally, we show that endogenous GLD-1 is a component of DRF. The conservation of the TGE control and the STAR family suggests that at least a subset of STAR proteins may work through the TGEs to control translation.

Keywords: *gld-1*/sex determination/STAR protein/*tra-2*/translation

Introduction

The precise temporal and spatial expression of key regulatory genes is crucial for normal development. It is now apparent that translational control by elements in the 3′-untranslated region (3′-UTR) play major roles in regulating developmentally important genes (Wickens *et al*., 1996). For example, elements in the 3′-UTR of the *Drosophila hunchback* and *oskar* mRNAs are necessary for repressing translation and hence controlling anterior–posterior axis formation (Wharton and Struhl, 1991; Kim-Ha *et al*., 1995; Rongo *et al*., 1995). While many 3′-UTR *cis*-acting elements are known, only a few *trans*-acting factors have been identified. As a result, the mechanisms underlying 3′-UTR translational controls are poorly understood. To comprehend these mechanisms better, we sought *trans*-acting factors that interact with the translational regulatory elements in the 3′-UTR of the *Caenorhabditis elegans* sex-determining gene, *tra-2*.

Caenorhabditis elegans has two sexes: hermaphrodite and male. Hermaphrodites are essentially female in the soma but make both sperm and oocytes in the germline. The primary signal for sex determination is the ratio of the number of X chromosomes to sets of autosomes, such that animals with two X chromosomes (XX) develop as hermaphrodites while animals with a single X chromosome (XO) develop as males (for a review see Meyer, 1997). The X to autosomal ratio controls the activity of a number of genes that act in a cascade to regulate sexual cell identity (Figure 1; see Meyer, 1997).

The sex-determining gene, *tra-2*, is required for female cell fates (Hodgkin and Brenner, 1977). *tra-2* is predicted to encode a large transmembrane protein, called TRA-2A, that is necessary to inhibit downstream male determinants (Kuwabara *et al*., 1992). TRA-2A is thought to be part of a signal transduction pathway that is important in ensuring that all the cells in an animal adopt the same sexual fate (Kuwabara *et al*., 1992). In males, TRA-2A activity is low and male development ensues (Hodgkin, 1980).

Proper male development requires that *tra-2* activity is repressed. Dominant gain-of-function (*gf*) mutations have been identified that result in excessive *tra-2* activity causing inappropriate female development in both XX and XO animals. XX animals develop as females (they make no sperm) and XO animals produce oocytes in the germline and yolk in the intestine (Doniach, 1986). The *gf* mutations all map to a direct repeat located in the *tra-2* 3′-UTR (Goodwin *et al*., 1993). This direct repeat consists of two regulatory elements, called TGEs (for *tra-2* and *GLI* elements), which control *tra-2* activity by repressing the translation of the *tra-2* mRNA (Goodwin *et al*., 1993; Jan *et al*., 1997). In addition, the TGEs bind a factor, called DRF (direct repeat factor), that is present in crude worm extracts. Previous analyses suggest that DRF is a repressor of translation (Goodwin *et al*., 1993; Jan *et al*., 1997). Our working model is that the binding of DRF to the TGEs results in translational repression of *tra-2*.

TGEs control translation not only in *C.elegans*, but also in the nematode *Caenorhabditis briggsae* and mammalian cells. Furthermore, the translation of at least three genes [*C.briggsae tra-2*, human *GLI* (Jan *et al*., 1997) and *C.elegans tra-1* (E.Jan, Y.Yoo and E.B.Goodwin, unpublished results)] is controlled by TGEs. These results indicate the TGE control is a conserved mechanism that may regulate the translation of a number of mRNAs.

To explore further the mechanism of how TGEs control sexual development by regulating the translation of *tra-2*,

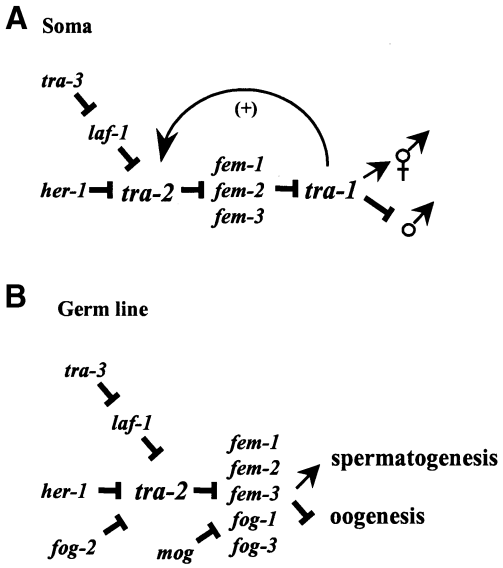

Fig. 1. Genetic control of sex determination in *C.elegans*. For simplicity, genes that act early to control both sex determination and dosage compensation are omitted (for a review see Meyer, 1997). (**A**) Sex determination in somatic tissues. Eight genes are critical determinants of somatic sexual fates: *her-1*, three *tra* genes, three *fem* genes and *laf-1*. In XO animals, *her-1* and *laf-1* inhibit *tra-2*; consequently, the *fem* genes inhibit *tra-1* and male development ensues. In XX animals, *her-1* is not active and *tra-3* represses *laf-1* activity; therefore, *tra-2* inhibits the *fem* genes allowing *tra-1* to promote female development. In addition, *tra-1* may feed back positively on to *tra-2* to amplify commitment to female development (Okkema and Kimble, 1991). (**B**) Sex determination in the germline. Seven of the genes that regulate somatic sexual fate also play a major role in regulation of germline sexual identity: *her-1, laf-1, tra-2, tra-3* and the *fem* genes. In addition, three *fog* genes (Schedl and Kimble, 1988; Barton and Kimble, 1990; Ellis and Kimble, 1995) and six *mog* genes (Graham and Kimble, 1993; Graham *et al*., 1993) affect germline but not somatic sexual fates. In XO animals, *her-1* and *laf-1* inhibit *tra-2*, permitting *fog-1*, *fog-3* and the *fem* genes to direct spermatogenesis. The XX germline is more complex because first sperm and then oocytes are made. The *her-1*, *fog-2* and *laf-1* genes are thought to repress *tra-2* 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 the terminal regulator in germline sex determination.

we screened for TGE-binding factors using the yeast three-hybrid system. We found that the protein GLD-1 (defective in germline development) specifically binds to the TGEs. GLD-1 is a member of the STAR (signal transduction and activation of RNA) family of RNA-binding proteins which are present in both invertebrates and vertebrates and are essential for many developmental decisions (Vernet and Artzt, 1997). The RNA targets of STAR proteins and how STAR proteins regulate RNA activity are poorly understood. Here, we show that GLD-1 is a translational repressor that acts through the TGEs to inhibit *tra-2* translation. The finding that the TGE control is a conserved mechanism raises the possibility that other STAR family members may act via TGEs to regulate translation.

Results

Identification of GLD-1 as a TGE-binding factor

To understand better the mechanism of the TGE control, we sought factors that bind to the *C.elegans tra-2* TGEs.

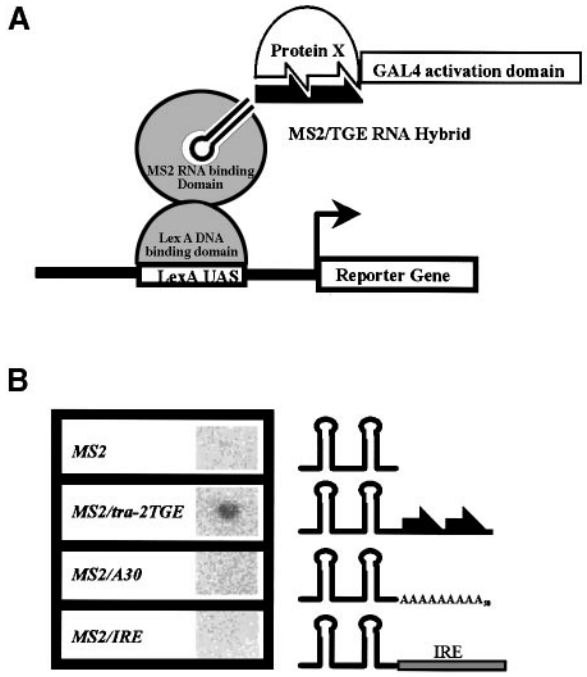

Fig. 2. Identification of GLD-1 as a TGE-binding factor using the yeast three-hybrid screen. (**A**) Model of the yeast three hybrid. Four constructs were used (SenGupta *et al*., 1996). The first construct, which is stably integrated into the yeast genome, consists of the LexA-binding site upstream of the reporter genes, *lacZ* and *HIS3*. The second construct expresses a fusion protein of the LexA DNA-binding domain and an MS2 viral coat protein. The MS2 coat protein binds specifically to a 21 nucleotide RNA stem–loop. The third construct expresses an RNA hybrid consisting of two 21 nucleotide stem–loops and the RNA 'bait'. In this study, the RNA 'bait' is the *C.elegans tra-2* TGEs (see Materials and methods for sequences). The fourth construct consists of the *C.elegans* cDNA library fused to the GAL4 DNA activation domain. When a protein (Protein X) expressed by the cDNA library binds the bait RNA, *lacZ* and *HIS3* reporter genes are transcriptionally activated. Colonies are tested for β-gal activity by a color assay and for the ability to grow on plates lacking histidine. (**B**) Identification of a clone that specifically requires TGEs to activate reporter transgene. Clones that required the RNA hybrid for activation of reporter transgene transcription were tested for RNA-binding specificity with several different RNAs. As shown, the test RNA hybrids carried either the two MS2 target 21 nucleotide stem–loops alone (*MS2*) or the MS2 stem–loops fused to the TGEs (*MS2/tra-2TGE*), to a poly(A)$_{30}$ (*MS2/A30*) or to an IRE (*MS2/IRE*). The IRE is found in untranslated regions of mRNAs encoding proteins involved in iron metabolism (for a review, see Rouault and Klausner, 1996). The IRE acts to control RNA translation and stability and is known to bind specifically to the IRE-binding protein. These test hybrids were transformed into yeast that contained the cDNA clones and tested for *lacZ* and *HIS3* expression in the presence of 5 mM aminotriazole. Shown is the only positive clone that activated transcription of *lacZ* when the RNA hybrid contained the TGEs but failed to activate transcription when the other RNA hybrids were used. The one positive clone was sequenced and was found to code for GLD-1.

Recently, a yeast three-hybrid screen was developed to identify RNA-binding proteins (SenGupta *et al*., 1996). The three-hybrid system selects for proteins that bind to specific RNA sequences. A diagram of the yeast three-hybrid screen is shown in Figure 2A. Briefly, a hybrid RNA is expressed that contains the MS2 coat protein-binding site, fused to an RNA 'bait', in our case the *tra-2* TGEs. For the 'bait', both *tra-2* TGEs arranged in tandem were used, which is a total of 60 nucleotides (see Materials and methods for sequences). This arrangement is precisely

how the TGEs are found in the *tra-2*(+) 3′-UTR (Goodwin *et al*., 1993). A fusion protein consisting of the MS2 coat protein and the LexA DNA-binding domain anchors the hybrid RNA to the promoter of either the *lacZ* or *HIS3* reporter gene. The binding of a protein expressed from the cDNA library to the TGEs results in the formation of a tripartite complex that activates the transcription of the reporter genes.

We used a *C.elegans* cDNA library to screen for proteins that interact with the *tra-2* TGEs. The library was transfected into yeast, and colonies that expressed β-gal and that grew on plates lacking histidine were selected. From a screen of 6×10^5 transformants, 87 positive colonies were isolated. Of these 87 positives, 20 were dependent on the presence of the hybrid MS2–*tra-2* TGE RNA. From these 20, we screened for clones that specifically required the TGEs to activate *lacZ* and *HIS3* reporter genes. Toward this end, we tested the ability of the 20 clones to activate transcription of *lacZ* and *HIS3* when the hybrid RNA contains the TGEs but not when it contains other 3′-UTR elements. Four target hybrid RNAs were used: the MS2-binding site alone or the MS2-binding site fused to the TGEs, to a poly$(A)_{30}$ or to an iron response element (IRE) (Figure 2B). Of the 20 positives, only one activated transcription when the hybrid bait contained the TGEs but failed to activate transcription when other RNA baits were used (Figure 2B).

Sequence analysis of the single positive clone revealed that it coded for the protein GLD-1. GLD-1 is a germline-specific cytoplasmic protein (Jones and Schedl, 1995), and is part of a family of RNA-binding proteins called the STAR family (for review see Vernet and Artzt, 1997). The STAR proteins are thought to link signal transduction pathways and RNA metabolism. The hallmarks of the STAR family are a single KH RNA-binding domain and conserved QUA1 and QUA2 domains. The STAR family includes the murine and human SAM68 (Darnell *et al*., 1994; Fumagalli *et al*., 1994; Taylor and Shalloway, 1994) and SF1 (Kramer, 1992; Toda *et al*., 1994; Agger and Freimuth, 1995; Arning *et al*., 1996), the murine, *Xenopus*, Zebrafish and human QUAKINGs (Ebersole *et al*., 1996; Vernet and Artzt, 1997; Zorn *et al*., 1997) and the *Drosophila* HOW/WHO proteins (Baehrecke, 1997; Zaffran *et al*., 1997). STAR proteins play important roles in a number of developmental events. They are necessary for embryogenesis and myelination in mice, as well as notochord differentiation in *Xenopus* embryos and muscle development in *Drosophila* (Ebersole *et al*., 1996; Zaffran *et al*., 1997; Zorn and Krieg, 1997). How STAR proteins perform these roles is still poorly understood.

GLD-1 has multiple roles in germline development (Francis *et al*., 1995a). GLD-1 is essential for oogenesis. In *gld-1*(*lf*) animals, the oocyte germline fails to progress through meiosis and re-enters mitosis, resulting in over-proliferation of germline cells and consequently a tumorous germline (Francis *et al*., 1995a). GLD-1 has non-essential roles in germline proliferation and sex determination (Francis *et al*., 1995a,b). With regard to sex determination, GLD-1 is necessary for hermaphrodite spermatogenesis. *gld-1*(*lf*) XX animals make few or no sperm. We hypothesize that spermatogenesis results from the repression of *tra-2* translation by GLD-1.

GLD-1 interacts specifically with TGE

To test whether GLD-1 directly interacts with the *C.elegans tra-2* TGEs, we used RNA gel mobility assays and asked whether purified bacterially expressed GST–GLD-1 fusion protein bound to the *tra-2* TGEs. Incubation of GST–GLD-1 with RNA containing the wild-type *tra-2*(+) 3′-UTR resulted in a slower migrating band, indicating complex formation (Figure 3A, compare lane 1 with 2–5; Figure 3C). GST–GLD-1 and the *C.elegans tra-2* 3′-UTR RNA had a binding constant of ~500 nM. Complex binding was not due to GST since GST alone did not bind RNA (data not shown). The binding of the *tra-2* 3′-UTR to GLD-1 was dependent upon the TGEs since GST–GLD-1 bound only weakly to mutant *tra-2* 3′-UTRs in which the TGEs were deleted (Figure 3A, compare lane 6 with 7–9; Figure 3C). We were unable to saturate binding to the mutant *tra-2* 3′-UTR RNAs, indicating that the binding constant is much greater than 500 nM and is probably due to non-specific binding. We also performed competition experiments and found that the *tra-2*(+) 3′-UTR but not mutant 3′-UTRs competed for GLD-1 binding to the *tra-2*(+) 3′-UTR (data not shown). The broadness of the GLD-1 shift with the wild-type *tra-2*(+) 3′-UTR may be due to oligomerization of the GLD-1 as previous studies have shown that GLD-1 can self-associate (Chen *et al*., 1997).

To explore further the binding specificity of GLD-1, we examined the ability of GLD-1 to bind small RNAs containing just the TGEs (Figure 3B and summarized in C). Similarly to the full-length *tra-2* 3′-UTRs, radiolabeled RNAs containing just the TGEs (EBG-9) bound GST–GLD-1 but RNAs in which the TGEs had been deleted (EBG-11) did not (Figure 3C). Previously, we identified functional TGEs in the 3′-UTR of the *C.briggsae tra-2* and human *GLI* mRNAs (Jan *et al*., 1997). One would predict that GLD-1 should also bind these elements. Indeed, we found that GST–GLD-1 specifically associated with small RNAs containing the *C.briggsae tra-2* (EJ-19) and *GLI* (EJ-38) TGEs (Figure 3B, lanes 1 and 2, and data not shown; Figure 3C), but did not form a complex with RNAs that contained a mutant *C.briggsae tra-2* TGE (EJ-32 and EJ-35, Figure 3B, lanes 2–6). EJ-35 contains a six nucleotide deletion within the 31 nucleotide *C.briggsae tra-2* TGE, and EJ-32 carries the same six nucleotide deletion as well as three base substitutions (see Materials and methods for sequences). In conclusion, GLD-1 binds specifically to TGEs.

If GLD-1 regulates *tra-2* activity by the TGEs, then previously identified mutations in GLD-1, which dramatically reduce GLD-1 activity, may disrupt the ability of GLD-1 to bind TGEs. Previously, Schedl and colleagues identified a point mutation, called GLD-1(q361) (Gly227 to Asp), in the KH domain that results in a strong loss-of-function phenotype (Jones and Schedl, 1995). *gld-1*(*q361*) homozygous animals do not produce sperm, and *gld-1*(*q361*)/+ heterozygous animals have a semi-dominant germline phenotype where some of the animals make only oocytes (Francis *et al*., 1995a). To test whether this point mutation altered the ability of GLD-1 to bind the TGEs, we asked whether GST–GLD-1(q361) mutant protein bound TGEs in a gel mobility shift assay. GST–GLD-1(q361) was not able to form a complex with the wild-type *tra-2* 3′-UTR (Figure 3D, compare lanes 2

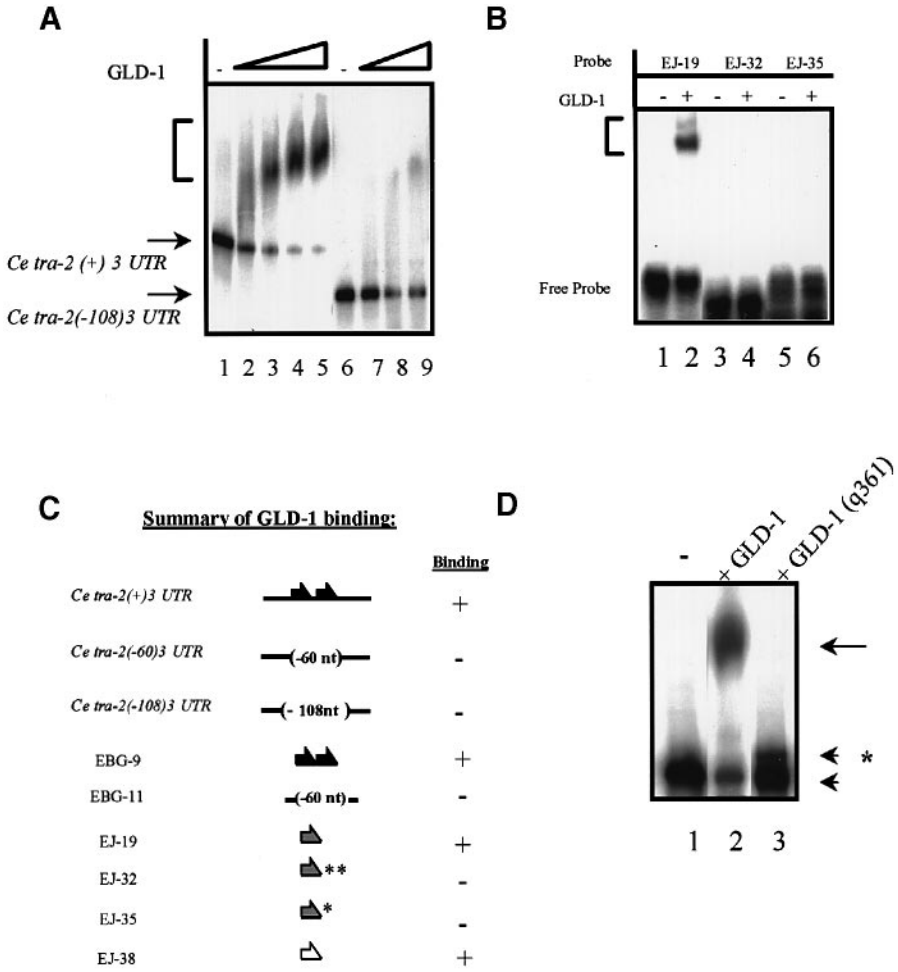

Fig. 3. GLD-1 binds specifically to the TGEs. Binding of GLD-1 to the TGEs was tested by RNA gel mobility shift analysis (Goodwin *et al*., 1997). (**A**) A 15 fmol aliquot of ^{32}P-labeled *C.elegans tra-2* 3′-UTR (lane 1) or mutant (lane 6) 3′-UTR in which the TGEs have been deleted was incubated alone or with increasing amounts of purified bacterially expressed GST–GLD-1 protein (lanes 2–5 and 7–9). The amounts of GST–GLD-1 protein added to the reactions are as follow: lanes 2–5: 0.1, 0.15, 0.2 and 0.25 μg; lanes 7–9: 0.15, 0.2 and 0.25 μg. Reactions were loaded and electrophoresed on a 4% non-denaturing polyacrylamide gel. The gel was dried and autoradiographed. Slower migrating bands represent complex formation (brackets); the faster migrating bands indicate free probe (arrows). (**B**) A 1 fmol aliquot of ^{32}P-labeled *C.briggsae tra-2* TGE (EJ-19) or mutant *C.briggsae tra-2* TGE (EJ-32 and EJ-35, see Materials and methods for sequences) was incubated alone (lanes 1, 3 and 5) or with 0.65 μg of GST–GLD-1 (lanes 2, 4 and 6). Slower migrating bands are due to complex formation (arrow); faster migrating bands are indicative of free probe. (**C**) Summary of GLD-1 binding. Binding to GST–GLD-1 was determined by RNA gel shifts using radiolabeled RNA and by competitive RNA gel shifts. Specific binding was scored positive if labeled RNA bound GST–GLD-1. Competitive RNA gel shifts were performed on full-length wild-type and mutant *C.elegans tra-2* 3′-UTRs and small RNAs which either contained or did not contain the *C.elegans tra-2* TGEs (data not shown). Left: names of RNAs (for sequences, see Materials and methods). Middle: diagrams of RNAs. Black arrows represent *Ce-tra-2* TGEs, stippled arrows represent the *Cb-tra-2* TGE, and open arrows represent the *GLI* TGE. The sizes of the deletions are indicated in parentheses. Right: the different RNAs were scored for the ability (+) or inability (–) to bind GLD-1. Asterisks beside arrows indicate mutant sequences. EJ-32 and EJ-35 carry small deletions or point mutations in the TGE that disrupt DRF binding to the *C.briggsae tra-2* TGE (unpublished results; for sequences see Materials and methods). (**D**) The KH domain of GLD-1 is required to bind to the TGEs. A 1 fmol aliquot of ^{32}P-labeled *tra-2* 3′-UTR was added alone (lane 1) or with 0.25 μg of GST–GLD-1 (lane 2) or 0.25 μg of GST–GLD-1(q361) (lane 3) in which the KH domain contains an amino acid substitution (Gly227→Asp; Jones and Schedl, 1995). Slower migrating bands are due to complex formation (arrow); the faster migrating bands indicate free probe (arrowhead). Non-specific binding is shown by the arrowhead with the asterisk.

and 3). These results show that the loss-of-function phenotype of GLD-1 correlates with loss of GLD-1 binding to the TGEs, supporting the idea that TGE binding is required for GLD-1 function.

GLD-1 is a component of DRF

Previous analyses suggest that DRF is a repressor of *tra-2* translation (Goodwin *et al*., 1993). Hence, one would predict that GLD-1 should be a component of DRF. To address this, we compared the migration of DRF in an RNA gel mobility assay in the presence and absence of GLD-1 antibody. Often DRF is a doublet, possibly indicating that it consists of multiple factors. Addition of GLD-1 antibody resulted in a reduction of DRF binding (Figure 4A, compare lanes 2 with 3). GLD-1 is probably a component of both complexes, since addition of antibody reduces both. Pre-absorbed GLD-1 antibody and an antibody to GST do not significantly affect the mobility of the DRF–*tra-2* 3′-UTR complex, indicating that the inhibition by GLD-1 antibody is specific (Figure 4A, lanes 4 and 5). The antibody results support the idea that GLD-1 is a component of DRF.

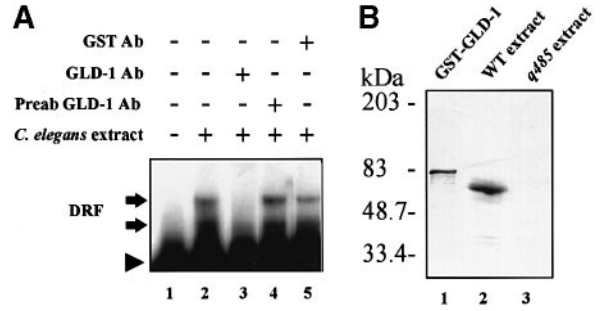

Fig. 4. GLD-1 is a component of DRF. (**A**) GLD-1 antibodies inhibit DRF activity. To test whether GLD-1 is a component of DRF, GLD-1 antibody was added to *C.elegans* extract in an RNA gel mobility assay. Radiolabeled RNAs containing the *C.elegans tra-2* 3′-UTR (lane 1) were incubated with either *C.elegans* adult extract (lane 2), adult extract and 0.4 μg of GLD-1 antibody (lane 3), adult extract and GLD-1 antibody pre-absorbed to GLD-1 (lane 4) or adult extract and 1 μg of GST antibody (lane 5). Reactions were loaded on a 6% polyacrylamide gel and autoradiographed. Slower migrating bands are DRF complexes (arrows); faster migrating band indicates free probe (arrowhead). (**B**) GLD-1 polyclonal antibodies are specific to GLD-1. Purified GST–GLD-1 (lane 1), wild-type *C.elegans* adult extract (lane 2) and mutant *C.elegans* adult extract from *gld-1*(*q485*lf) animals were loaded on an 8% SDS–polyacrylamide gel. Shown is a Western blot using GLD-1 antibody. A single band is detected for GST–GLD-1 (66 kDa) and in *C.elegans* extract (58 kDa). However, no band is detected in mutant *gld-1*(*q485*null) extracts: *gld-1*(*q485*lf) animals do not produce GLD-1 protein (lane 3; Jones *et al*., 1996).

GLD-1 represses tra-2 activity via the TGEs in vivo

If GLD-1 is important in regulating translation, then it should control the activity of mRNAs that contain TGEs *in vivo*. To address this, we asked whether the expression of GLD-1 could inhibit the activity of reporter transgenes that carried TGEs. Presently, it is not possible to assay transgenes in the germline of *C.elegans*. Consequently, we performed this analysis by ectopically expressing GLD-1 in the soma. To express GLD-1 in the soma, a construct containing the heat shock promotor (*hsp16-41*) fused to the entire GLD-1-coding region (*hsp::GLD-1*) was made. Four reporter transgenes were used: all coded for the *lacZ* gene and contained either the wild-type *tra-2* 3′-UTR [*lacZ::tra-2(+)3′UTR*], a mutant *tra-2* 3′-UTR in which one [*lacZ::tra-2(–32)3′UTR*] or both TGEs [*lacZ::tra-2(–60)3′UTR*] were removed, or a 108 nucleotide deletion [*lacZ::tra-2(–108)3′UTR*] that removes the TGEs plus flanking sequences. The use of the *lacZ::tra-2(–32)3′UTR* transgene is a particularly sensitive assay for regulation, since a single TGE is able to partially repress translation (Goodwin *et al*., 1997). The transgenes were controlled by the inducible heat shock promoter (*hsp16-41*). Transgenic animals carrying *hsp::GLD-1* and either *lacZ::tra-2(+)3′UTR*, *lacZ::tra-2(–32)3′UTR*, *lacZ::tra-2(–60)3′UTR* or *lacZ::tra-2(–108)3′UTR* transgenes were heat shocked and the percentage of transgenic animals with intestinal β-gal staining were scored.

We found that ectopic expression of GLD-1 in animals carrying TGEs resulted in a dramatic decrease in intestinal β-gal staining. In the absence of GLD-1, 7 and 59% of transgenic animals carrying *lacZ::tra-2(+)3′UTR* and *lacZ::tra-2(–32)3′UTR*, respectively, had β-gal staining in intestinal cells (Figure 5A, Table I). In contrast, when GLD-1 was expressed in the soma, 0% of *lacZ::tra-2(+)3′UTR* and only 18% of *lacZ::tra-2(–32)3′UTR* transgenic animals had intestinal β-gal staining (Figure 5B, Table I). Ectopic expression of GLD-1 had little or no effect on the β-gal expression of *lacZ::tra-2(–60)3′UTR* and *lacZ::tra-2(–108)3′UTR* (Figure 5C and D, Table I).

If GLD-1 represses *tra-2* translation, then expression of GLD-1 in the soma may result in masculinization of somatic structures. We analyzed the phenotype of animals that carried *hsp::GLD-1* that were heat shocked once a day from embryogenesis to adults. Approximately 12% of animals in which GLD-1 was expressed in the soma had truncated tails, indicative of somatic masculinization (Figure 6B, Table II). However, no masculinization was detected when the mutant GLD-1(q361) protein was expressed (Table II). These results correlate with GLD-1 repressing *tra-2* activity.

If somatic GLD-1 expression is promoting male development by repressing *tra-2*, then GLD-1 should act upstream of the *tra-2* TGE control in a genetic hierarchy. Toward this end, we overexpressed GLD-1 using the *hsp::GLD-1* construct in XX and XO animals that were loss of function for *her-1* or *fem-3* (Figure 1, Table II). *her-1* and *fem-3* are required for male development, and loss of their activities causes XO animals to be feminized (Meyer, 1997). Genetic analysis indicates that *tra-2* 3′-UTR regulation acts downstream of *her-1* and upstream of *fem-3* to regulate sexual identity (see Figure 1). If GLD-1 is repressing *tra-2* translation, then overexpression of GLD-1 would masculinize *her-1*(*lf*) animals but would not affect *fem-3*(*lf*) animals. We found that similarly to wild-type animals, 23% of XX and XO *her-1*(*lf*) animals in which GLD-1 was overexpressed developed truncated tails (Table II). In contrast, GLD-1 expression did not alter the sexual development of the somas of XX *fem-3*(*lf*) animals (Table II). These data are consistent with GLD-1 functioning between *her-1* and *fem-3* in a genetic hierarchy to inhibit male development.

As discussed above, GLD-1 is required for hermaphrodite spermatogenesis. XX animals that lack GLD-1 activity make no or a few sperm (Francis *et al*., 1995a). In contrast, a subset of *gld-1*(*gf*) mutations, called *gld-1*(*mog*) (masculinization of the germline), cause XX animals to make only sperm and no oocytes (Francis *et al*., 1995a). It is possible that the Mog phenotype results from an increase in the ability of GLD-1 to repress *tra-2* translation in the germline. To address this possibility, we used double mutant analysis to ask what is the germline phenotype of XX *gld-1*(*mog*); *tra-2(gf*) double mutant animals. Earlier work had shown that *gld-1*(*q93*mog)*; tra-2*(*q122*gf) animals made sperm, suggesting that GLD-1 is not regulating spermatogenesis by the TGEs (Francis *et al*., 1995b). However, *tra-2*(*q122*gf) still retains a single TGE that is capable of partially regulating translation (Goodwin *et al*., 1997). Therefore, we made the double mutant between *gld-1*(*q93*Mog) and *tra-2*(*e2020*gf). *tra-2*(*e2020*gf) removes both TGEs and does not retain TGE control (Goodwin *et al*., 1993). The *gld-1*(*q93*Mog)*; tra-2*(*e2020*gf) double mutant animals do not make sperm (n = 53), agreeing with GLD-1 acting via the TGEs to control spermatogenesis. In conclusion, the observations that GLD-1 requires *fem-3* activity to masculinize somatic tissue, and that *gld-1*(*q93*Mog)*; tra-2*(*e2020*gf*)* animals do not make sperm, are consistent with GLD-1 acting upstream of the TGEs to control sexual cell identity by repressing *tra-2* translation.

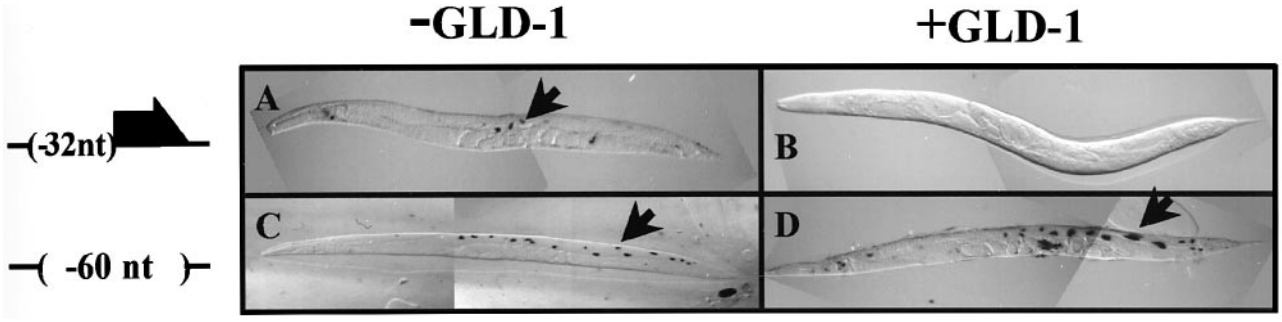

Fig. 5. GLD-1 represses *tra-2* activity via the TGEs *in vivo*. Lateral views of *C.elegans* adult animals with anterior to the left. The reporter *lacZ* gene is driven by the *C.elegans* heat shock promotor (*hsp16-41*; Stringham *et al*., 1992) and is fused to the nuclear localization signal, such that β-gal staining is primarily nuclear. Left: different *tra-2* 3′-UTRs inserted downstream of the *lacZ* reporter gene. (A and C) Transgenic *C.elegans* animals carrying the different 3′-UTR reporter transgenes in the absence of GLD-1. (B and D) Transgenic *C.elegans* animals containing the different reporter transgenes and the *hsp::GLD-1* transgene that expresses ectopic GLD-1 in the soma. The *hsp::GLD-1* contains the heat shock promotor which drives the expression of GLD-1 from the *gld-1* cDNA. (**A**) Animals carrying *lacZ::tra-2(–32)3′UTR* in which one TGE is deleted. β-Gal activity is detected in four intestinal cells (arrow). (**C**) Animals carrying *lacZ::tra-2(–60)3′UTR* in which both TGEs were deleted. β-Gal activity is detected in 15 intestinal cells (arrow)). When GLD-1 is expressed, there is a decrease in β-gal intestinal staining in *C.elegans* animals carrying the *lacZ::tra-2(–32)3′UTR* (**B**), but not in animals carrying *lacZ::tra-2(–60)3′UTR* (**D**). β-Gal activity is detected in 18 intestinal cells (arrow).

Table I. GLD-1 represses the TGE control *in vivo*

Reporter transgene[a]	GLD-1 transgene[b]	% animals with intestinal β-gal staining[c]
lacZ::tra-2(+)3′UTR	none	7% (n = 59)
	hsp::GLD-1	0% (n = 26)
	hsp::GLD-1(q361)	5% (n = 22)
lacZ::tra-2(–32)3′UTR	none	59% (n = 80)
	hsp::GLD-1	18% (n = 56)
	hsp::GLD-1(q361)	60% (n = 52)
lacZ::tra-2(-60)3′UTR	none	68% (n = 105)
	hsp::GLD-1	53% (n = 58)
	hsp::GLD-1(q361)	74% (n = 46)
lacZ::tra-2(-108)3′UTR	none	52% (n = 82)
	hsp::GLD-1	48% (n = 33)
	hsp::GLD-1(q361)	n.d.

[a]Reporter transgenes containing the *C.elegans* heat shock promotor (*hsp16-41*) upstream of the reporter *lacZ* gene. The reporter transgenes contain an NLS. Wild-type *tra-2* or mutant *tra-2* 3′-UTRs were inserted downstream of the *lacZ* gene. In all experiments, adult transgenic worms were heat shocked for 2 h at 33°C and allowed to recover for an additional 2 h at 20°C before being fixed and stained for β-gal activity.
[b]Transgenic *C.elegans* animals containing different reporter transgenes as shown on the left were crossed into transgenic *C.elegans* animals containing *hsp::GLD-1* or *hsp::GLD-1(q361)*. Both *hsp::GLD-1* and *hsp::GLD-1(q361)* are controlled by the heat shock promotor (*hsp16-41*) and carry the *unc-54* 3′-UTR. *hsp::GLD-1* contains the coding region for wild-type GLD-1 and *hsp::GLD-1(q361)* carries the coding region for a mutant GLD-1 in which there is an amino acid substitution from Gly227→Asp.
[c]Transgenic animals were scored as positive if blue precipitate was detectable in intestinal cells at 630× magnification. Intestinal cells were scored since genetic evidence indicates that TGE regulation is present in these cells (Doniach, 1986). Percentiles represent the values of one typical transgenic line. Other lines gave similar results. n = total number of animals scored from at least four different experiments. n.d., not determined.

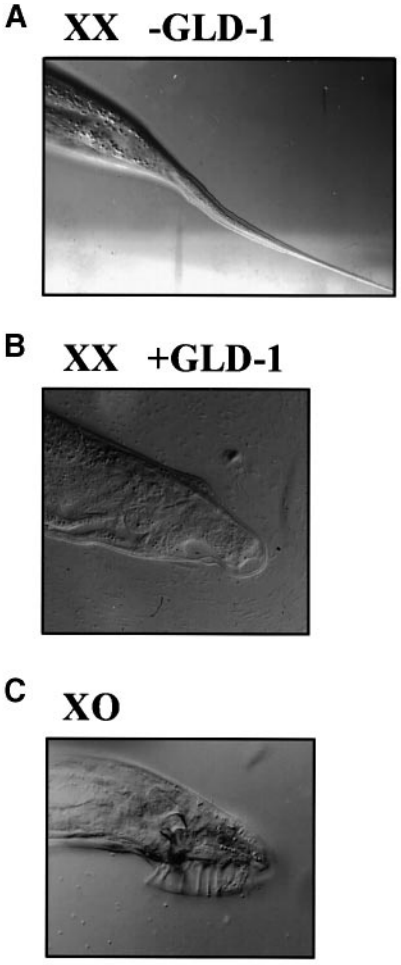

Fig. 6. Ectopic expression of GLD-1 results in somatic masculinization. (**A**) Adult wild-type XX hermaphrodite and (**C**) XO male tails. (**B**) Transgenic *C.elegans* XX animals carrying the *hsp::GLD-1* transgene were heat shocked once a day from embryogenesis to adult. The truncated tail is indicative of somatic masculinization.

TRA-2 expression is affected in a gld-1(lf) background

To address whether GLD-1 governs *tra-2* translation *in vivo*, we examined whether TRA-2A protein levels were altered in a *gld-1*(*lf*) background, using immunofluorescence analyses. TRA-2A polyclonal antibodies that recognize the N-terminal portion of the protein were used to compare levels of TRA-2A in wild-type and *gld-1* (null) mutations. These antibodies detect TRA-2A since mutations that dramatically reduce *tra-2* mRNA also greatly reduce the levels of TRA-2A (L.Graves and E.B.Goodwin, unpublished results). One would predict

Table II. *gld-1* lies between *her-1* and *fem-3* in a genetic hierarchy

Genotype[a]	GLD-1 transgene[b]	% masculinized animals[c]
wild-type	none	0% (n = 93)
wild-type	*hsp::GLD-1*	12% (n = 110)
wild-type	*hsp::GLD-1(q361)*	0% (n = 171)
fem-3(*e1996*lf)	none	0% (n = 153)
fem-3(*e1996*lf)	*hsp::GLD-1*	0% (n = 97)
her-1(*e1518*lf)	none	0% (n = 193)
her-1(*e1518*lf)	*hsp::GLD-1*	23% (n = 127)

[a]Wild-type adult animals were N2 hermaphrodites. For *fem-3*(*e1996*lf) experiments, we examined the Unc (uncoordinated) self-progeny from an *unc-24(e138) fem-3(e1996)/DnT1* strain that either did or did not carry the *hsp::GLD-1* transgene. *unc-24* is a mutation that causes an uncoordinated phenotype, and DnT1 is a balancer for chromosome IV. For *her-1(e1518)* analysis, we examined the self-progeny from a *him-8(e1488); her-1(e1518)* strain. *him-8* mutations cause non-disjunction of the chromosomes during mieosis and consequently 50% of the progeny are XX and 50% are XO.
[b]*C.elegans* animals with different genotypes were mated with animals carrying the transgene *hsp::GLD-1 or hsp::GLD-1(q361).* In all experiments, adult transgenic worms were heat shocked for 2 h at 33°C every day from embryogenesis to adulthood.
[c]Adult animals that had truncated tails, which is indicative of masculinization, were scored positive. Percentiles represent at least three experiments.

that if GLD-1 is controlling the translation of *tra-2* there would be an increase in TRA-2A protein levels in *gld-1* (null) animals as compared with wild-type. To examine this, we used immunocytochemistry to compare the levels of TRA-2A in the gonads of third larval stage (L3) worms in wild-type and *gld-1*(null) backgrounds. L3 gonads were used since at this time *tra-2* must be repressed to allow for spermatogenesis (Hodgkin, 1986). As predicted, there were higher levels of TRA-2A in the *gld-1*(null) animals as compared with wild-type (Figure 7A). The increase in TRA-2A levels is not due to the presence of abnormal germline, since the germline is normal at this time of development (Francis *et al*., 1995a). We also measured the amount of *tra-2* mRNA to ensure that the increased TRA-2A was not due to an increase in mRNA levels. We found that *tra-2* mRNA levels are lower in the gonads of *gld-1*(*lf*) animals as compared with wild-type gonads (Figure 7B). The fact that lower *tra-2* mRNA levels are observed yet there is an increase in the amounts of TRA-2A in the mutant backgrounds suggests that *tra-2* is translated more actively in *gld-1*(null) backgrounds as compared with wild-type, and that GLD-1 binding is required to repress *tra-2* translation. The lower levels of *tra-2* mRNA may indicate a role for TGEs and GLD-1 in stabilizing the mRNA, as deletions that remove the TGEs also result in decreased levels of *tra-2* mRNA (Goodwin *et al*., 1993).

GLD-1 directly represses translation via the TGEs in vitro

We directly asked whether GLD-1 represses translation via the TGEs by assaying whether purified GLD-1 can inhibit translation in an *in vitro* translation assay. Using yeast extracts, we assayed the translation of reporter RNAs containing different 3′-UTRs. Yeast extracts were used since translation in these extracts is sensitive to poly(A) tail length (Iizuka *et al*., 1994), and previously we found

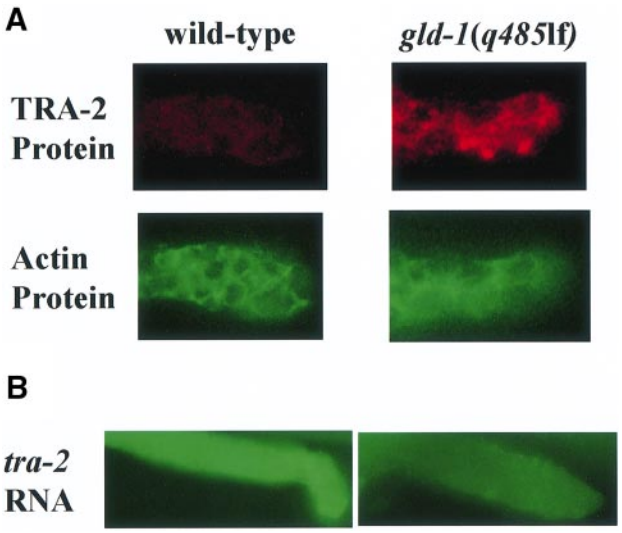

Fig. 7. TRA-2 expression is altered in a *gld-1*(*q485*lf) background. Protein and RNA levels were assayed by immunofluorescence and *in situ* hybridization experiments, respectively. Shown are the distal ends of extruded gonads of third stage larvae XX animals; wild-type (left) and *gld-1*(*q485*lf) (right) animals were prepared side-by-side on a single slide to allow comparison. (**A**) Animals were double-stained using antibodies to TRA-2A and actin proteins. TRA-2A protein levels, assayed using polyclonal antibodies to the N-terminal portion of TRA-2A, are higher in *gld-1*(*q485*lf) animals as compared with wild-type. Actin protein levels, assayed using a monoclonal antibody, were also measured to ensure equal permeabilization of the animals. The animals depicted are representative of the total assayed (n = 32). (**B**) *tra-2* mRNA levels, assayed by *in situ* hybridization using a probe specific to the 4.7 kb *tra-2* transcript, are lower in *gld-1*(*q485*lf) animals as compared with wild-type. The animals shown are representative of the total assayed (n = 37).

that TGEs may control translation by regulating the length of the poly(A) tail (Jan *et al*., 1997). The reporter RNAs encoded *lacZ* and carried wild-type *tra-2* 3′-UTR [*tra-2*(+)] or mutant *tra-2* 3′-UTRs in which one [*tra-2(–32)*] or both TGEs plus some flanking sequences [*tra-2(–108)*] were deleted. The RNAs were capped and contained a poly(A) tail of 30 A residues. The reporter RNAs were added to the yeast extract with or without a 70-fold molar excess of purified GST–GLD-1 protein and the expression of *lacZ* was quantitated at specific times. The addition of GST–GLD-1 protein to reactions containing RNAs carrying *tra-2*(+) or *tra-2(–32)* 3′-UTRs resulted in a decrease in *lacZ* expression (Figure 8A, squares, and data not shown). However, incubation of GLD-1 protein with RNAs carrying *tra-2(–108)* 3′-UTR did not affect *lacZ* expression (Figure 8B, circles). Northern analysis showed that the addition of GLD-1 did not significantly alter RNA levels during the course of the experiment (data not shown), indicating that differences in *lacZ* expression are due to differences in translation. The repression is specific to wild-type GLD-1 since addition of mutant GLD-1(q361) protein did not repress translation (Figure 8A, triangles). Our data demonstrate that GLD-1 represses translation via the TGEs *in vitro*.

Discussion

Sexual development in *C.elegans* is controlled in part by the translational regulation of the sex determination gene *tra-2*. The TGE control regulates the translation of multiple

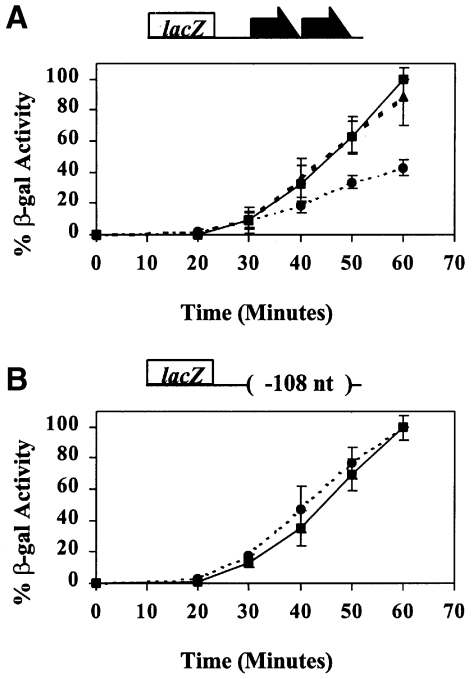

Fig. 8. GLD-1 represses translation via the TGEs *in vitro*. Translation of reporter RNAs was assayed in yeast extracts. (**A**) Incubation of reporter *lacZ* RNAs carrying 4 nM *tra-2(+)3′UTR* with 280 nM GST–GLD-1 protein (●) resulted in a 2- to 3-fold decrease in β-gal activity as compared with incubations of RNAs alone (■). Increasing GST–GLD-1 protein (>280 nM) did not decrease β-gal activity further. Similarly to RNAs carrying *tra-2*(+) 3′-UTRs, addition of GST–GLD-1 protein to reactions containing RNAs carrying *tra-2(–32)* 3′-UTRs resulted in a decrease in *lacZ* expression (data not shown). However, there was no effect of adding 280 nM GST–GLD-1(q361) protein (▲) to RNAs carrying *tra-2(+)3′UTR*. (**B**) Similarly, there was no effect of adding 280 nM GST–GLD-1 protein (●) to RNAs carrying *tra-2(–108)3′UTR* as compared with RNA alone (■). Above each graph is a cartoon of the 3′-UTRs present in the reporter RNA. Expression of the reporter β-gal was measured by a colorimetric assay. Data are plotted as a percentage of total β-gal activity made at the 60 min time point of reactions containing only the reporter RNAs. Aliquots of the reaction mixture were taken at the indicated time points. All yeast extracts showed cap and poly(A) synergism in translational efficiency. All presented data are averages and standard deviations of at least five independent experiments.

mRNAs and is present in both invertebrates and vertebrates (Jan *et al*., 1997). Here, we demonstrate that the STAR protein GLD-1 controls sexual fate by repressing the translation of *tra-2* via the TGEs.

GLD-1 and translational control of tra-2

GLD-1 plays multiple roles in germline development. It is essential in oogenesis, and is also necessary for spermatogenesis and inhibition of germline proliferation (Francis *et al*., 1995a). We propose that the GLD-1 is necessary for repressing *tra-2* translation in the germline to allow spermatogenesis to occur. The oogenic and proliferation phenotypes of GLD-1 may result from misregulation of additional mRNAs that contain TGEs.

Normal sexual development of both XX and XO animals requires that *tra-2* is translationally repressed in both the germline and the soma (Doniach, 1986). However, GLD-1 is germline specific (Jones *et al*., 1996). These findings suggest that other gene product(s) function in the soma to repress *tra-2* translation, and that the TGE control is regulated by tissue-specific factors. For example, there may be a somatic factor that is a homolog of GLD-1. Indeed, several GLD-1-like sequences have been found in the *C.elegans* database that may be the somatic factor for *tra-2* translational control. Alternatively, translational control may require multiple factors, and some of these factors may be tissue specific and others not. Two results possibly support this hypothesis. First, GLD-1 has a moderate affinity for the TGEs (500 nM). This may be due to the fusion protein, as the first 80 amino acids are missing. Alternatively, it may indicate that high affinity binding of GLD-1 to TGEs requires another factor(s). Secondly, the yeast *in vitro* assay showed that purified GLD-1 is sufficient to inhibit translation 2.5- to 3-fold. Although *tra-2* is dosage sensitive and small changes in activity have significant effects on phenotype (Doniach, 1986), it is possible that *in vivo* GLD-1 interacts with other factors to increase its ability to repress translation.

The sex determination gene, *laf-1*, is an excellent candidate for a factor that interacts with GLD-1 (Goodwin *et al*., 1997). *laf-1* is required for the translational repression of *tra-2*, and affects the TGE control in the germline (Goodwin *et al*., 1997). However, *laf-1* may also interact with a somatic factor, perhaps a GLD-1-like factor, since *laf-1* affects *tra-2* translation in the soma (Goodwin *et al*., 1997). Presently, it is unclear whether *laf-1* is a component of DRF or promotes DRF binding. It is possible that a homolog of *laf-1* in yeast is participating with GLD-1 to repress translation in the *in vitro* translation assay. Spermatogenesis also requires that the germline-specific gene *fog-2* inhibits *tra-2* activity (Schedl and Kimble, 1988; Figure 1). It is possible that *fog-2* may act with GLD-1 in the germline to repress *tra-2* translation. Alternatively, *fog-2* may act at another level to regulate TRA-2A.

gld-1 mutant phenotypes may be due to loss of TGE control

Previous genetic studies have suggested that mutations in the KH domain of GLD-1 result in a dramatic loss of GLD-1 activity. Three missense mutations (*q361*, *oz89* and *q93oz55*) that change the absolutely conserved Gly227 to Asp in the GLD-1 KH motif result in a loss-of-function phenotype that in homozygous XX animals abolishes sperm production (Jones and Schedl, 1995). Here, we show that the *q361* mutation causes a loss of GLD-1 binding to TGEs. This result agrees with previous data that show that a similar mutation in the related STAR protein, SAM68, eliminates specific RNA binding (Lin *et al*., 1997). Moreover, we show that the mutant GLD-1 (q361) protein is not able to repress the activity of a reporter RNA *in vivo* or *in vitro*. These results suggest that the loss of sperm in *gld-1*(*q361*) mutants is due to a reduction in GLD-1 repression of *tra-2* translation.

These same three alleles also have a semi-dominant Fog phenotype (feminization of germline) (Francis *et al*., 1995a); a percentage of heterozygous animals produce only oocytes. There are several possibilities for this phenotype. The semi-dominant phenotype may result from the sensitivity of sexual development to levels of *tra-2* activity (Doniach, 1986). Alternatively, Francis *et al.* (1995a) proposed that the semi-dominant Fog phenotype may result from a stable mutant protein either titrating out a limited supply of factor(s) or poisoning a GLD-1 multimer. Interestingly, other STAR family proteins bind

to one another (Chen *et al*., 1997). It is possible that GLD-1 likewise self-associates and that this self-association is necessary for proper GLD-1 function.

Jones and Schedl identified two classes of gain-of-function GLD-1 mutations, one class which result in a Fog phenotype and the other in a Mog phenotype (Jones and Schedl, 1995). It is possible that Mog mutations cause an increase in the ability of GLD-1 to bind TGEs. Alternatively, GLD-1 (Mog) protein may alter the interaction of GLD-1 with a regulator and, consequently, there is an increase in GLD-1 activity. GLD-1 (Fog) protein may reduce GLD-1 binding to the *tra-2* TGEs or affect the interaction of GLD-1 with factors that regulate its activity.

How is GLD-1 regulating the translation of tra-2?

There are several possibilities of how GLD-1 represses *tra-2* translation. One model is that GLD-1 may bind directly or indirectly to translational initiation factors and consequently inhibit their activities. Alternatively, GLD-1 may sequester or mask the *tra-2* transcript from the translational machinery. Finally, GLD-1 may influence the lengths of the poly(A) tail. Support for this last model comes from our studies that show that the presence of the TGEs correlates with a short poly(A) tail and the absence of the elements with a long poly(A) tail (Jan *et al*., 1997).

A number of factors have been identified that bind 3′-UTR elements and are implicated in repressing translation. Two related proteins work via elements in the 3′-UTR to inhibit mRNA activity in *C.elegans* and *Drosophila*. In *C.elegans*, FBF-1 inhibits the activity of the *fem-3* mRNA (Zhang *et al*., 1997), and in *Drosophila* Pumilio inhibits the translation of *hunchback* mRNA (Murata and Wharton, 1995; Wharton *et al*., 1998). Interestingly, database analysis suggests that FBF-1 and Pumilio are part of a larger family of proteins that may be translational regulators (Zhang *et al*., 1997). Also in *Drosophila*, Bruno and a 55 kDa protein repress *oskar* translation (Kim-Ha *et al*., 1995; Gunkel *et al*., 1998), Bicoid inhibits *caudal* translation (Dubnau and Struhl, 1996; Rivera-Pomar *et al*., 1996), and Smaug inhibits the translation of *nanos* (Smilbert *et al*., 1996). In *C.elegans*, a non-coding RNA, called *lin-4*, is a translational repressor of the heterochronic genes *lin-28* and *lin-14* (Wightman *et al*., 1993; Moss *et al*., 1997).

Although a number of factors have been identified that bind to 3′-UTR elements and are required for translational control *in vivo*, only two factors have been shown to inhibit translation *in vitro*. Ostareck *et al.* (1997) demonstrated that purified hnRNP K and E1 can bind to LOX 3′-UTR elements and repress translation in a reticulocyte cell-free translation assay. Here, we demonstrate that purified GLD-1 represses translation in a TGE-dependent manner in a yeast cell-free translation system. Interestingly, although hnRNP K, E1 and GLD-1 repress translation via elements in the 3′-UTR, LOX 3′-UTR translational control occurs independently of changes in poly(A) tail length (Ostareck-Lederer *et al*., 1994), whereas the *tra-2* 3′-UTR translational control correlates with changes in poly(A) tail lengths (Jan *et al*., 1997). These differences indicate that hnRNP K and E1 and GLD-1 may act by different mechanisms to inhibit translation. This is interesting since regulation of a number of 3′-UTR translational control elements, such as *fem-3* (Ahringer and Kimble, 1991), *bicoid* (Salles *et al*., 1994) and *hunchback* (Wreden *et al*., 1997), correlate with changes in poly(A) tail lengths, and others such as *nanos* (Salles *et al*., 1994) and *oskar* (Webster *et al*., 1997) do not.

Possible relationship of TGE and the STAR family of proteins

The TGE control regulates the translation of several mRNAs in both invertebrates and vertebrates (Jan *et al*., 1997). The STAR proteins are also present in a number of organisms and are important in many developmental decisions, including embryogenesis and myelination in mice, notochord differentiation in *Xenopus* embryos and muscle development in *Drosophila* (Vernet and Artzt, 1997). However, the RNA targets and mechanisms by which they control RNA activity are poorly understood. The STAR proteins can be separated into several subfamilies that may indicate distinct functions. For example, the mammalian STAR family member, SF1, and its yeast homolog, BBP, are splicing factors and bind to or very near the conserved branchpoint sequence (Abovich and Rosbash, 1997; Berglund *et al*., 1997). SF1/BBP are distant from other STAR members (Vernet and Artzt, 1997). They contain only half of the QUA1 domain and it is not well conserved (Vernet and Artzt, 1997). Moreover, they have a zinc-knuckle RNA-binding motif (Berglund *et al*., 1997). GLD-1 is most similar to the subfamily that includes the *Drosophila* HOW/WHO (Baehrecke, 1997; Zaffran *et al*., 1997) and vertebrate QUAKING proteins (Ebersole *et al*., 1996; Vernet and Artzt, 1997; Zorn and Krieg, 1997). The fact that the TGE control is conserved raises the possibility that other STAR family members, perhaps some of the family members most similar to GLD-1, act via TGEs to control translation in different organisms.

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.

The following mutant *C.elegans* alleles were used in this study: (i) LGI, *unc-13*(*e51*), *gld-1*(q485*lf*) (Francis *et al*., 1995a), *gld-1*(*q93*Mog) (Francis *et al*., 1995a); (ii) LGII: *tra-2*(*e2020gf*) (Doniach, 1986); (iii) LGIV: *unc-24(e138)fem-3(e1996lf)/DnT1* (Hodgkin, 1986), *him-8 (e1488)*; and (iv) LGV: *her-1(e1518lf)* (Hodgkin, 1980).

Yeast three-hybrid system

The RNA hybrid contains the *tra-2* TGEs arranged in tandem upstream of the MS2 stem–loops. The sequence of the *tra-2* TGEs produced by the RNA hybrid: UAUUUAAUUUCUUAUCUACUCAUAUCUA and CUCAUAUUUAAUUUCUUAUCUACUCAUAUCUA. To construct the hybrid RNA plasmid, the *tra-2* TGEs were amplified from cDNA using PCR primers EBG-74 and EBG-75 that contained a *Sma*I site (see below for primer sequences). The resulting PCR products were subcloned into the unique *Sma*I site of pIII/MS2-2 (a gift of Dr M.Wickens) that contains the *URA3* gene. This chimeric RNA plasmid was called pIII/DRE-MS2. pIII/DRE-MS2 was transformed into the yeast strain, L40-coat (SenGupta *et al*., 1996; gift of Dr M.Wickens). In this strain, the *HIS3* and *lacZ* genes are under the control of the LexA-binding sites. A strain carrying the hybrid RNA plasmid subsequently was transformed with a mixed stage *C.elegans* cDNA library, PRB-2 (kindly provided by Dr R.Barstead). Transformants were selected for growth on plates lacking histidine and uracil plus 5 mM 3-aminotriazole. After several days of growth, the colonies were lifted onto nitrocellulose and stained for β-gal activity. Only colonies that expressed β-gal were analyzed further.

The binding specificity of a clone was tested by transforming the yeast strain L4-coat with the test cDNA plasmid and plasmids that encoded different hybrid RNAs. These plasmids coded for the MS2-

binding site or the MS2-binding site fused with either the *C.elegans tra-2* TGEs, an IRE or a poly(A) tract containing 30 A residues. Yeast triple transformants were assayed for β-gal activity and for the ability to grow on plates lacking histidine. Only one clone was found to bind specifically to the TGEs and it was given the name pBG515. The insert of pBG515 was sequenced and found to encode the full-length GLD-1 coding region.

RNA gel mobility shifts

The vectors, pGEX3-14N (gift of Dr T.Schedl) and pGEXq361, were used to produce wild-type and mutant GLD-1 proteins fused with GST (Jones *et al*., 1996). pGEX3-14N and pGEXq361 encode amino acids 84–457 which is 82% of the coding region (nucleotides 465–1370) (Jones *et al*., 1996). Fusion proteins were isolated as described (Ausubel *et al*., 1989). The mutant GLD-1 protein results from a Gly227 to Asp change (Jones and Schedl, 1995). pGEXq361 was made by cloning an *Nde*I and *Mfe*I fragment from pMALq361 (gift of Dr T.Schedl) into the same restriction sites of pGEX3-14N. Wild-type and mutant GLD-1 fusion proteins were expressed and purified identically.

^{32}P-labeled and unlabeled RNA probes containing different 3′-UTRs were produced by standard methods. The different 3′-UTRs were subcloned in KSII (+) pBluescript vector (Stratagene). The 3′-UTR-containing pBluescript vectors were linearized and the sense 3′-UTR RNAs were transcribed *in vitro* by either T3 or T7 RNA polymerase. Other ^{32}P-labeled and unlabeled RNA probes [EBG-9, EJ-19, EJ-32, EJ-35, EJ-38 and EBG-11; see Jan *et al*. (1997) for description and below for sequences] were produced using the method of Milligan and Uhlenbeck (1989). Cold RNA probes were produced by the RiboMAX kit (Promega). Quantitation of the cold RNA probes was measured by spectrophotometry at OD_{260}.

The sequences of the wild-type (EJ-19) and mutant (EJ-32 and EJ-35) *C.briggsae tra-2* TGEs are:

EJ-19, CAGAT**CTCA**CTTTCCT**ACTTTCCT**GCCTAGT**TTTCT**GAACACA;
EJ-32, CAGAT**CTGT**GTTTCCT**ACTTTCCT**GCCTAG( )GAACACA;
EJ-35, CAGAT**CTCA**CTTTCCT**ACTTTCCT**GCCTAG( )GAACACA.

The conserved nucleotides are in bold, underlined nucleotides represent mutations and parentheses indicate deletions.

Apparent binding constants were determined by titrating increasing amounts of purified GST–GLD-1 with a constant amount of radiolabeled RNAs. Reactions were loaded on a 3.75% native acrylamide gel. The gels were dried and the radioactive bands were quantified by a phosphoimager (FUJIX BAS 2000). For each lane, radioactivity migrating above free probe was indicative of binding and was quantified. The apparent binding constants were defined as the protein concentration at which half of the total amount of RNA was bound.

Analysis of endogenous GLD-1 binding to tra-2 3′-UTR

Purified bacterially expressed GST–GLD-1 was used to immunize rabbits (Cocalico Inc., Reamstown, PA). GLD-1 antibodies were purified using a GST–GLD-1 affinity column (Bio-Rad, Affigel). To test whether GLD-1 is a component of DRF, RNA gel shifts were performed as described (Goodwin *et al*., 1993). GLD-1 antibodies were incubated with *C.elegans* extract for 5 min at room temperature before adding to the reaction mix containing the radiolabeled RNAs. Reactions were loaded on a 6% polyacrylamide gel and autoradiographed. GLD-1 antibodies were pre-absorbed with GST–GLD-1 protein prior to incubation with *C.elegans* extract. Depletion of the GLD-1 antibodies was confirmed by Western blot analysis. Western blot analysis was performed as described (Harlow and Lane, 1988).

Transgene analysis

All β-gal reporter transgenes were derived from the same parent vector, pPC16.41 (gift of Dr Peter Candido). This vector contains the *C.elegans* inducible promoter, *hsp16-41*, the *lacZ* coding sequence and a polylinker. The construction of pBG2 [*lacZ::tra-2(+)3′UTR*], pBG3 [*lacZ::tra-2(–32)3′UTR*] and pBG4 [*lacZ::tra-2(–60)3′UTR*] are described in Goodwin *et al*. (1997). *hsp::GLD-1* contains the heat shock-inducible promoter, *16-41*, the entire full-length *gld-1* protein-coding region and the *unc-54* 3′-UTR. The *hsp16-41* promoter plus the HSP initiating methionine was amplified from genomic DNA by PCR using oligos CKM-5 and CKM-6 that contain *Cla*I sites. The subsequent product was cloned into the *Cla*I site of KSII (+) Bluescript, resulting in the plasmid pBG516. The *unc-54* 3′-UTR was amplified from genomic DNA with primers CKM-7 and EBG-45, which contained translational stop codons in three frames, and both primers contained *Apa*I sites. The PCR product was cloned into the *Apa*I site of pBG516, resulting in pBG517. To insert the full-length *gld-1* coding region, pBG515 (see above) was cleaved with *Xho*I and the resulting fragment was cloned into the *Xho*I site of pBG517, resulting in the plasmid pBG518, which is also referred to as *hsp::GLD-1*. *hsp::GLD-1(q361)* was constructed by cloning an *Nde*I and *Mfe*I fragment from pMALq361 into the same restriction sites of *hsp::GLD-1*.

Transgenic *C.elegans* animals were generated using standard methods (Mello and Fire, 1995). *hsp::GLD-1* or *hsp::GLD-1(q361)* (100 ng/μl) were injected into wild-type *C.elegans* with 100 ng/μl of pRF4 and 50 ng/μl *emb-9::GFP* (a kind gift from C.Witkowski and J.Kramer). *emb-9::GFP* contains the coding region of the green fluorescent protein (GFP) directed by the *emb-9* promoter. This construct will be expressed predominantly in the muscle of the adult animal. Transgenic animals carrying extra-chromosomal arrays containing *hsp::GLD-1* and *emb-9::GFP* were crossed to transgenic animals carrying *lacZ* reporter transgenes that carry different *tra-2* 3′-UTRs. For studies on *lacZ::tra-2 (–60)3′UTR*, 100 ng/μl of *hs::GLD-1* with 50 ng/μl *emb-9::GFP* were injected into transgenic animals carrying *lacZ::tra-2(–60)3′UTR*. Only progeny expressing GFP in the adult muscle were analyzed for β-gal activity.

Cytochemistry and RNA in situ analyses

Immunofluorescence and *in situ* hybridization experiments were performed on the extruded gonads of L3 animals. The gonads were permeabilized by freeze-cracking in liquid nitrogen. For immunofluorescence, animals were then fixed in cold methanol for 10 min, followed by 10 min in cold acetone. The polyclonal antibodies used in the experiment were generated by immunization of rabbits with a GST–TRA-2 fusion consisting of the N-terminal portion of the TRA-2A protein. The resulting antibodies were affinity purified as described for GLD-1. Characterization of TRA-2 antibodies will be described elsewhere (L.E.Graves and E.B.Goodwin, in preparation). For *in situ* hybridizations, animals were treated as described (Seydoux and Fire, 1994). A single-stranded, digoxigenin-labeled DNA probe was synthesized using PCR run-off from a linearized plasmid containing a 774 bp fragment of the *tra-2* cDNA. This probe is specific to the 4.7 kb *tra-2* transcript. The probe was detected using a fluorescein-conjugated anti-digoxigenin antibody (Boehringer Mannheim). An anti-mouse actin monoclonal antibody was used (ICN Biomedicals).

Yeast in vitro translation assay

Yeast lysates were produced as described previously (Iizuka *et al*., 1994) and modified as described (Preiss and Hentze, 1998), where extracts were not treated with micrococcal nuclease. The *in vitro* translation assay was performed as described (Iizuka *et al*., 1994). Capped RNAs containing the *lacZ* gene and different 3′-UTRs were produced by a standard *in vitro* transcription reaction (Ausubel *et al*., 1989). The ratio of cap analog to GTP was 5:1. pBG51, pBG52 and pBG53 correspond to constructs containing the *lacZ* gene and wild-type *tra-2*(+) 3′-UTR, a mutant *tra-2(–32)* 3′-UTR in which one TGE was deleted or a mutant *tra-2(–108)* 3′-UTR in which both TGEs plus some flanking sequences were deleted, respectively. All three plasmids contain a poly$(A)_{30}$ tract downstream of the 3′-UTRs. An *Nsi*I site is immediately 3′ to the poly$(A)_{30}$ tract. The constructs were linearized using *Nsi*I and transcribed using a Promega SP6 Ribomax kit. RNAs were quantitated by spectrophotometry and checked for purity on an agarose gel. RNA was added to the yeast lysates to a final concentration of 4 nM. GST–GLD-1 was added to reaction to a final concentration of 280 nM. At specific time points, aliquots were taken from each reaction and frozen by liquid nitrogen to stop the reaction or added to TRIZOL (Gibco-BRL). β-gal activity was assayed using a colorimetric assay (Tropix) and a luminometer (Monolight 2010 Luminometer). RNA levels were quantitated by Northern blot analysis. All presented data are representative of at least five independent experiments.

Oligo sequences

EBG-9, 5′-GGACGATTAGATATGAGATGATAAGAAATTAAATATG-AGTAGATATGAGTAGATAAGAAATTAAATAATGAAATGGAAAT-TGTCGCCCTATAGTGAGTCGTATTA-3′; EBG-11, 5′-TGGACGATT-ATGAAATGGAAATTGTACAAATAATAGAAACGAAAATGAGTAA-GAAATGAAATTTTGGAACCAAATTCTCGCCCTATAGTGAGTCGT-ATTA-3′; EJ-19, 5′-GTGTTCAGAAAACTAGGCAGGAAAGTAGGA-AAGTGAGATCTGTTAATCGCCCTATAGTGAGTCGTATTA-3′ (the underlined nucleotides represent the nucleotides deleted and/or mutated in EJ-32 and EJ-35); EJ-24, 5′-GGAAGGATAGAAACCCCTTAGGA-AATGCGATCTGTGATGGATGAGATTCCCTCGCCCTATAGTGAGT-

CGTATTA-3′; EJ-32, 5′-TACAAGATCTGTGTTCCTAGGCAGGAAA-GTAGGAAACACAGATCTGTTCGCCCTATAGTGAGTCGTA-TTA-3′; EJ-35, 5′-GAATTCTCGAGTACAAGATCTGTGTTCCTAGG-CAGGAAAGTAGGAAAGTGAGATCTGTTCGCCCTATAGTGAGTC-GTATTA-3′; EJ-38, 5′-TGCAGCTCCCCCAATTTTTCTGGAAGGA-TAGAAACCCCTTAGGAAATGCGATCTGTGATGGATGAGATTC-CCTCGCCCTATAGTGAGTCGTATTA-3′; EBG-45, 5′-CTAGGGGC-CCTGCGGTTTTTTCTATGAT-3′; EBG-74, 5′-TCCCCCGGGGGAT-TTGTACAATTTCCATTTCAT-3′; EBG-75, 5′-TCCCCCGGGGGAA-GTTGAGGTCGAGTGGACGAT-3′; CKM-5, 5′-CCATCGATGCATT-TTCGAAGTTTTTTAGAT-3′; CKM-6, 5′-CCATCGATGGCGAGCTG-CTTGTTGCAAAAGG-3′; CKM-7, 5′-GCTAGGGCCCTAACTAAG-TAATAGGGGCCGCTGTCATCA-3′.

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