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Conservation of the *C.elegans tra-2* 3′UTR translational control

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The *Caenorhabditis elegans* sex-determination gene, *tra-2*, is translationally regulated by two 28 nt elements (DREs) located in the 3′UTR that bind a factor called DRF. This regulation requires the *laf-1* gene activity. We demonstrate that the nematode *Caenorhabditis briggsae tra-2* gene and the human oncogene *GLI* are translationally regulated by elements that are functionally equivalent to DREs. Here, we rename the DREs to TGEs (*tra-2* and *GLI* elements). Similarly to the *C.elegans tra-2* TGEs, the *C.briggsae tra-2* and *GLI* TGEs repress translation of a reporter transgene in a *laf-1* dependent manner. Furthermore, they regulate poly(A) tail length and bind DRF. We also find that the *C.elegans* TGEs control translation and poly(A) tail length in *C.briggsae* and rodent cells. Moreover, these same organisms contain a factor that specifically associates with the *C.elegans* TGEs. These findings are consistent with the TGE control being present in *C.briggsae* and rodent cells. Three lines of evidence indicate that *C.briggsae tra-2* and *GLI* are translationally controlled *in vivo* by TGEs. First, like *C.elegans tra-2* TGEs, the *C.briggsae tra-2* and *GLI* TGEs control translation and poly(A) tail lengths in *C.briggsae* and rodent cells, respectively. Second, the same factor in *C.briggsae* and mammalian cells that binds to the *C.elegans tra-2* TGEs binds the *C.briggsae tra-2* and *GLI* TGEs. Third, deletion of the *GLI* TGE increases *GLI*'s ability to transform cells. These findings suggest that TGE control is conserved and regulates the expression of other mRNAs.

Keywords: *C.elegans*/*GLI*/TGE/*tra-2*/translational control

Introduction

Translational controls are critical for a variety of developmental decisions (for review see Wickens *et al.*, 1996). In many organisms, *cis*-acting regulatory elements in the 3′ untranslated region (3′UTR) govern such major developmental events as embryonic axis formation, maternal mRNA expression and sex determination (Wickens *et al.*, 1996). Many developmental pathways are highly conserved between simple organisms such as flies and worms, and complex organisms such as mice and humans. For example, the *hedgehog* pathway controls cell fate decisions in both *Drosophila* and mice, indicating its fundamental importance in development (Goodrich *et al.*, 1996). In this paper, we ask if the 3′UTR control that governs the translation of the *Caenorhabditis elegans* sex-determining gene, *tra-2*, is a conserved mechanism that controls the translation of mRNAs in nematodes as well as in mammals.

In *C.elegans*, sex-determination is governed by a cascade of regulatory genes that specify one of two sexual fates (Hodgkin, 1990; Villeneuve and Meyer, 1990; Kuwabara *et al.*, 1992). The primary signal for sex-determination is the ratio of the number of X chromosomes to sets of autosomes (Kuwabara *et al.*, 1992; Figure 1). Animals that contain two X chromosomes (XX) develop as hermaphrodites, whereas XO animals develop as males. Hermaphrodites are essentially females that produce both sperm and oocytes.

The *tra-2* gene promotes female cell fates (Hodgkin and Brenner, 1977). Loss of *tra-2* activity causes XX animals to develop as males. *tra-2* has been cloned and is predicted to encode a large transmembrane protein, called TRA-2A, which is thought to function by inhibiting downstream male determinants and by coordinating neighboring cells to adopt the same fate (Okkema and Kimble, 1991; Kuwabara *et al.*, 1992). In the male, *tra-2* activity is low and male development ensues (Hodgkin, 1980).

Development of both hermaphrodites and males depends upon the negative regulation of *tra-2*. Dominant gain-of-function mutations (*gf*) of *tra-2* express increased *tra-2* activity, resulting in the transformation of hermaphrodites into females (Doniach, 1986). Whereas hermaphrodites make both sperm and oocytes, females only make oocytes. The *tra-2(gf)* mutations also feminize XO animals; the intestine produces yolk and the germ line produces oocytes.

The *tra-2(gf)* mutations map to a 60 nt direct repeat located in the 3′UTR. The direct repeat consists of two 28 nt elements (DREs) separated by a 4 nt spacer (Goodwin *et al.*, 1993). The DREs control *tra-2* activity by repressing the translation of *tra-2* mRNA (Goodwin *et al.*, 1993). Recently, we have demonstrated that DREs control poly(A) tail length (S.Thompson, E.B.Goodwin and M.Wickens, unpublished data). These results suggest that DREs may repress translation by inhibiting polyadenylation. A factor, called DRF, specifically binds to the DREs (Goodwin *et al.*, 1993). Our working model is that the binding of DRF to DREs represses translation and thereby inhibits female development.

Two genes are required for normal translational control of *tra-2*. The newly identified sex-determining gene, *laf-1*, is necessary for repressing *tra-2* translation (Figure 1; Goodwin *et al.*, 1997), and may in fact encode DRF. In addition, the sex-determining gene, *tra-3*, appears to

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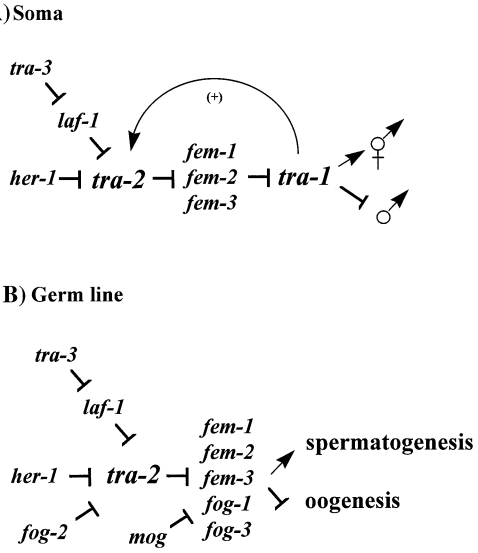


Fig. 1. Genetic control of sex determination in *C.elegans*. For simplicity, genes that act early to control both sex determination and dosage compensation are omitted [for review and detailed references, see Villeneuve and Meyer (1990)]. (**A**) Sex determination in somatic tissues. Eight genes are 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*, 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* represses the *fem* genes and *tra-1* promotes 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 germ line. Seven of the genes that regulate somatic sexual fate also play a major role in regulation of germ line 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 germ line 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 germ line is more complex because first sperm and then oocytes are made. The *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 germ line sex determination. Although *tra-1* influences germ line sex determination in both XX and XO animals, its role is not yet clear (de Bono *et al.*, 1995; Hodgkin, 1987; Schedl *et al.*, 1989).

promote female development by freeing *tra-2* from DRE repression (Figure 1; Goodwin *et al.*, 1997). *tra-3* has been cloned and is predicted to encode a calpain-like protease (Barnes and Hodgkin, 1996). One simple model is that *tra-3* destroys the activity of *laf-1*/DRF by proteolytic cleavage, resulting in the translation of *tra-2* and female development.

In this paper, we find that the *tra-2* DREs may be members of a highly conserved family of regulatory elements that control translation of other mRNAs in a variety of organisms. We show that two genes, the *Caenorhabditis briggsae tra-2* and the human oncogene *GLI*, are translationally regulated in *C.briggsae* and mammalian cells, respectively, by elements that are functionally equivalent to DREs. These findings suggest that this translational control is conserved and is present not only in nematodes but in mammals as well. To reflect the broader role of these control elements in biology, we rename the DRE TGE (*tra-2* and *GLI* element) after the genes in which the elements were first found. We will refer to these elements as TGEs for the remainder of the paper.

Results

The 3′ UTRs of Cb-tra-2 and GLI can control translation in C.elegans

To ask whether the TGE control regulates the translation of other genes, we searched the 3′UTRs of a number of genes for sequences with homology to the *C.elegans tra-2* (*Ce-tra-2*) TGEs. Database searches failed to identify other genes that contained strong similarity to the *Ce-tra-2* TGEs. However, by close eye inspection of a small set of 3′UTRs (see below), two genes were identified that had 3′UTR sequences with similarity to TGEs: the nematode *C.briggsae tra-2* gene (*Cb-tra-2*) and the human oncogene *GLI* (Figure 2). The *Cb-tra-2* gene, like *Ce-tra-2*, is predicted to encode a large transmembrane protein (Kuwabara *et al.*, 1992; Kuwabara, 1996b). Reduction of *Cb-tra-2* activity results in masculinization of *C.briggsae* animals, indicating that the *Cb-tra-2* gene, like *Ce-tra-2*, is involved in specifying sexual cell fates (Kuwabara, 1996b). *GLI* codes for a zinc-finger transcription factor of the *Krüppel* family and was originally identified by its amplification and high levels of expression in a human glioblastoma (Kinzler *et al.*, 1987; Ruppert *et al.*, 1988). Other members of this family include the human *GLI2* and *GLI3* genes, the *C.elegans* sex determining gene, *tra-1* (Zarkower and Hodgkin, 1992) and the *Drosophila* segmentation gene, *ci* (Orenic *et al.*, 1990). *GLI* is also amplified in some human malignant gliomas, osteosarcomas and rhabdomyosarcomas (Kinzler *et al.*, 1987; Roberts *et al.*, 1989). Furthermore, *GLI* in cooperation with the adenovirus E1A protein can transform rat kidney fibroblast cells (Ruppert *et al.*, 1991). *GLI* is expressed in both ectoderm and mesoderm derived tissues, suggesting that it may play multiple roles during post-implantation development (Walterhouse *et al.*, 1993).

Since the database search failed to identify other genes with TGEs, we reasoned that if additional genes are regulated by the TGE control the sequence identity between different elements may be low. Fortuitously, we had found that the *C.elegans* sex determining gene *tra-1* is regulated by *laf-1* (E.Jan and E.B.Goodwin, unpublished results). This suggested to us that the *tra-1* 3′UTR may contain a TGE. Analysis of the *tra-1* 3′UTR revealed a sequence with similarity to the *Ce-tra-2* TGEs. Since *tra-1* is homologous to *Drosophila ci* and the human oncogene, *GLI*, we searched these 3′UTRs for TGEs. We found that the *GLI* 3′UTR but not, apparently, the *ci* 3′UTR contains TGE-like sequences. In the course of our analysis the sequence of the *Cb-tra-2* gene was determined (Kuwabara, 1996a). The fact that important regulatory elements are often conserved between species led us to inspect the *Cb-tra-2* 3′UTR.

The sequences that are similar between the *Cb-tra-2* and *GLI* 3′UTRs and the *Ce-tra-2* TGEs consist of the CUCA ‘spacer’ and a pyrimidine-rich sequence (Figure 2A; boxed and underlined). Furthermore, *Cb-tra-2* and *GLI* 3′UTRs share a second pyrimidine rich sequence (UUUCU), which is absent in the *Ce-tra-2* TGEs (Figure 2A). However, unlike the 3′UTR of *Ce-tra-2*, which

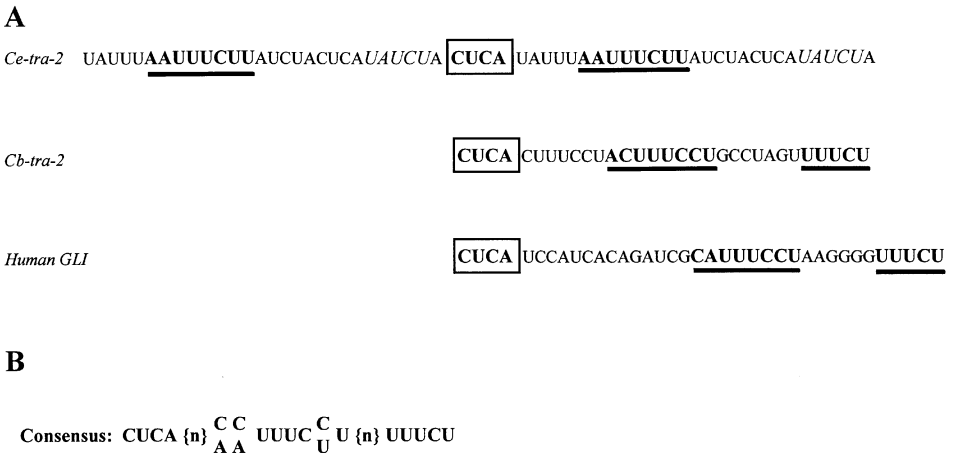


Fig. 2. *Caenorhabditis briggsae tra-2* and *GLI* 3′UTRs contain sequences with homology to the *C.elegans tra-2* TGE. (**A**) Shown are the alignments of the *C.elegans tra-2* TGEs with similar sequences in the *C.briggsae tra-2* and *GLI* 3′UTRs. Bold and large fonts are the regions of strong homology between the different elements. This includes the CUCA 'spacer' and a pyrimidine rich motif. There is a second pyrimidine rich motif (UUUCU) shared between the *C.briggsae tra-2* and *GLI* TGEs. The CUCA spacer is boxed and the pyrimidine rich motifs are underlined. The *Ce-tra-2* TGEs also have a second pyrimidine rich element (UAUCU) (in italics and large fonts), in which four out of five nucleotides are identical to the second UUUCU pyrimidine rich element in *Cb-tra-2* and *GLI* regulatory elements. (**B**) TGE consensus sequence.

contains two identical elements, the *Cb-tra-2* and *GLI* 3′UTRs contain a single regulatory element.

The similarity between these 3′UTR sequences raises the possibility that *Cb-tra-2* and *GLI* may be translationally controlled by TGE regulation. To address this question, we asked if the *Cb-tra-2* and *GLI* 3′UTRs could translationally repress a reporter transgene in *C.elegans*. Four different reporter transgenes were made. All coded for the *lacZ* gene and had either the wild-type *Cb-tra-2* or *GLI* 3′-UTRs [*lacZ::Cb-tra-2(+)3′UTR* or *lacZ::GLI(+)3′UTR*, respectively] or mutant 3′UTRs in which the *Cb-tra-2* or *GLI* putative regulatory elements were deleted [*lacZ::Cb-tra-2(–38)3′UTR* or *lacZ::GLI(–60)3′UTR*, respectively]. The transgenes were controlled by the inducible heat shock promoter (*hsp16–41*; Stringham *et al.*, 1992). The expression levels of these transgenes were compared with previously characterized transgenes that carried either the wild-type *Ce-tra-2* 3′UTR (*lacZ::Ce-tra-2(+)3′UTR*), or mutant *Ce-tra-2* 3′UTRs in which one TGE (*lacZ::Ce-tra-2(–32)3′UTR*) or both TGEs (*lacZ::Ce-tra-2(–60)-3′UTR*) were deleted (see Figure 3A and B and Table I; Goodwin *et al.*, 1997).

We found a dramatic difference between the transgenes with wild-type 3′UTRs as compared with transgenes with mutant 3′UTRs. For *lacZ::Cb-tra-2(+)3′UTR* and *lacZ::GLI(+)3′UTR*, only 10–11% of transgenic animals had β-gal staining in intestinal cells (Figure 3B and C and Table I). However, for *lacZ::Cb-tra-2(–38)3′UTR*, 59%, and for *lacZ::GLI(–60)3′UTR*, 51% of transgenic animals had intestinal β-gal staining (Figure 3E and F and Table I). For each experiment, we analyzed three independent lines that gave similar results. The total amount of β-gal activity was also measured using an *in vitro* enzyme assay (Table I). Similar to the *in vivo* analysis, transgenes with the wild-type 3′UTRs had less β-gal activity than transgenes with mutant 3′UTRs. RNase protection analysis indicated that the different transgenes produced similar amounts of RNA (Table I). Therefore, the difference in β-gal activity is not likely to be due to differences in production or stability of the RNA, but due to differences in translation. These results indicate that the *Cb-tra-2* and *GLI* 3′UTRs can repress translation of a reporter transgene in *C.elegans*, and that this inhibition requires regulatory sequences that have homology to the *Ce-tra-2* TGEs.

The Cb-tra-2 and GLI regulatory elements are functionally equivalent to the Ce-tra-2 TGEs

If the *Cb-tra-2* and *GLI* sequences are functionally equivalent to the *Ce-tra-2* TGEs then they should have similar properties. Mutations in the *laf-1* gene should disrupt the ability of the elements to repress translation (see Introduction; Goodwin *et al.*, 1993). In addition, the elements should regulate poly(A) tail lengths and bind DRF (see Introduction; Goodwin *et al.*, 1993).

The dependence of the regulation by the *Cb-tra-2* and *GLI* control elements upon *laf-1* activity was tested by asking whether *laf-1* mutations could affect the translation of *lacZ::Cb-tra-2(+)3′UTR* and *lacZ::GLI(+)3′UTR*. *In vivo* assays demonstrated a striking increase in β-gal expression in *laf-1(q267)/+* mutant animals carrying the *lacZ::Cb-tra-2(+)3′UTR* or *lacZ::GLI(+)3′UTR*; 58% and 23% of animals showed intestinal β-gal expression, respectively (Table I). *laf-1(q267)/+* did not alter the activity of *lacZ::Cb-tra-2(–38)3′UTR* or *lacZ::GLI(–60)-3′UTR* (Table I), indicating that the effect of the *laf-1* mutation is dependent upon the presence of the regulatory elements. The *laf-1* mutation did not alter the steady-state levels of reporter RNA (Table I). Therefore, *laf-1* mutations can disrupt the translational control by the *Cb-tra-2* and *GLI* regulatory elements. Conversely, *laf-1* mutations did not affect the 3′UTR regulation of the *C.elegans* heterochronic gene *lin-14* or the *C.elegans* sex-determination gene, *fem-3* (data not shown), which are both controlled by elements in the 3′UTRs (Ahringer and Kimble, 1991; Wightman *et al.*, 1993), further supporting the idea that translational control by *laf-1* is specific to TGE control.

The ability of the *Cb-tra-2* and the *GLI* elements to control poly(A) tail lengths was examined by PAT analysis [Poly(A) Test; see Materials and methods]. In these experiments, an oligo(dT) primer that contains a unique

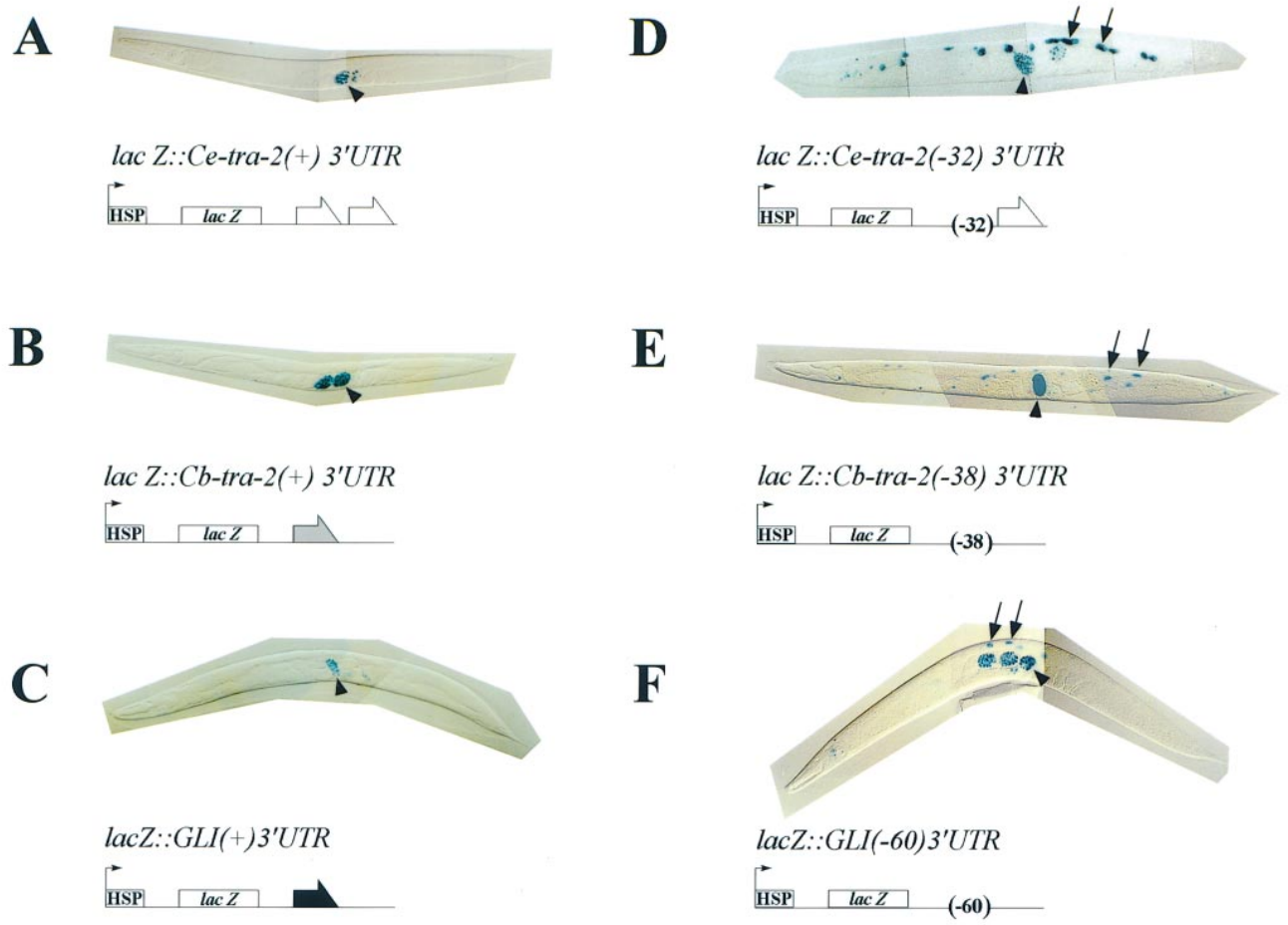


Fig. 3. *Caenorhabditis briggsae tra-2* and *GLI* regulatory elements can repress translation in *C.elegans*. Lateral views with anterior to the left; names and diagrams of particular transgenes are shown below each animal. The reporter *lac*Z gene is driven by the *C.elegans* heat shock promoter (*hsp16-41*) and is fused to the nuclear localization signal, such that β-gal staining is primarily nuclear. In *C.elegans* animals carrying the (**A**) *lacZ::Ce-tra-2(+) 3′UTR*, (**B**) *lacZ::Cb-tra-2(+) 3′UTR* or (**C**) *lacZ::GLI(+) 3′UTR* transgene, no β-gal activity is observed in the intestine, but embryos show strong β-gal staining (arrowheads). Wild-type *C.elegans* animals carrying the mutant transgenes: (**D**) *lacZ::Ce-tra-2(–32) 3′UTR*, (**E**) *lacZ::Cb-tra-2(–38) 3′UTR* or (**F**) *lacZ::GLI(–60) 3′UTR*. β-gal staining is not only observed in the embryos (arrowheads), but is also observed in the intestine [arrows; 13, 9 and 4 large intestinal nuclei are visible in (D), (E) and (F), respectively] and in the nuclei of several unidentified cells.

5′ anchor sequence was used to reverse transcribe mRNA into cDNA. Subsequently, an anchor and a gene specific primer were used to amplify the 3′ end of the cDNA. The poly(A) tail lengths of transgenes with regulatory elements were compared with those of transgenes in which the elements were deleted. Specifically, the poly(A) tail lengths of RNA from *lacZ::Cb-tra-2(+)3′UTR* and *lacZ::GLI(+) 3′UTR* were compared with RNA from *lacZ::Cb-tra-2(–38)3′UTR* and *lacZ::GLI(–60)3′UTR*. We found that *lacZ::Cb-tra-2(+)3′UTR* and *lacZ::GLI(+)3′UTR* mRNA had between 50 and 100 fewer A residues than *lacZ:: Cb-tra-2(–38)3′UTR* and *lacZ::GLI(–60)3′UTR* mRNA (Figure 4A; compare the arrowhead in lanes 2, 4 and 6 with the bracket in lanes 3, 5 and 7), indicating that the *Cb-tra-2* and *GLI* regulatory elements can control the length of the poly(A) tail. Occasionally, other PCR products of varying sizes were detected. However, these bands were not reproducible and probably do not represent true products.

The ability of DRF to bind *Cb-tra-2* and *GLI* regulatory elements was determined by gel retardation analysis. We first assayed for the presence of a factor in *C.elegans* that bound RNA containing the *Cb-tra-2* or *GLI* element. Incubation of a crude *C.elegans* protein extract with labeled small RNA that contained the *Cb-tra-2* (EJ-19) element resulted in the appearance of a slower-moving complex (Figure 5B; arrow). To remove non-specific binding, the reaction contained a large excess of mutant *Ce-tra-2* 3′UTR in which the TGEs were deleted. In addition, wild-type *Cb-tra-2* 3′UTR formed a complex, while a mutant *Cb-tra-2* 3′UTR in which the regulatory element was removed did not (Figure 5E), indicating that the *Cb-tra-2* 3′UTR is sufficient for binding. In contrast, labeled small RNA containing the *GLI* element was not able to specifically bind a factor in *C.elegans*. However, the entire wild-type *GLI* 3′UTR did bind a factor (Figure 5C, arrow in lane 2). This binding was specific for the *GLI* element since a mutant *GLI* 3′UTR in which the regulatory element was deleted did not form a complex (Figure 5E). Thus, the *Cb-tra-2* and *GLI* elements can bind a factor in *C.elegans*.

Next, we asked whether the factor that bound the *Cb-tra-2* and *GLI* elements was DRF (Goodwin *et al.*, 1997). We added an excess of cold RNA that either contained or

Table I. The *C.briggsae tra-2* and *GLI* 3′UTRs can control translation of a reporter transgene in *C.elegans*

Genotype[a]	Transgene[b]		% animals with β-gal staining[c]	β-gal activity[d]	β-gal:Actin[e]
Wild-type	*lacZ::Ce-tra-2(+)3′UTR*	(*n* = 72)	7	0.03	0.46 ± 0.14
Wild-type	*lacZ::Ce-tra-2(–32)3′UTR*	(*n* = 123)	65	0.14	0.74 ± 0.16
Wild-type	*lacZ::Ce-tra-2(–60)3′UTR*	(*n* = 83)	59	0.10	0.32 ± 0.11
Wild-type	*lacZ::Cb-tra-2(+)3′UTR*	(*n* = 71)	10	0.03	0.56 ± 0.28
Wild-type	*lacZ::Cb-tra-2(–38)3′UTR*	(*n* = 127)	59	0.13	0.61 ± 0.33
laf-1(q267)/+	*lacZ::Cb-tra-2(+)3′UTR*	(*n* = 57)	58	n.d.	0.98 ± 0.02
laf-1(q267)/+	*lacZ::Cb-tra-2(–38)3′UTR*	(*n* = 31)	68	n.d	1.2 ± 0.12
Wild-type	*lacZ::GLI(+)3′UTR*	(*n* = 222)	11	0.03	0.84 ± 0.34
Wild-type	*lacZ::GLI(–60)3′UTR*	(*n* = 95)	51	0.15	0.39 ± 0.14
laf-1(q267)/+	*lacZ::GLI(+)3′UTR*	(*n* = 74)	23	n.d.	1.0 ± 0.34
laf-1(q267)/+	*lacZ::GLI(–60)3′UTR*	(*n* = 32)	38	n.d.	0.30 ± 0.16

[a]Wild-type adult animals were N2 hermaphrodites; *laf-1/+* animals were progeny from *laf-1*(*lf*)*/qC1*. 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]Seven different transgenes were constructed. All seven transgenes carry the *lacZ* coding region under control of the inducible heat shock promoter [*hsp16–41*; (Stringham *et al.*, 1992)]. *lacZ::Ce-tra-2(+) 3′UTR* has the wild-type *Ce-tra-2* 3′UTR which contains two TGEs; *lacZ::Ce-tra-2(–32) 3′UTR* has a mutant *Ce-tra-2* 3′UTR in which one TGE has been removed; *lacZ::Ce-tra-2(–60)3′UTR* has a mutant *Ce-tra-2* 3′UTR in which both TGEs have been deleted. *lacZ::Cb-tra-2(+) 3′UTR* has the wild-type *Cb-tra-2* 3′UTR, and *lacZ::Cb-tra-2(–38) 3′UTR* has a mutant *Cb-tra-2* 3′UTR in which the putative TGE has been removed. *lacZ::GLI(+) 3′UTR* has the wild-type *GLI* 3′UTR, and *lacZ::GLI(–60) 3′UTR* has a mutant *GLI* 3′UTR in which the putative TGE has been removed.

[c]Transgenic animals were scored as positive if blue precipitate was detectable in intestinal cells at 630× magnification; genetic evidence suggests that control by the *C.elegans tra-2* 3′UTR functions in intestinal cells (Doniach, 1986). Percentiles represent the percent of transgenic animals with blue precipitate in intestinal cells and also represent the values of one representative transgenic line. At least three lines were examined for each construct, which all gave similar results. *n* = total number of animals scored from at least four different experiments.

[d]Numbers represent β-gal activity present in crude lysates of adult worms. Adult transgenic animals were harvested, lysed and the total β-gal-activities measured. Since some transgenic lines carried extrachromosomal arrays, the β-gal activities were normalized for the percent transgenic animals produced by each line. Units are change of OD_{574} from CPRG hydrolysis per min per mg protein, and are mean values of at least three different experiments. Standard deviations are in parentheses.

[e]RNase protection analysis was used to measure the amount of transgenic RNA made from the different transgenes after a 2 h heat shock. As an internal control, mRNA from the *act-1* was measured. Shown is the mean ratio of the amount of protected fragment from the transgene to the amount of protected fragment from *act-1*. Total RNA from each adult transgenic line was extracted as described (see Materials and methods). Relative β-gal to actin RNA levels were normalized for the percent transgenic animals produced by each line.

did not contain the *Ce-tra-2* TGEs and assayed for loss of complex formation. We found that increasing molar amounts of a small RNA containing the *Ce-tra-2* TGEs (EBG-9) (Figure 5B, compare lane 2 with lanes 3 and 4) competed with a small RNA containing the *Cb-tra-2* element (EJ-19) for complex formation. However, a small mRNA in which the *tra-2* TGEs were deleted did not compete for binding (Figure 5B, lane 5). We found similar results with respect to the *GLI* element. Increasing molar amounts of RNA containing the entire *Ce-tra-2* 3′UTR but not mutant *Ce-tra-2* 3′UTR, carrying a 108 nt deletion that removed the TGEs plus some flanking sequence, competed with the *GLI* 3′UTR for complex formation (Figure 5C, compare lane 2 with lanes 3–5). A mutant *Ce-tra-2* 3′UTR with a 60 nt deletion that precisely deletes the TGEs also failed to compete for binding (Figure 5E). Therefore, *Cb-tra-2* and *GLI* regulatory elements can bind DRF and this binding is dependent upon the presence of the regulatory sequence.

In summary, the regulatory sequences of *Cb-tra-2* and *GLI* behave in a strikingly similar manner to the *C.elegans tra-2* TGE. They inhibit translation of a reporter transgene in a *laf-1* dependent manner. Furthermore, they regulate poly(A) tail lengths and bind DRF. We propose that these sequences are functional TGEs.

TGE control is present in C.briggsae

The fact that the *Cb-tra-2* TGE represses translation in *C.elegans* suggests that TGE control is present in *C.briggsae*. Toward this end, we asked whether the *Ce-tra-2* and *Cb-tra-2* TGEs could control the translation of reporter transgenes in *C.briggsae*. Four reporter constructs were made. All constructs encoded a fusion of *lacZ* with the Green Fluorescence Protein (GFP) and had either wild-type *Ce-tra-2* or *Cb-tra-2* 3′UTRs [*GFP::Ce-tra-2(+)3′UTR* and *GFP::Cb-tra-2(+)3′UTR*, respectively] or mutant 3′UTRs in which the TGEs were deleted [*GFP::Ce-tra-2(–60)3′UTR* and *GFP::Cb-tra-2(–38)-3′UTR*, respectively]. The transgenes were controlled by the *C.elegans* gut-specific *ges-1* promoter, which drives transcription in *C.briggsae* (Kennedy *et al.*, 1993).

As in *C.elegans*, the expression of the wild-type and mutant transgenes in *C.briggsae* differ dramatically. For *GFP::Ce-tra-2(+)* 3′UTR 0% and for *GFP::Cb-tra-2(+)3′UTR* 8% of transgenic animals had β-gal staining. However, 56% of *GFP::Ce-tra-2(–32)3′UTR* and 70% of *GFP::Cb-tra-2(–38)3′UTR* had β-gal staining (Table II). Similar results were obtained when total β-gal activity was measured using an *in vitro* enzyme assay (Table II). RNase protection analysis demonstrated that the different transgenes produced similar amounts of RNA (Table II). These results indicate that both the *Ce-tra-2* and *Cb-tra-2* TGEs can repress translation in *C.briggsae.*

If the mechanism by which the TGE inhibits translation in *C.briggsae* is similar to that in *C.elegans*, then the *Ce-tra-2* and *Cb-tra-2* TGEs should regulate the length of the poly(A) tail, and a factor should be present in *C.briggsae* that specifically associates with the TGEs.

The ability of the *Cb-tra-2* and *Ce-tra-2* TGEs to regulate poly(A) tail lengths in *C.briggsae* was examined

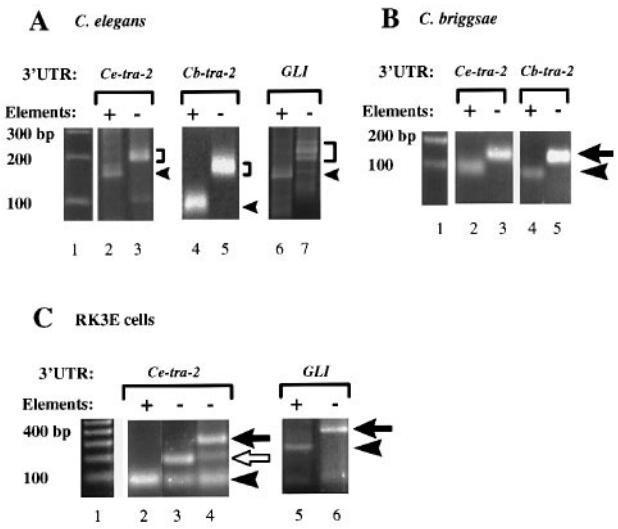


Fig. 4. The *C.briggsae tra-2* and *GLI* regulatory elements control poly(A) tail length. (**A**) 100 bp DNA ladder (lane 1). RT–PCR products of mRNAs from *C.elegans* animals transformed with either *lacZ::Ce-tra-2(+)3′UTR* (lane 2), *lacZ::Ce-tra-2(–60)3′UTR* (lane 3), *lacZ::Cb-tra-2(+)3′UTR* (lane 4), *lacZ::Cb-tra-2(–38)3′UTR* (lane 5), *lacZ::GLI(+)3′UTR* (lane 6) or *lacZ::GLI(–60)3′UTR* (lane 7) (see Materials and methods; PAT). RT–PCRs were loaded on a 2% agarose gel. Reporter mRNA transcripts that have no poly(A) tail would yield an RT–PCR product of 100 bp for *Ce-tra-2* and *GLI* 3′UTRs and an RT–PCR product of 63 bp for *Cb-tra-2* 3′UTR. Arrowhead indicates RT–PCR products from wild-type transgene mRNAs. Bracket indicates RT–PCR products from mutant transgene mRNAs. The RT–PCR products from transcripts with wild-type 3′UTRs (lanes 2, 4, and 6) generate a poly(A) tail length of ~50–70 A residues. The RT–PCR product from transcripts with mutant 3′UTRs (lanes 3, 5 and 7) had a poly(A) tail of ~100–150 A residues, indicating that the regulatory elements control poly(A) tail length in *C.elegans*. (**B**) 100 bp DNA ladder (lane 1). RT–PCR products of mRNAs from *C.briggsae* animals that carry either *GFP::Ce-tra-2(+)3′UTR* (lane 2), *GFP::Ce-tra-2(–60)3′UTR* (lane 3), *GFP::Cb-tra-2(+)3′UTR* (lane 4) or *GFP::Cb-tra-2(–38)3′UTR* (lane 5). If the mRNA was not adenylated, the expected size of the RT–PCR product would be 58 bp and 63 bp for *Ce-tra-2* and *Cb-tra-2*, respectively. RT–PCR products from transcripts with wild-type 3′UTRs (arrowhead) have a poly(A) tail of ~30–40 A residues (lanes 2 and 4). Similar to *C.elegans*, transgenes mRNAs with mutant 3′UTRs (arrow) have a poly(A) tail of ~100 A residues (lanes 3 and 5). (**C**) 100 bp DNA ladder (lane 1). RT–PCR products of mRNA reporter constructs that were transiently transfected into RK3E cells with either *luc::Ce-tra-2(+)3′UTR* (lane 2), mutant *luc::Ce-tra-2(–32)3′UTR* (lane 3), *luc::Ce-tra-2(–60)3′UTR* (lane 4), *luc::GLI(+)3′UTR* (lane 5) or *luc::GLI(–90)3′UTR* (lane 6). The expected PCR product of an mRNA with no poly(A) tail is 58 bp and 112 bp for *Ce-tra-2* and *GLI*, respectively. RT–PCR products from transcripts with wild-type *Ce-tra-2* and *GLI* 3′UTRs (arrowheads) have a poly(A) tail of ~30 and 180 A residues, respectively (lanes 2 and 5). RT–PCR products from transcripts with mutant *Ce-tra-2* and *GLI* 3′UTRs (arrows) have a poly(A) tail of ~130–230 and 280 A residues, respectively (lanes 3, 4 and 6). The open arrow indicates the RT–PCR product from the mutant transgene, *luc::Ce-tra-2(–32)3′UTR*, in which one TGE has been deleted. RT–PCR products from wild-type reporter transcripts that contain TGEs are indicated by plus signs and those from mutant reporter transcripts that do not contain TGEs are indicated by minus signs.

using the PAT assay (see Materials and methods). Similar to the results in *C.elegans*, transgenic RNA that contained wild-type 3′UTRs had ~50 less A residues than did transgenic RNA in which the TGE had been removed (Figure 4B; compare lanes 2 and 4 with lanes 3 and 5, respectively). Therefore, the *Cb-tra-2* and *Ce-tra-2* TGEs can regulate the lengths of poly(A) tails in *C.briggsae*.

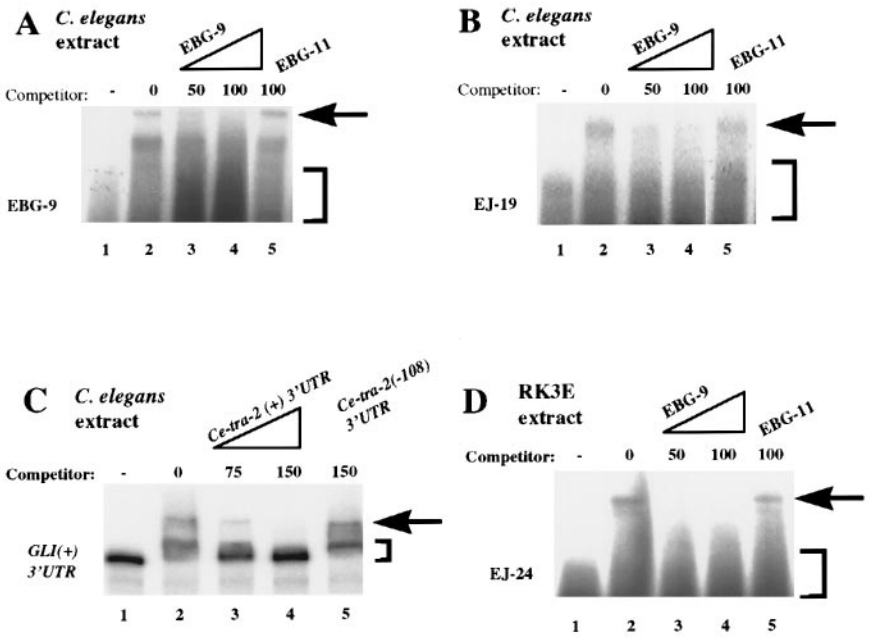
Using RNA gel shift analysis, we assayed crude *C.briggsae* worm extract for a factor that specifically bound to the *Ce-tra-2* and *Cb-tra-2* TGEs. Incubation of crude extract with RNA containing the *Ce-tra-2* (EBG-9) or *Cb-tra-2* (EJ-19) TGEs resulted in the appearance of a slower-moving complex, indicating that there is a factor in *C.briggsae* that binds to TGEs (Figure 5B, lane 2 and Figure 5E). Factor binding is dependent upon the presence of the TGEs, since excess molar amounts of cold EBG-9 or EJ-19 could compete for binding (Figure 5B, lanes 3 and 4 and Figure 5E). However, increasing amounts of a mutant *Ce-tra-2* 3′UTR RNA in which the *Ce-tra-2* TGE had been removed did not interfere with complex formation (Figure 5B, lane 5), indicating that the *Ce-tra-2* and *Cb-tra-2* TGEs are sufficient for binding of factor in *C.briggsae*. To verify these results, we radioactively labeled a variety of RNAs that either contained or did not contain the TGEs and assayed for complex formation. All RNAs that contained TGEs formed a complex, while RNAs in which TGEs had been removed did not form a complex (Figure 5E). Therefore, extracts of *C.briggsae* have a factor that binds specifically to TGEs. We propose that this factor is the *C.briggsae* homologue of DRF.

In summary, the fact that the *Ce-tra-2* and *Cb-tra-2* TGEs can regulate translation and poly(A) tail length and specifically bind a factor present in *C.briggsae* is consistent with the TGE control being present in *C.briggsae.*

TGE control is present in mammalian cells

We next asked whether *Ce-tra-2* and *GLI* TGEs could control translation of a reporter construct in mammalian cells. Translational control in a rat kidney fibroblast cell line (RK3E) that had been stably transfected with E1A was assayed by transient transfection of different reporter constructs. 3′UTRs that contained either *Ce-tra-2* or *GLI* 3′UTRs [*luc::Ce-tra-2(+)3′UTR* or *luc::GLI(+)3′UTR*, respectively] or mutant 3′UTRs in which the TGEs were deleted [*luc*::*Ce-tra-2(–60) 3′UTR* and *luc*::*GLI(–90) 3′UTR*, respectively] were subcloned into the mammalian reporter vector, pGL3 (Promega). A 90 nt deletion of the *GLI* 3′UTR was used in this experiment, since we were unable to clone the 60 nt deletion into the pGL3 vector. The pGL3 vector contains the reporter luciferase gene driven by the SV40 promoter. All experiments were co-transfected with a β-gal plasmid to correct for transfection efficiencies.

As shown in *C.elegans* and in *C.briggsae*, the expression of the wild-type and mutant reporter constructs in RK3E cells differ significantly. For *luc*::*Ce-tra-2(–60)3′UTR* and *luc*::*GLI(–90)3′UTR*, there was an ~3-fold increase in luciferase expression over the wild-type *luc*::*Ce-tra-2(+)3′UTR* and *luc*::*GLI(+)3′UTR* constructs, respectively (Figure 6). Interestingly, *luc::tra-2(–32)3′UTR* transgenes in which one TGE had been deleted showed an intermediate increase of ~2-fold as compared with the wild-type *luc::Ce-tra-2(+)3′UTR* and mutant *luc::Ce-tra-2(–90)3′UTR*, indicating that one TGE can partially regulate translation in RK3E cells. Previously, we had shown that a single TGE can partially control translation in *C.elegans* (Goodwin *et al.*, 1993), further indicating that the TGEs were behaving similarly in both organisms. RNase protection analysis indicates that the steady-state RNA levels of the reporter constructs are similar (see



E

Summary of DRF binding in:

	C. elegans	*C. briggsae*	RK3E	Hela
Ce tra-2(+) 3'UTR	+	+	+	+
Ce tra-2(-108) 3'UTR	-	-	-	-
Ce tra-2(-60) 3'UTR	-	-	-	n.d.
EBG-9	+	+	+	+
EBG-11	-	-	-	-
Cb tra-2(+) 3'UTR	+	+	n.d.	n.d.
Cb tra-2(-38) 3'UTR	-	-	n.d.	n.d.
EJ-19	+	+	n.d.	n.d.
GLI (+) 3'UTR	+	n.d.	+	+
GLI (-60) 3'UTR	-	n.d.	-	-
EJ-24	-	n.d.	+	+

Fig. 5. DRF binds to the *C.briggsae tra-2* and *GLI* regulatory elements and may be present in *C.briggsae* and mammalian extracts. (**A**) 20 fmol of ^{32}P-labeled *Ce-tra-2* TGEs (EBG-9) were incubated alone (lane 1) or with 5 μg of crude *C.elegans* adult extract (lane 2). Reactions were loaded on a 6% nondenaturing polyacrylamide gel. The gel was dried and autoradiographed. Slower-migrating bands are due to complex formation (arrow); the faster migrating bands indicate free probe (bracket). 50- and 100-fold molar excess of cold EBG-9 (lanes 3 and 4) competed for complex formation while 100-fold molar excess of an RNA in which the *Ce-tra-2* TGEs were removed (EBG-11) did not (lane 5), indicating that DRF binding requires the TGEs. (**B**) 20 fmol of ^{32}P-labeled RNA containing the *Cb-tra-2* regulatory element (EJ-19) were added alone (lane 1) or with 5 μg of crude *C.elegans* adult extract (lane 2). The slower migrating band (lane 2, arrow) is indicative of complex formation. A 50- or 100-fold molar excess of cold EBG-9 could compete for binding (lanes 3 and 4), whereas a 100-fold molar excess of EBG-11 did not compete, indicating that the *Cb-tra-2* TGE binds DRF. The faster migrating band is free probe (bracket). (**C**) 6 fmol of ^{32}P-labeled *GLI* 3′UTR were added alone (lane 1) or with 5 μg of *C.elegans* adult extract (lane 2). The retarded band indicates complex formation (arrow). A 75- and 150-fold molar excess of cold *Ce-tra-2(+)* 3′UTR could compete with *GLI* 3′UTR for complex formation, while 150-fold molar excess of cold *Ce-tra-2* 3′UTR RNA in which the TGEs were deleted did not, indicating that the *GLI* 3′UTR binds specifically to DRF. The faster-migrating band is free probe (bracket). (**D**) 20 fmol of ^{32}P-labeled RNA containing the *GLI* regulatory element (EJ-24) were added alone (lane 1) or with 5 μg of RK3E cell extract (lane 2). The slower-migrating band is indicative of complex formation (arrow). A 50- or 100-fold molar excess of cold EBG-9 could compete for factor binding (lanes 3 and 4), but a 100-fold molar excess of cold EBG-11 did not, indicating that the *GLI* and *Ce-tra-2* TGEs bind the same mammalian factor. We propose that this factor may be a homologue of DRF. The faster-migrating band is free probe (bracket). (**E**) Summary of binding experiments in which different RNAs were tested for their ability to bind factor in crude *C.elegans*, *C.briggsae*, RK3E or HeLa cells. Binding was assayed by two methods. First, binding was examined by labeling a particular RNA and directly measuring complex formation. Second, to test whether the *Ce-tra-2*, *Cb-tra-2* and *GLI* TGEs were binding the same factor, an excess of cold test RNA was tested for its ability to compete for binding of factor to the *Ce-tra-2* TGEs. Specificity of binding was determined by adding increasing amounts of RNAs that either did or did not contain the *Ce-tra-2* TGEs. In every case, RNAs containing TGEs were able to bind factor, but RNAs lacking TGEs could not. (Left) Names of RNAs (see Materials and methods for sequences). (Middle) Diagrams of RNAs. Open arrows represent *Ce-tra-2* TGEs, stippled arrows represent the *Cb-tra-2* TGE and black arrows represent the *GLI* TGE. The sizes of the deletions are indicated in brackets. (Right) The different RNAs were scored for the ability (plus) or inability (minus) to bind DRF.

Table II. *Ce-tra-2* and *Cb-tra-2* TGEs repress translation in *C.briggsae*

Transgene[a]		% animals with β-gal staining[b]	β-gal activity[c]	β-gal/*lag-1*[d]
GFP::Ce-tra-2(+)3′UTR	(*n* = 54)	0	0.04	0.78 ± 0.12
GFP::Ce-tra-2(–32)3′UTR	(*n* = 22)	56	0.12	0.61 ± 0.06
GFP::Cb-tra-2(+)3′UTR	(*n* = 38)	8	0.04	1.13 ± 0.08
GFP::Cb-tra-2(–38)3′UTR	(*n* = 62)	70	0.30	1.28 ± 0.33

[a]Four different transgenes were constructed. All four transgenes carry a fusion of the *lacZ*/GFP coding region under control of the *C.elegans ges-1* promoter. *GFP::Ce-tra-2(+) 3′UTR* has the wild-type *Ce-tra-2* 3′UTR which contains two direct repeats separated by a 4 nt spacer; *GFP::Ce-tra-2(–32)3′UTR* has a mutant *Ce-tra-2* 3′UTR in which one direct repeat plus the 4 nt spacer has been removed. *GFP::Cb-tra-2(+) 3′UTR* has the wild-type *Cb-tra-2* 3′UTR; *GFP::Cb-tra-2(–38)3′UTR* has a mutant *Cb-tra-2* 3′UTR in which the putative TTE plus some flanking sequences have been removed.

[b]In these experiments, L1 animals were scored as positive if blue precipitate was detectable in intestinal cells at 630× magnification. The *ges-1* promoter expressed the highest at L1 stage of development. Percentiles represent the values of one representative transgenic line. At least two lines were examined for each construct, which gave similar results. Since some transgenic lines carried extrachromosomal arrays and only a fraction of animals carried the array, the β-gal activities were normalized for the percent transgenic animals produced by each line. *n* = total number of animals scored.

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

[d]RNase protection analysis was used to measure the amount of transgenic RNA made from the different transgenes. As an internal control, mRNA from the *C.briggsae lag-1* gene was measured. Shown is the ratio of the amount of protected fragment from the transgene to the amount of protected fragment from *lag-1*. Relative β-gal to *lag-1* RNA levels were normalized for the percent transgenic animals produced by each line.

Figure 6 legend). Since the luciferase activity is linear, there is a direct correlation between luciferase activities and RNA levels. Therefore, the luciferase activities were corrected for differences in reporter RNA levels. In addition, luciferase activities were corrected for transfection efficiency by normalizing the activity to the expression of the co-transfected *lacZ* plasmid. These results demonstrate that TGE control is present in at least one mammalian cell line.

To investigate whether the translational control in RK3E cells may be TGE regulation, we analyzed poly(A) tail lengths of reporter RNAs and tested whether a factor in RK3E and HeLa extract can specifically bind to the *Ce-tra-2* and *GLI* TGEs. Using the PAT analysis, we found that the mutant transgenes had a longer poly(A) tail than the wild-type transgenes (Figure 4C, compare lanes 2 and 5 with lanes 3, 4 and 6). Interestingly, the transgene with the mutant *Ce-tra-2* 3′UTR that carries one TGE had an intermediate poly(A) tail length as compared with the transgenes with the *Ce-tra-2* wild-type 3′UTR or a mutant 3′UTR in which both TGEs were deleted (Figure 4C, lane 3, open arrow). This intermediate length correlates remarkably well with the observation that a single TGE can partially regulate translation (see above: Goodwin *et al.*, 1993). Therefore *Ce-tra-2* and *GLI* TGEs can control poly(A) tail lengths in RK3E cells.

Using RNA gel shift analysis, we found that small RNAs that code for the *Ce-tra-2* (EBG-9) and *GLI* (EJ-24) TGEs bind to a factor in RK3E and HeLa cell extracts (Figure 5D, lane 2 arrow and Figure 5E), and that an excess of cold *Ce-tra-2* TGEs (EBG-9), but not the mutant *Ce-tra-2* 3′UTR in which the TGEs were deleted, competed with labeled probe for binding (Figure 5D, lanes 3–5). In addition, radioactively labeled wild-type *Ce-tra-2* and *GLI* 3′UTRs bound specifically to a factor in RK3E and HeLa cell extract, whereas the mutant 3′UTRs in which the TGEs were deleted did not (Figure 5E). This suggests that a factor in RK3E and HeLa cell extracts binds specifically to the TGEs and that both the *Ce-tra-2* and *GLI* TGEs are sufficient for binding. We propose that this factor is the mammalian homologue of DRF.

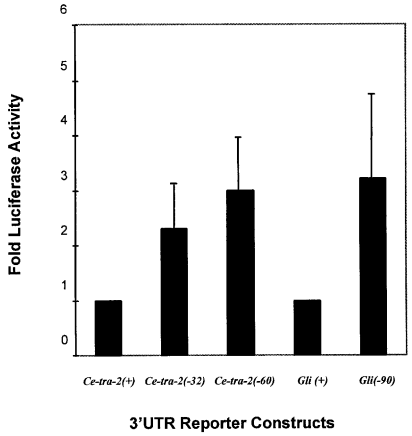


Fig. 6. The *C.elegans tra-2* and *GLI* TGEs control translation in RK3E cells. The reporter constructs *luc::Ce-tra-2(+)3′UTR, luc::Ce-tra-2(–32)3′UTR, luc::Ce-tra-2(–60)3′UTR, luc::GLI(+)3′UTR* or *luc::GLI(–90)3′UTR* were transiently transfected into RK3E cells (see Materials and methods). Shown are the relative fold increases in luciferase activities of the mutant constructs over the wild-type constructs (*n* = three experiments). The activities of the wild-type *luc::Ce-tra-2(+)3′UTR* and *luc::GLI(+)3′UTR* transgenes were set at 1. To account for differences in transfection efficiencies, luciferase activities were normalized to expression of an internal *lacZ* expression plasmid. Since the luciferase activities are linear, the luciferase activities were normalized to reporter RNA levels. The relative luciferase RNA to β-gal RNA levels were determined by RNase protection assays. The relative RNA levels with respect to *luc::Ce-tra-2(+)3′UTR* and *luc::GLI(+)3′UTR* which were set at an arbitrary value of 1 are: *luc::Ce-tra-2(–32)3′UTR*= 0.88 ± 0.15; *luc::Ce-tra-2(–60)3′UTR* = 0.69 ± 0.22; *luc::GLI(–90)3′UTR* = 0.83 ± 0.14.

Interestingly, the *GLI* TGE is sufficient for binding in RK3E cell extracts but it is not sufficient in *C.elegans* extracts (see above). The fact that the *GLI* TGE is sufficient for binding in RK3E cells suggests that this element has most of the sequences required for DRF binding. It is possible that the evolutionarily distant *C.elegans* DRF

Table III. Disruption of TGE control in *GLI* leads to increased foci formation

Overexpression construct[a]	Number of foci formed[b]
Control no construct	0, 0, 0, 0
Control antisense *GLI* cDNA	0, 0, 0, 0
Wild-type *GLI* cDNA (with TGE)	7, 10, 17, 14
Mutant *GLI* cDNA (without TGE)	47, 53, 30, 27

[a]*GLI* protein was overexpressed from either a control antisense *GLI* cDNA, wild-type *GLI* cDNA or a mutant *GLI* cDNA in which the TGE was deleted (see Materials and methods). Expression of these cDNAs was driven by a Moloney-Murine Leukemia virus long terminal repeat. Expression plasmids containing the wild-type or mutant cDNA were transfected into RK3E cells which had been stably transfected with E1A. Cells were transfected by lipofectamine and incubated at 37°C until foci formed.
[b]Numbers represent individual experiments.

binds less strongly to the *GLI* TGE as compared with the *Ce-tra-2* and *Cb-tra-2* TGEs, and that flanking 3′UTR sequences help stabilize a structure required for DRF recognition. Alternatively, the isolated *GLI* TGE may lack other 3′UTR sequences required for binding by *C.elegans* DRF.

If the TGE control regulates *GLI* expression in mammalian cells *in vivo*, then loss of regulation should enhance *GLI* activity. We tested whether deletion of the TGE in the *GLI* 3′UTR could lead to an increase in transformation potential of RK3E cells. Over expression of *GLI* in RK3E cells leads to foci formation and can form tumors in nude mice (Ruppert *et al.*, 1991). We have overexpressed in RK3E cells a wild-type *GLI* cDNA or a mutant *GLI* cDNA which lacks the TGE and asked whether removal of the TGE resulted in increased foci formation. RK3E cells which over-expressed the mutant *GLI* cDNA formed ~2- to 6-fold more foci than cells which over-expressed the wild-type *GLI* cDNA (Table III). This result strongly supports the hypothesis that *GLI in vivo* is translationally regulated by TGE control.

Discussion

The *C.elegans* sex determining gene, *tra-2*, is translationally regulated by TGEs located in its 3′UTR (Goodwin *et al.*, 1993). In this paper, we demonstrate that two genes, the *C.briggsae tra-2* gene and the human oncogene *GLI*, are translationally regulated *in vivo* and that this may be occurring via TGE control. Our data suggest that TGE regulation is present in mammals as well as nematodes, indicating that TGE control may be a widespread mechanism for regulating gene activity.

Several lines of evidence support the conclusion that *Cb-tra-2* and *GLI* 3′UTRs contain TGEs. The *Cb-tra-2* and *GLI* TGEs inhibit translation in *C.elegans*, and this repression is dependent upon *laf-1* activity, a known regulator of TGE control. Also, in *C.elegans*, the *Cb-tra-2* and *GLI* TGEs control the length of the poly(A) tail and bind DRF, as do the *Ce-tra-2* TGEs. DRF binding is specific to the TGEs since the *fem-3* 3′UTR is not able to bind DRF (Goodwin *et al.*, 1993).

In this paper, we find that the *Ce-tra-2* TGEs can regulate translation not only in *C.elegans* but also in *C.briggsae* and mammalian cells. Moreover, in these organisms, the *Ce-tra-2* TGEs also regulate the length of the poly(A) tail and specifically bind a factor. It is possible that these factors are the homologues of DRF. These findings are consistent with TGE regulation being a highly conserved mechanism for controlling gene expression.

Four lines of evidence indicate that *Cb-tra-2* and *GLI* are translationally regulated by TGE control *in vivo*. First, similarly to the *Ce-tra-2* TGEs, the *Cb-tra-2* and *GLI* TGEs can repress translation of reporter constructs in *C.briggsae* and in mammalian cells, respectively. Second, as is the case with the *Ce-tra-2* TGEs, the *C.briggsae* and *GLI* TGEs regulate the length of poly(A) tails in their respective organisms. Third, deletion of the *GLI* TGEs increases the ability of *GLI* to transform cells. Fourth, the *Cb-tra-2* and *GLI* TGEs bind specifically to the same factor in *C.briggsae* and mammalian extracts that binds the *Ce-tra-2* TGEs. Since DRF has not been cloned from either *C.elegans* or mammals, we cannot exclude the possibility that the mechanism that functions in *C.elegans* is different from that in mammals.

Comparison of the different TGEs reveals conserved sequences that may be crucial for control. The CUCA 'spacer' is conserved, suggesting that it may be functionally significant (Figure 2). In addition, a pyrimidine-rich sequence is conserved. Furthermore, the *Cb-tra-2* and *GLI* share an additional pyrimidine-rich sequence (UUUCU). The *Ce-tra-2* TGEs also have a second pyrimidine-rich element (UAUCU) in which four out of five nucleotides are identical to the UUUCU element, suggesting that it may be functionally similar (see Figure 2). Alignment of the different elements reveals a possible consensus sequence for a TGE that contains the CUCA motif and the two pyrimidine-rich regions (Figure 2B). Presently, it is unclear whether the distance or sequences that separate the conserved regions is important for control. It is possible that these apparently non-conserved regions are necessary for a secondary structure that is required for translational repression.

Translational repression by the *Ce-tra-2* 3′UTR requires two TGEs arranged as a direct repeat, but regulation by the *Cb-tra-2* and *GLI* 3′UTRs requires a single TGE. Of the different TGEs, the two *Ce-tra-2* TGEs are the poorest match with the consensus (Figure 2). This may indicate that the *Ce-tra-2* TGEs are weak regulatory elements and therefore two are required for full regulation. The fact that full regulation by the *Ce-tra-2* 3′UTR requires two TGEs, but by the *Cb-tra-2* or *GLI* 3′UTRs requires only a single TGE, suggests that the *Ce-tra-2* direct repeat is the more recently derived element. It is possible that the *Ce-tra-2* direct repeat evolved from a duplication event. Subsequently, mutations occurred that resulted in both elements becoming essential for control. Gene conversion would have assured that both TGEs maintained the same sequence. Therefore, the *Cb-tra-2* and *GLI* elements may be more similar to the ancestral TGE and more typical of other TGEs.

Regulation of tra-2 activity in C.elegans and C.briggsae is conserved

In *C.elegans*, development of both hermaphrodites and males depends upon negative regulation of *tra-2*. Development of XO animals requires TGE control and the *her-1* gene (Hodgkin, 1990; Goodwin *et al.*, 1993). HER-1 is

predicted to be a secreted protein that is thought to inhibit *tra-2* activity by binding to TRA-2A extracellular domain (Kuwabara and Kimble, 1992; Perry *et al.*, 1993). Hermaphrodite spermatogenesis requires three different regulatory mechanisms: translational control by TGEs, an apparent post-translational regulation of *tra-2* identified by the *tra-2(mx)* mutations (P.Kuwabara, P.Okkema and J.Kimble, unpublished) and repression by the *fog-2* gene (Schedl and Kimble, 1988). The *tra-2(mx)* mutations are missense mutations in a small region of TRA-2A which cause XX animals to develop as females (P.Kuwabara, P.Okkema and J.Kimble, unpublished).

Comparison of the cDNA sequences of *Ce-tra-2* and *Cb-tra-2* indicates that the regions of *tra-2* required for proper regulation are conserved between the two species (Kuwabara, 1996a; this paper). *C.briggsae* is a hermaphrodite/male species that diverged from *C.elegans* between 20 and 50 million years ago (Kennedy *et al.*, 1993). The TGE control, the HER-1 binding site and the *tra-2(mx)* region of the protein are present in *C.briggsae* (Kuwabara, 1996a; this paper).

The ability of an essentially female animal to produce sperm was one of the critical events for evolution of a hermaphrodite/male species from a female/male species. Hermaphroditism may have required the evolution of both the mx and TGE control. Alternatively, the evolution of only one of the controls may have resulted in the hermaphrodite sex.

Translational control of GLI expression

Little is known about the regulation of *GLI* expression. As discussed previously, *GLI* is a member of a gene family that includes the human genes *GLI2* and *GLI3*, the *Drosophila* segment polarity gene *ci* and the *C.elegans* sex-determining gene *tra-1* (Kinzler *et al.*, 1988; Ruppert *et al.*, 1988; Orenic *et al.*, 1990; Zarkower and Hodgkin, 1992). These genes encode proteins that are highly similar in their DNA binding domains but share little homology outside this region (Kinzler *et al.*, 1988). *GLI* was originally identified by its amplification in certain glioblastomas (Kinzler *et al.*, 1987). *GLI*, in cooperation with E1A protein, can transform rat kidney fibroblast cells and cause tumor growth in nude mice (Ruppert *et al.*, 1991). Presently, it is unclear whether it is the increased expression or misexpression of *GLI* that leads to carcinogenesis.

Here, we demonstrate that *GLI* is translationally controlled, and that this regulation may be important in suppressing tumorigenesis. The translation of *GLI* is regulated by the TGE control. Presently, it is unknown how this regulation affects the developmental expression of *GLI*. The TGE control may act in all cells in which *GLI* is transcribed. Alternatively, the TGE control may regulate the tissue or temporal pattern of *GLI* activity to repress translation in a subset of cells that transcribe *GLI*.

The *C.elegans* homologue of *GLI*, *tra-1*, contains a TGE-like sequence. Recent work indicates that *tra-1* is also regulated by the TGE control (E.Jan and E.B.Goodwin, unpublished results). Perhaps the common ancestral gene of *GLI* and *tra-1* was regulated by the TGE control, or the two genes could have independently obtained the TGE regulation during evolution.

Interestingly, *ci* is also regulated at the post-transcriptional level (Motzny and Holmgren, 1995). However, it is not known if this occurs by controlling translation or protein stability. If *ci* is translationally regulated, it is unlikely to be by the TGE control, since the *ci* 3′UTR does not appear to contain a TGE and is not capable of repressing translation of a reporter transgene in *C.elegans* (E.Jan and E.B.Goodwin, unpublished results).

Regulation of translation by elements in the 3′UTR is important for controlling gene activity in a variety of organisms (for review see Wickens *et al.*, 1996). To date, there is only limited information on how conserved different 3′UTR controls are. One example of 3′UTR control that is functionally conserved is regulation by cytoplasmic polyadenylation elements (CPEs). CPEs are present in many mammalian and *Xenopus* transcripts and control translation by regulating poly(A) tail lengths (Verrotti *et al.*, 1996).

Previous work has suggested that the translation of the *Drosophila* gene, *hunchback* (*hb*) and the *C.elegans* gene, *glp-1*, may be controlled by similar mechanisms (Evans *et al.*, 1994). The 3′UTR of *hb* contains a nanos response element (NRE) that is necessary to repress *hb* translation in the posterior of the *Drosophila* embryo (Dahanukar and Wharton, 1996; Smibert *et al.*, 1996). The region of the *glp-1* 3′UTR required to repress *glp-1* translation in the posterior blastomeres of the four-cell embryo contains a sequence with similarity to the NREs (Evans *et al.*, 1994). However, it has not been established whether the *glp-1* element is functionally equivalent to NREs.

We have demonstrated that TGE control may be a conserved process that is present in nematodes and mammals. This range of conservation indicates that TGE regulation is quite old and functionally important. It is possible that TGE control was present before the split of vertebrates and invertebrates, or it could have evolved several times. In addition, we have identified two genes, *Cb-tra-2* and *GLI*, whose translation is governed by TGEs. These findings suggest that TGE control is a general mechanism for regulating gene expression and that more genes controlled in this fashion may exist.

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 *C.elegans* mutant alleles were used in this study: LGIII, *laf-1(q267)* and the balancer *qC1*. *qC1* suppresses recombination over much of chromosome III.

Construction of transgene reporter constructs

All transgenes used to investigate translational control in *C.elegans* were derived from the same parent vector, pPC16.41 (a kind gift of Dr Peter Candido). This vector contains the *C.elegans* inducible heat-shock promoter, *hsp16–41*, the *lacZ* coding sequence and a polylinker (Stringham *et al.*, 1992). To construct the 3′UTR reporter transgenes, 3′UTRs were PCR amplified and inserted into restriction sites in the polylinker. The construction of pBG2 [*lacZ::Ce-tra-2(+)3′UTR*], pBG3 [*lacZ::Ce-tra-2(–32)3′UTR*] and pBG4 [*lacZ::Ce-tra-2(–60)3′UTR*] are described in Goodwin *et al.* (1997). For pBG5 [*lacZ::Cb-tra-2(+)3′UTR*], the *C.briggsae tra-2* 3′UTR was PCR amplified from *C.briggsae* genomic DNA using primers EBG-40 and EBG-42 (see below for sequences). For pBG6 [*lacZ::GLI (+)3′UTR*], the human *GLI* 3′UTR was PCR amplified from HeLa genomic DNA using primers EBG-52 and EBG-53. The resulting PCR fragments were subcloned into *Stu*I and *Apa*I sites of pPC16.41. pBG7 [*lacZ::Cb-tra-2(–38)3′UTR*] was constructed by digesting pBG5 with *Bgl*II and religating the resulting vector. pBG8 [*lacZ::GLI(–60)3′UTR*] was constructed by amplifying

pBG6 using primers EJ-12 and EBG-53, and the resulting PCR product was subcloned into *Spe*I and *Apa*I sites of pPC16.41.

Transgenes for analyzing 3′UTR control in *C.briggsae* were constructed using the parent vector, pSG1 (a kind gift of Steve Gendreau and Dr Joel Rothman). pSG1 contains the *C.elegans* gut-specific *ges-1* promoter, encodes a GFP–*lacZ* fusion protein, and the *C.elegans unc-54* 3′UTR. For pBG9 [*GFP::Ce-tra-2(+)3′UTR*], pBG10 [*GFP::Ce-tra-2(–32)3′UTR*], pBG11 [*GFP::Cb-tra-2(+)3′UTR*] and pBG12 [*GFP::Cb-tra-2(–38)3′UTR*], pBG1, pBG2, pBG6 and pBG7, respectively, were digested with *Bss*hII and *Apa*I, and the resulting fragments were subcloned into the same sites of pSG1. pBG9 and pBG10 were kindly provided by Cindy Motzny.

Reporter constructs to assay 3′UTR regulation in mammalian cells were constructed using the mammalian vector, pGL3 Promoter Vector (Promega). pGL3 contains the SV40 promoter, the luciferase gene and the SV40 poly(A) signal. For pBG13 [*luc::Ce-tra-2(+)3′UTR*], pBG14 [*luc::Ce-tra-2(–60)3′UTR*] and pBG15 [*luc::Ce-tra-2(–60)3′UTR*], the *C.elegans tra-2* 3′UTRs were PCR amplified from pBG2, pBG3 and pBG4, respectively, using primers EJ-4 and EBG-21. For pBG16 [*luc::GLI(+)3′UTR*], *GLI* 3′UTR was PCR amplified from pBG6 using primers EJ-23 and EBG-21. For pBG17 [*luc::GLI(–90)3′UTR*], a portion of the *GLI* 3′UTR was PCR amplified from pBG6 using primers EJ-22 and EJ-21. The resulting PCR fragments were subcloned into *Xba*I and *Bam*HI sites of pGL3.

Transgenic assays

Transgenic *C.elegans* and *C.briggsae* animals were generated using standard methods (Mello *et al.*, 1991). For *C.elegans*, the injection solution contained either 25 or 50 ng/μl of test plasmid and 200 ng/μl of plasmid pRF4, which contains the dominant *rol-6* marker. For *C.briggsae*, the injection solution contained 125 ng/μl of test plasmid and 75 ng/μl of RF46. Expression of β-gal was assayed as described (Fire, 1992).

Transfection and luciferase assay

RK3E cells (ATCC CCL2) were maintained in minimal essential medium (MEM, Gibco-BRL) supplemented with 10% heat-inactivated FBS, penicillin (100 units/ml) and streptomycin (100 μg/ml) in 5% CO_2 at 37°C.

Cells were plated at 4×10^5 cells per 60 mm tissue culture dish. On the following day, a total of 4 μg DNA was used to transfect the cells in each experiment; 2000 ng of the reporter constructs, 500 ng of transfection efficiency construct and 1500 ng of pBluescript plasmid DNA. Transfection, luciferase and β-galactosidase activities were performed by the manufacturer's protocol (Promega) with minor modifications.

Transformation and foci formation assays

Transformation assays were performed using the LTR-2 expression vector (Ruppert *et al.*, 1991). The LTR-2 vector drives the expression of wild-type and mutant *GLI* cDNAs using the Moloney-Murine Leukemia virus long terminal repeat. The LTR-2 vector containing the wild-type *GLI* cDNA is described elsewhere (Ruppert *et al.*, 1991). The LTR-2 vector carrying the mutant *GLI* cDNA in which the TGE has been deleted was constructed as follows. A 5′ PCR fragment of the *GLI* cDNA was PCR amplified using EJ-14 and EJ-15 from a pBluescript vector containing the *GLI* cDNA. A 3′ PCR fragment of the *GLI* cDNA was PCR amplified using EJ-16 and EJ-17 from the same construct. The two 5′ and 3′ fragments were then cloned into the LTR-2 vector, subsequently producing a mutant *GLI* cDNA which introduces a 60 nt deletion of the 3′UTR.

Transformations were carried out using the manufacturer's protocol (Promega) with minor modifications. To count foci, cells were fixed with L-glutaraldehyde and stained with Hematoxylin reagent 2–4 weeks after transfection.

β-galactosidase assays

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

RNase protection assays

RNA was isolated by the method of Chomczynski and Saachi (1987). RNase protection assays were performed using an Ambion HybRPA kit, a modification of the method of Lee and Costlow (1987). The β-gal-^{32}P probe was made from pBG18 linearized with *Hin*dIII using T7 polymerase. pBG18 was constructed by digesting pPC16.41 with *Hin*dIII and *Hpa*I, and subcloning the resulting fragment into the *Hin*dIII and *Sma*I of KS(+) pBluescript. *Caenorhabditis elegans act-1* RNA probe was synthesized from an *act-1*-specific clone linearized with *Eco*RI (kindly provided by M.Krause) using T3 RNA polymerase. The reactions were run on a 5% denaturing urea-polyacrylamide gel. The gels were dried and the signals were quantified using a phosphoimager (FUJIX BAS 2000). *Caenorhabditis briggsae lag-1* RNA probe was synthesized from a *C.briggsae lag-1*-specific clone (kindly provided by V.Kodyiani and J.Kimble) linearized with *Nhe*I using T7 RNA polymerase.

For mammalian cells, a luciferase RNA probe was synthesized from a luciferase- specific clone (kindly provided by S.Terhune and L.Laimins).

RNA gel shift analysis

RNA gel shifts were performed as described (Goodwin *et al.*, 1993). ^{32}P-labeled and unlabeled RNA probes containing the different 3′UTRs were produced by standard methods. The different full length and mutant 3′UTRs were subcloned into KSII(+) pBluescript vector. 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-24, EBG-11) 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}.

Poly(A) tail assays

The poly(A) tail lengths were measured by the PAT analysis (Salles and Strickland, 1995). RNA was isolated as described above. For each experiment, cDNA was reverse-transcribed using RACE-1 from total RNA. For each experiment, one round of PCR using RACE-1 and a primer specific to the coding region of the reporter gene was performed followed by a nested PCR using RACE-1 and a 3′UTR specific primer. For *lacZ* and GFP reporter constructs, the first PCR used the primers RACE-1 and EBG-62. For luciferase transgenes, the first PCR products were amplified using RACE-1 and EJ-37. For constructs containing the *Ce-tra-2* 3′UTR, the second PCR reaction was performed using the primers, RACE-1 and EBG-84. For transgenes containing the *Cb-tra-2*, the PCR products were re-amplified using RACE-1 and EJ-18, and for transgenes with the *GLI* 3′UTRs, the PCR products were re-amplified using RACE-1 and EJ-22.

Primer sequences

EBG-9: 5′-TGGACGATTAGATATGAGATGATAAGAAATTAAATA-TGAGTAGATATGAGTAGATAAGAAATTAAATAATGAAATGGAA-ATTGTCGCCCTATAGTGAGTCGTATTA-3′

EBG-11: 5′- TGGACGATTATGAAATGGAAATTGTACAAATAATA-GAAACGAAAATGAGTAAGAAATGAAATTTTGGAACCAAATTC-TCGCCCTATAGTGAGTCGTATTA-3′

EBG-21: 5′-AAATTTTATAGATCTTTTATTAACAAGAAAACAAAA-3′

EBG-40: 5′-CTATAGGCCTTAGAATGCTCATTTCCACAGTTTT-3′

EBG-42: 5′-TCAGGGGCCCACAAGACAATAAATTTATTAAGAA-GTG-3′

EBG-52: 5′-CTATAGGCCTAAAGAGTAGGGAATCTC-3′

EBG-53: 5′-TCAGGGCCCCTGATGCAGTTCCTTTATTAT-3′

EBG-62: 5′-AGTATCGGCGGAATTCCAACT-3′

EBG-84: 5′-ATCGTCCACTCGACCTCAACTTGTAAT-3′

EJ-4: 5′-TTTATTTCTAGAAATGTCTGTTTCCTTTTTCAG-3′

EJ-12: 5′-TCAACTAGTAAAAATTGGGGGAGCTGCAG-3′

EJ-14: 5′-CCATGATCAGCGGGGCAG-3′

EJ-15: 5′-CCCC AATTTTTCTTAGGCACTAGAGTTGAGGAA-3′

EJ-16: 5′-TCTAGTGCCTAAGAAAAATTGGGGGAGCTGCA-3′

EJ-17: 5′-AGAACTAGTGGATCCCCCG-3′

EJ-18: 5′-CTTGTAATTAAATATGAATTCC-3′

EJ-19: 5′-TGTGTTCAGAAAACTAGGCAGGAAAGTAGGAAAGT-GAGATCTGTTAATCGCCCTATAGTGAGTCGTATTA-3′

EJ-21: 5′-GCATGGATCCCTGATGCAGTTCCTTTATTAT-3′

EJ-22: 5′-TCAACTAGTCAGGGATGGGAGGTATGG-3′

EJ-24: 5′-GGAAGGATAGAAACCCCTTAGGAAATGCGATCTGTG-ATGGATGAGATTCCCTCGCCCTATAGTGAGTCGTATTA-3′

RACE-1: 5-GCGAGCTCCGCGGCCGCGTTTTTTTTTTTT-3′

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