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
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
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(+)/H11032 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 (hsp106-41; 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(+)/H11032 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(+)/H11032 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(+)/H11032 3'UTR and lacZ::GLI(+) 3'UTR. In vivo assays demonstrated a striking increase in β-gal expression in lacZ::lafl(q267)+/ H11032 mutant animals carrying the lacZ::Cb-tra-2(+)/H11032 3'UTR or lacZ::GLI(+) 3'UTR; 58% and 23% of animals showed intestinal β-gal expression, respectively (Table I). lacZ::lafl(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 sequence that is complementary to a poly(A) rich sequence in the 3'UTR of the reporter transgene was used to detect poly(A) tail lengths. PAT analysis showed that the Cb-tra-2 and GLI 3'UTRs contain sequences with homology to the Ce-tra-2 TGEs. The Cb-tra-2 and GLI 3'UTRs contain a single regulatory element.
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 lacZ 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-23′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 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 1. The C.briggsae tra-2 and GLI 3’UTRs can control translation of a reporter transgene in C.elegans

<table>
<thead>
<tr>
<th>Genotypea</th>
<th>Transgeneb</th>
<th>% animals with β-gal stainingc</th>
<th>β-gal activityd</th>
<th>β-gal:Actined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>lacZ::Ce-tra-2(+)/3’UTR (n = 72)</td>
<td>7</td>
<td>0.03</td>
<td>0.46 ± 0.14</td>
</tr>
<tr>
<td>Wild-type</td>
<td>lacZ::Ce-tra-2(-32)/3’UTR (n = 123)</td>
<td>65</td>
<td>0.14</td>
<td>0.74 ± 0.16</td>
</tr>
<tr>
<td>Wild-type</td>
<td>lacZ::Cb-tra-2(+)/3’UTR (n = 43)</td>
<td>59</td>
<td>0.10</td>
<td>0.32 ± 0.11</td>
</tr>
<tr>
<td>Wild-type</td>
<td>lacZ::Cb-tra-2(-32)/3’UTR (n = 71)</td>
<td>10</td>
<td>0.03</td>
<td>0.56 ± 0.28</td>
</tr>
<tr>
<td>Wild-type</td>
<td>lacZ::Cb-tra-2(-38)/3’UTR (n = 127)</td>
<td>59</td>
<td>0.13</td>
<td>0.61 ± 0.33</td>
</tr>
<tr>
<td>laf-1(q267)/+</td>
<td>lacZ::Cb-tra-2(+/+)/3’UTR (n = 57)</td>
<td>58</td>
<td>n.d.</td>
<td>0.98 ± 0.02</td>
</tr>
<tr>
<td>laf-1(q267)/+</td>
<td>lacZ::Cb-tra-2(-38)/3’UTR (n = 31)</td>
<td>68</td>
<td>n.d.</td>
<td>1.2 ± 0.12</td>
</tr>
<tr>
<td>Wild-type</td>
<td>lacZ::GLI(+)/3’UTR (n = 222)</td>
<td>11</td>
<td>0.03</td>
<td>0.84 ± 0.34</td>
</tr>
<tr>
<td>Wild-type</td>
<td>lacZ::GLI(-60)/3’UTR (n = 95)</td>
<td>51</td>
<td>0.15</td>
<td>0.39 ± 0.14</td>
</tr>
<tr>
<td>laf-1(q267)/+</td>
<td>lacZ::GLI(+)/3’UTR (n = 74)</td>
<td>23</td>
<td>n.d.</td>
<td>1.0 ± 0.34</td>
</tr>
<tr>
<td>laf-1(q267)/+</td>
<td>lacZ::GLI(-60)/3’UTR (n = 32)</td>
<td>38</td>
<td>n.d.</td>
<td>0.30 ± 0.16</td>
</tr>
</tbody>
</table>

aWild-type adult animals were N2 hermaphrodites; laf-1/+ animals were progeny from laf-1(00)/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.
bSeven different transgenes were constructed. All seven transgenes carry the lacZ coding region under control of the indehiscible 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.

Conservation of tra-2 3’UTR control

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 Cb-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 Cb-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 Cb-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 Cb-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 Cb-tra-2 TGEs to regulate poly(A) tail lengths in C.briggsae was examined...
Ce-tra-2 residues, respectively (lanes 2 and 5). RT–PCR products from reporter transcripts that do not contain TGEs are indicated by minus.

RT–PCR products from wild-type reporter transcripts, luc::Ce-tra-2(–32)3 mutant transgene, poly(A) tail of ~130–230 and 280 A residues, respectively (lanes 3, 4 and 5), indicating that the regulatory elements control poly(A) tail length in C.elegans. RT–PCR products of mRNA reporter constructs that contain TGEs formed a complex, while 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 Ce-tra-2 and Ch-tra-2 TGEs can regulate translation and poly(A) tail length and specifically bind a factor present in C.briggsae that 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), 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 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 Ct-tra-2 and Ce-tra-2 TGEs can regulate the lengths of poly(A) tails in C.briggsae.
Conservation of tra-2 3’ UTR control

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 32P-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% non-denaturing 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 32P-labeled RNA containing the Ch-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 Ch-tra-2 TGE binds DRF. The faster migrating band is free probe (bracket). (C) 6 fmol of 32P-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 32P-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 DRF 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, Ch-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 Ch-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.
and C.elegans 3′UTR contains two direct repeats separated by a 4 nt spacer; luc::Ce-tra-2(–32)3′UTR has a mutant Ce-tra-2 3′UTR in which the putative TTE plus some flanking sequences have been removed.

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.

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.

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.

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 C.elegans 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.

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

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

<table>
<thead>
<tr>
<th>Transgenea</th>
<th>% animals with β-gal stainingb</th>
<th>β-gal activityc</th>
<th>β-gal/lag-1d</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP::Ce-tra-2(+3)′UTR (n = 54)</td>
<td>0</td>
<td>0.04</td>
<td>0.78 ± 0.12</td>
</tr>
<tr>
<td>GFP::Ce-tra-2(–32)3′UTR (n = 22)</td>
<td>56</td>
<td>0.12</td>
<td>0.61 ± 0.06</td>
</tr>
<tr>
<td>GFP::Ch-tra-2(+3)′UTR (n = 38)</td>
<td>8</td>
<td>0.04</td>
<td>1.13 ± 0.08</td>
</tr>
<tr>
<td>GFP::Ch-tra-2(–38)3′UTR (n = 62)</td>
<td>70</td>
<td>0.30</td>
<td>1.28 ± 0.33</td>
</tr>
</tbody>
</table>

aFour 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::Ch-tra-2(+) 3′UTR has the wild-type Ch-tra-2 3′UTR; GFP::Ch-tra-2(–38)3′UTR has a mutant Ch-tra-2 3′UTR in which the putative TTE plus some flanking sequences have been removed.

bIn 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.

cNumbers 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.

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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 C.elegans 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.

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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

<table>
<thead>
<tr>
<th>Overexpression constructa</th>
<th>Number of foci formedb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control no construct</td>
<td>0, 0, 0, 0</td>
</tr>
<tr>
<td>Control antisense GLI cDNA</td>
<td>0, 0, 0, 0</td>
</tr>
<tr>
<td>Wild-type GLI cDNA (with TGE)</td>
<td>7, 10, 17, 14</td>
</tr>
<tr>
<td>Mutant GLI cDNA (without TGE)</td>
<td>47, 53, 30, 27</td>
</tr>
</tbody>
</table>

aGLI 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 EIA. Cells were transfected by lipofectamine and incubated at 37°C until foci formed.
bNumbers represent individual experiments.

Table III indicates that disruption of TGE control in GLI leads to increased foci formation. We tested whether deletion of the TGE in vivo leads to increased foci formation. Over expression of a wild-type GLI cDNA or a wild-type mutant GLI cDNA which over-expressed the mutant GLI cDNA in which the TGE was deleted resulted in increased foci formation. RK3E cells lead to increased 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 TGEs 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 CUCACU ‘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 CUCACU 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 polyclinker (Stringham et al., 1992). To construct the 3'UTR reporter transgenes, 3'UTRs were PCR amplified and inserted into restriction sites in the polyclinker. The construction of pBG2 [lacZ::*Ce-tra-2(–32)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(–50)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)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 *StuI* and *ApaI* sites of pPC16.41. pBG7 [lacZ::*Cb-tra-2(–38)3'UTR] was constructed by digesting pBG5 with *BglII* 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 SpeI and ApsI 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–IacZ fusion protein, and the C elegans unc-54 3′UTR. For pBG9 [GFP::Ce-tra-2(+/-)3′UTR], pBG10 [GFP::Ce-tra-2(-325)3′UTR], pBG11 [GFP::Cb-tra-2(+/-)3′UTR] and pBG12 [GFP::Cb-tra-2(-383)3′UTR], pBG1, pBG2, pBG6 and pBG7, respectively, were digested with BshIII and Apal, 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′UTR 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(-/-)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 Xhol and BamHI 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 25 or 50 ng/μl of test plasmid and 200 ng/μl of plasmid pH4, 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% CO2 at 37°C. Cells were plated at 4×104 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; 200 ng of the reporter constructs, 500 ng of tranfection 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 was used in all experiments performed in the transgenic strain H11032. 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 1-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 OD540 over time by the amount of total protein in each extract.

RNase protection assays
RNase was isolated by the method of Chomczynski and Salachi (1987). RNase protection assays were performed using an Ambion Hy3RPA kit, a modification of the method of Lee and Costlow (1987). The β-gal–3′UTP probe was made from pBG18 linearized with HindIII using T7 polymerase. pBG18 was constructed by digesting pPC16.41 with HindIII and HpaI, and subcloning the resulting fragment into the HindIII and Smal of KS (+) pBluescript. Caenorhabditis elegans act-1 RNA probe was synthesized from an act-1-specific clone linearized with EcoRI (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.Hinkle) linearized with XbaI 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). 32P-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 KsI (+) 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 32P-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 OD260.

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-64. For transgenes containing the Ce-tra-2 3′UTR, 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′-TTCACTGATAGATCAGCTACATGAGAGGAAAATTGAATACCGAATGGAACTTTGAGCCCTAGTATGCTGATTTA-3′
EBG-11: 5′-TTGCACATTAGTGGATAATAGGAAATATGATACCAATAAGAAGAAATGGGGATCCTGTGAAGGATGTAAT-3′
EBG-21: 5′-AAATTTTATAGATCCTTTATTTAACAAGAGAAAAAACA-3′
EBG-40: 5′-CTAAGCCCTTGAAGTCTCATTCCACAGTTTT-3′
EBG-42: 5′-TCAGGGGCCCCAACAGAAGAAAAATTATTAGGAAAGTG-3′
EBG-52: 5′-CATAGGCCCTTAAAGGATGGAATCTC-3′
EBG-53: 5′-TCAGGCCCCCTTGTAGCAGTCTCCTTATAT-3′
EBG-62: 5′-AGTATCGCGGGAATCCAC-3′
EBG-84: 5′-ATGTCGACCTGACCTCCAACCTTGTAAT-3′
EJ-4: 5′-TTATTTCTTGAAAGTCTGTTTTCTTTTGCTCGAG-3′
EJ-12: 5′-CACAAGGATAAAGTGGGGAGCCTGAC-3′
EJ-14: 5′-CCATGATCGGCGGGAGC-3′
EJ-15: 5′-CCCAATAATTTGCTTGAAAGTTGAGGGAAGTCGTA-3′
EJ-16: 5′-CTAGTCGCTAGAAAATTTGCGAGGACTGCA-3′
EJ-17: 5′-AGAAGAATCAGGATCCCGG-3′
EJ-18: 5′-TTTGAATATTTTGAATGATAT-3′
EJ-19: 5′-GTGTTGTCAGAAAGATCGGAGAATGAGAGGATGAGGATGAGCTCTAGTGTTG-3′
EJ-21: 5′-GCATGGATCCGAGCTGATTTTATATAT-3′
EJ-22: 5′-TCACATGTCAGGGAGGATGGAGAT-3′
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References


Conservation of tra-2 3'UTR control


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