Targeted translational regulation using the PUF protein family scaffold

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Regulatory complexes formed on mRNAs control translation, stability, and localization. These complexes possess two activities: one that binds RNA and another—the effector—that elicits a biological function. The Pumilio and FBF (PUF) protein family of RNA binding proteins provides a versatile scaffold to design and select proteins with new specificities. Here, the PUF scaffold is used to target translational activation and repression of specific mRNAs, and to induce specific poly(A) addition and removal. To do so, we linked PUF scaffold proteins to a translational activator, GLD2, or a translational repressor, CAF1. The chimeric proteins activate or repress the targeted mRNAs in Xenopus oocytes, and elicit poly(A) addition or removal. The magnitude of translational control relates directly to the affinity of the RNA-protein complex over a 100-fold range of Kd. The chimeric proteins act on both reporter and endogenous mRNAs: an mRNA that normally is deadenylated during oocyte maturation instead receives poly(A) in the presence of an appropriate chimera. The PUF-effector strategy enables the design of proteins that affect translation and stability of specific mRNAs in vivo.

CAF1/RNA stability | GLD2/polyadenylation

Messenger RNA control is pervasive. Regulatory complexes that control translation, stability and localization form on elements in the 3′UTR of target mRNAs (1–8). These regulatory complexes possess two activities: one that provides RNA specificity and another, the effector, which determines the outcome. These activities can reside in the same or different molecules in the complex. Effector domains can be analyzed by tethering the protein to the 3′UTR of a reporter mRNA. This general approach, sometimes termed a “tethered function” assay, has been used to identify the activities of known regulators, subdivide them into smaller regulatory regions, and analyze their mechanisms of action (9–11). One advantage of the approach is the separation of RNA binding and effector activities. Typically, recruitment of the protein to be analyzed is accomplished using a well-characterized, high-affinity RNA–protein interaction, such as that of MS2 coat protein or λ N-protein with its binding site. For many experiments, 3′UTRs are an ideal location to insert the binding sites: their plasticity tolerates insertion of foreign sequences and they recruit many natural regulatory proteins.

In this article, we explore the use of the Pumilio and FBF (PUF) protein scaffold as a device to tether translational effector domains in living cells. PUF proteins bind specific, short RNA sequences (7). The RNA sequence specificities of several PUF proteins have been determined experimentally, as has the structure of several PUF-RNA complexes. All PUF proteins examined to date comprise a similar scaffold, in which a ramped triangle of three α-helices is repeated eight times (Fig. 1) (12–14). Together, these repeats form an extended arc. On one face of the arc are eight α-helices that bind RNA, called RNA recognition helices. PUF proteins with new RNA specificities. Mutations can be rationally designed to alter RNA specificity, based on the known structures of natural PUF-RNA complexes (12, 16, 18, 21–24). New specificities can be obtained by transferring a segment from one protein to another, by targeted mutagenesis, or by genetic selections from protein libraries (18, 23). Thus, the PUF scaffold may provide a tractable tool to design proteins with new specificities, for a variety of purposes.

The effector proteins, GLD2 and CAF1, control translation and poly(A) tail length. GLD2 is a cytoplasmic poly(A) polymerase. It activates translation of specific mRNAs during early development and in the nervous system (25–30). CAF1 is a deadenylase and shortens poly(A) tails (31, 32). In addition, CAF1 possesses an intrinsic translational repression activity that persists even in the absence of its deadenylase activity (33). Both GLD2 and CAF1 are recruited to specific mRNAs through the action of other proteins that bind RNA, as they do not bind RNA with high affinity on their own.

Xenopus laevis oocytes provide an excellent system to test the utility of the PUF scaffold as a tethering device. The endogenous translational machinery translates microinjected RNAs and the production of protein can be easily monitored (34, 35). Injected RNAs typically are stable, even if they lack poly(A), which simplifies analysis of translational activity. A variety of activation and repression domains have been identified in oocytes (9, 10, 26, 28, 33, 36, 37), and cytoplasmic poly(A) addition and removal events are well documented (30, 38–44).

Here, we test whether the PUF scaffold can be used to target effector domains to specific mRNAs in vivo. We use natural and mutant PUF proteins to tether translational effector domains to both reporter and endogenous mRNAs. Our findings demonstrate that translational activity can be increased or decreased by design. We discuss this approach for targeted modulation of mRNA activity in the cytoplasm.

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**Results**

**FBF-2/GLD-2 Fusion Proteins Enhance Translation.** To examine whether PUF scaffolds could be used to cause mRNA activation, we first linked FBF-2, a *C. elegans* PUF protein, to GLD-2, a *C. elegans* poly(A) polymerase (29, 45). An HA-tagged FBF-2/GLD-2 chimera was expressed in *Xenopus* oocytes. The same oocytes then received a mixture of two reporter mRNAs: a polyadenylated firefly luciferase mRNA containing an FBE binding element (FBE) in its 3′ UTR and a *Renilla* luciferase mRNA that lacked an FBE (Fig. 2A). Data were expressed as the ratio of firefly to *Renilla* luciferase.

The FBF-2/GLD-2 chimera increased translation of firefly luciferase (Fig. 2B). Enhancement was specific, in that it required the presence of a wild-type FBE (Fig. 2B). Activation was not significantly enhanced by increasing the number of sites in the reporter mRNA (Fig. S1). In addition, activation required the GLD-2 segment of the chimera. Thus, FBF-2 alone had no effect, and was effectively neutral. The abundance of the FBF-2/GLD-2 protein was no greater than that of FBF-2 alone (Fig. 2B). We conclude that FBF-2 alone does not alter translation, but can cause activation when joined to GLD-2.

**FBF-2/GLD-2 Fusion Proteins Direct Polyadenylation.** To test whether the FBF-2/GLD-2 chimera caused specific polyadenylation, we injected a short radiolabeled RNA as a substrate. The RNA corresponded to the 3′ UTR of the mRNA used in Fig. 2A, and carried a single FBE. The FBF-2/GLD-2 fusion expressed in oocytes added poly(A) to the reporter RNA, as judged by gel electrophoresis (Fig. 2C). Both orientations of FBF-2 and GLD-2 were functional (lanes 3 and 4). Polyadenylation required the GLD-2 portion of the chimera (lane 2 vs. 3 and 4), and was abolished by mutations in the GLD-2 active site (lane 5). We conclude that FBF-2 can tether GLD-2 to promote polyadenylation and translation.

**PUF Proteins Provide Specificity.** The RNA binding specificity of *C. elegans* PUF-8 differs from that of FBF-2 by a single nucleotide: FBF-2 requires an “extra” nucleotide in the middle of the eight-nucleotide PUF-8 binding site, termed the 8BE (18). Exchanging PUF-8 for FBF-2 in the GLD-2 chimera switched the chimera’s specificity (Fig. 3A). The GLD-2/FBF-2 chimera enhanced translation of reporter RNA that contained the FBE (bar 1 vs. 3) and had no effect on reporter RNA with an 8BE (bar 2 vs. 4). PUF-8 had the opposite specificity, enhancing translation only of the reporter RNA with an 8BE (bar 5 vs. 6). The chimeric proteins were expressed at similar levels (Fig. 3A, Lower). These data demonstrate that the presence or absence of the “extra” base can be discriminated by these PUF protein tethers.

**Point Mutations Alter Specificity Predictably.** Rationally designed mutations in the PUF domain of FBF-2 change its RNA binding specificity predictably (18). For example, the wild-type protein binds the sequence UGU GCC AU (WT in Fig. 3B) more tightly than UG44 CCC AU (Mutant in Fig. 3B), but a designed mutant of FBF-2, carrying two point mutations in the sixth recognition helix, prefers the latter sequence (18). These mutations alter the amino acids that make edge-on contacts with the base.
The difference in the specificities of these wild-type and mutant proteins enabled selective polyadenylation (Fig. 3B). A wild-type FBF-2/GLD-2 chimera added poly(A) to reporter RNA with a wild-type FBE (lane 1 vs. 2) and had only a modest effect on the mutant RNA (lane 3 vs. 4) (18). Conversely, the mutant (R6-SE) FBF-2/GLD-2 chimera added poly(A) to reporter RNA with a mutant FBE (lane 7 vs. 8) but had only a small effect on wild-type RNA (lane 5 vs. 6) (18). We conclude that point mutations in the PUF scaffold predictably alter the specificity of polyadenylation in vivo.

PUF Tethers CAF1b to Induce Repression and Deadenylation. To test whether PUF scaffolds could also be used to cause mRNA repression, we linked FBF-2 to Xenopus CAF1b (see introductory section) (32, 33). CAF1b is one of two forms of the CAF1 protein in Xenopus (33). The chimera decreased translation of reporter RNA (Fig. 4A). Repression was dependent on the fusion protein, and both parts of the chimera were necessary. The abundance of FBF-2/CAF1b was no greater than that of FBF-2 or CAF1b (Fig. 4A).

Chimeras Can Oppose Deadenylation of Endogenous mRNAs. In oocytes injected into oocytes expressing FBF-2/GLD-2 chimera counteracted deadenylation (Fig. 3B). The FBF-2/CAF1b chimera removed poly(A) from a short radiolabeled reporter RNA (Fig. 4B). The reporter RNA carried a single FBE and corresponded to the 3′ UTR of the mRNA used in Fig. 4A. As with translational repression, the deadenylation activity was enhanced by the presence of FBF-2 moiety, and required the CAF1b segment (Fig. 4B). Previous data has shown deadenylation by tethered CAF1 requires its active site (33). We conclude that FBF-2 can tether CAF1b to promote deadenylation and translational repression.

Translational Output Is Related to Binding Affinity. We tested the relationship of affinity to translational activation by systematically varying the affinity of the PUF-RNA complex. To do so, we used a series of mutations in either human Pumilio 1 or its RNA binding site (PBE). The apparent Kd of each of these complexes had been determined previously in vitro (Tables S1 and S2) (21).

The Kd of hPUM1-RNA complex mirrored the translation effect across a wide range of Kd (Fig. 5A and B). As Kd increased, the effect on translation decreased. Differences in translational output were not the result of protein expression, as shown by Western blotting (Fig. S2). Combining both sets of data reveals a consistent correlation from subnannomolar to ~20 nM Kd (Fig. 5C). Above ~20 nM, translational activation was not observed (Fig. 5C).

The FBF-2/CAF1b chimera removed poly(A) from a short radiolabeled reporter RNA containing the 3′ UTR of the ribosomal protein L1 carrying a 50 adenosine poly(A) tail. L1 RNA undergoes deadenylation pathway removes poly(A) tails from RNAs that lack cytoplasmic polyadenylation elements (39, 44). To test whether PUF/GLD-2 chimeras could compensate for this deadenylation activity in vivo, we attempted to add poly(A) to the same mRNA molecules that naturally would undergo deadenylation.

To test the strategy, we first engineered a strain of bacteria expressing reporter RNA containing the 3′ UTR of the ribosomal protein L1 carrying a 50 adenosine poly(A) tail. L1 RNA undergoes default deadenylation during oocyte maturation (42). It fortuitously contains a cryptic FBF-2 binding sequence that is not recognized by endogenous Xenopus PUF proteins because the site contains an extra base required for FBF-2 binding (18). We injected the L1 3′ UTR reporter RNA into oocytes expressing FBF-2/GLD-2. Two hours following injection, progesterone was added to mature the oocytes.

The FBF-2/GLD-2 chimera counteracted deadenylation during maturation (Fig. 6A). The L1 reporter, RNA alone, was deadenylated during oocyte maturation (lane 1 and 2). However, in oocytes expressing FBF-2/GLD-2, the same RNAs received poly(A) tails instead (lane 3). Mutations that disrupted the UGU
of the putative binding element eliminated the polyadenylation activity, and allowed the endogenous deadenylation activity to proceed unimpaired (lanes 4–6). We next tested whether the FBF-2/GLD-2 protein would polyadenylate endogenous L1 mRNA and prevent its deadenylation in vivo. We expressed FBF-2 or FBF-2/GLD-2 proteins in oocytes, and then induced meiotic maturation. The lengths of poly(A) tails on endogenous L1 mRNAs were determined using a PCR-based poly(A) tail (PAT) assay (Fig. 6B) (46). In the absence of any expressed protein, L1 RNA was substantially deadenylated during maturation (Fig. 6C, lanes 1 and 2). FBF-2 alone did not dramatically alter this pattern (Fig. 6C, lanes 3 and 4). In contrast, FBF-2/GLD-2 protein resulted in longer poly(A) tails than were present before maturation began (Fig. 6C, lane 1 vs. 6; the band in lane 6 indicated by an asterisk is a PCR artifact). FBF-2 and FBF-2/GLD-2 were expressed (Fig. S3). We conclude that the FBF-2/GLD-2 protein directed polyadenylation of the endogenous mRNA, compensating for that mRNA’s natural deadenylation.

Discussion

MS2 coat protein (10) and bacteriophage N protein (11) have been widely used to tether effector proteins to mRNAs, typically with the aim of studying the effector (9). Here, we instead have concentrated on developing a versatile tethering device through the use of PUF proteins. PUF proteins possess several advantages over previously used tethers for targeted regulation. Most importantly, their modular scaffold affords the opportunity to manipulate their sequence specificity by design. We have shown that the PUF scaffold can be used to target specific mRNAs for translational activation or repression, and for poly(A) addition and removal. PUF proteins with new RNA binding specificities, which we refer to as “neo-PUFs,” can be used to target both natural and reporter mRNAs. Manipulations of translation, turnover, and localization should all be accessible using this strategy.

The general approach we envision, and have demonstrated here, is summarized in Fig. 7. A neo-PUF protein designed to bind selectively to the 3’ UTR of a specific mRNA is joined to an effector domain that elicits a change in that mRNA’s activity. The method is not confined to mRNAs; however, the evolutionary flexibility of 3’ UTRs and the large number of regulatory proteins that act through them, makes 3’ UTRs particularly attractive.

The general strategy can be used in both the cytoplasm and nucleus. In this article, we have manipulated cytoplasmic events, including translation and changes in poly(A) length. Two reports recently used a similar strategy to control pre-mRNA splicing in the nucleus, engineering PUF proteins to cause shifts in splicing patterns (19, 47). In those cases, the effector domain was derived from an SR- or G-rich protein, and activated or repressed splicing, respectively.

The affinity of the PUF protein for its RNA binding site, measured in vitro, are directly related to the activity observed (Figs. 3 and 5). FBF-2 and PUF-8 fusions with GLD-2 only activate RNAs with cognate sites (Fig. 3A). The two sites differ by the presence or absence of a single nucleotide, yielding a 10- to 20-fold specificity for each protein’s cognate vs. noncognate site (18). The noncognate pairs exhibit K_D of ~300 nM (18); from our studies here, these are expected to yield no effect in vivo (Fig. 5), and they do not. Conversely, the cognate pairs, with K_D of 15 nM (FBF-2) or 30 nM (PUF-8) (18), are within the range of K_D required for biological effects (Fig. 5).

The general feasibility of the approach hinges on the ease with which neo-PUFs can be designed, and how completely their specificities can be switched experimentally. Studies of multiple natural and mutant PUF proteins have led to a biochemical and structural understanding of how RNA selectivity can be achieved (12, 16, 18, 21, 22). Furthermore, introducing rationally designed or selected mutations can alter specificities (12, 16, 18, 21, 23). In some instances, mutations shift the balance in affinities for two RNAs, rather than cause a complete switch in specificity. In this situation, as illustrated by Fig. 4, activity on noncognate RNAs is diminished, but not abolished. The same issue arises with zinc-finger domains designed to regulate transcription (48–51). The specificity problem for targeting mRNAs is mitigated by the low complexity of the mRNA population relative to that of genomic DNA.

Our experiments exploited the fact that neither C. elegans FBF-2, PUF-8, nor human Pumilio—on their own—caused deadenylation or repression in Xenopus oocytes; they were effectively neutral as tethers. In experiments with other PUFs and cell types, the intrinsic activities of the PUF protein will need to be evaluated and, if necessary, eliminated by mutagenesis.

The approach described here enables enhanced expression of the mRNA, either through effects on translation or stability. In this regard, it differs from several methods to decrease expression, such as RNAi. As a result, this approach provides new opportunities for studies of RNA control.

Materials and Methods

DNA Constructs. pc52+3HA series. Three hemagglutinin (HA) tag sequences were inserted into the BamHI and EcoRI restriction sites of the pc52+ vector. An NcoI restriction site was introduced before the EcoRI site (pc52+3HA). The FBF-2 RNA binding domain (121-632 aa) (26), full-length CAF1b (33), and the 3’ fragment of GLD-2 (532–1,113 aa) (26) were PCR-amplified and ligated into the NcoI and Stul restriction sites of pc52+3HA to create pc52+3HA:FBF-2, pc52+3HA:CAF1b, and pc52+3HA:GLD-2, respectively. An XmaI restriction site was introduced before the Stul restriction site in the pc52+3HA:FBF-2 and pc52+3HA:GLD-2 plasmids.

PUF fusion proteins. The 3’ fragment of GLD-2 (26) was PCR amplified and ligated into the XmaI and Stil restriction sites of pc52+3HA:FBF-2 with the addition of a stop codon (pc52+3HA:FBF-2:GLD-2). Additional restriction sites were added to the pc52+3HA:FBF-2 vector by annealing, phosphorylating, and ligating oligos AP003 (ccgagggactgtagaagaagactcttca-
constructs were linearized by Tail + vector by replacing the NRE-2 sequence between Tail end of total RNA by RNA P1’ | A www.pnas.org/cgi/doi/10.1073/pnas.1105151108 UTR with UTR, AP126 (aactcccagagcgttatctcatgttc- P1’ | A

Fig. 6. An FBF-2/GLD-2 chimera opposes default deadenylation. (A) Injected reporter RNA analysis. 32P-RNA was injected into oocytes expressing the indicated proteins, which then were induced to mature using progesterone. Control oocytes did not receive progesterone. Lengths of poly(A) are indicated to the right and markers to the left. (lanes 1–4) Wild-type L1 RNA, with the indicated FBE; (lanes 5–8) a mutant RNA with the sequence indicated. (lanes 1 and 5) RNA before maturation; (lanes 2 and 6) RNA after maturation, no protein expressed; (lanes 4 and 8) RNA before maturation, FBF-2/GLD-2 expressed; (lanes 3 and 7) RNA after maturation, FBF-2/GLD-2 expressed. (B) PAT assay (46). Steps are numbered: 1, The P1 primer was ligated to the 3’ end of total RNA by RNA ligase; 2, the P1’ primer was used to make cDNA; 3, a radiolabeled gene-specific primer (P2*) was used to amplify the L1 mRNA; 4, a radiolabeled nested primer (P3*) was used to enrich the L1 product from the first PCR. (C) Analysis of endogenous L1 mRNA. Oocytes expressed the proteins indicated above the lanes, and were either induced to mature with progesterone or incubated without progesterone. Lengths of poly(A) are indicated to the right and markers to the left. The asterisk (lane 6) indicates a band detected in the no reverse-transcriptase (RT) control.

gacttgttagaagg) and AP004 (ctctctagactgttagaagtcctctcttagaggttccc) into the XmaI and StuI restriction sites (pCS2+3HA:FBF-2+PL). CAF1b (33) was PCR-amplified and inserted into the C1 and BglII restriction sites of pCS2+3HA:FBF-2+PL to create pCS2+3HA:FBF-2:CAF1b. The construct was further modified by the addition of a flexible protein linker consisting of four repeats of a GGGGS peptide. The linker was inserted into the XmaI and ClaI restriction sites using an oligo (ccggggagggggcggctc-tgagggaggctgctggggagggggcggctcctctctctctctctctc).

FBF-2, PUF8 (AA142-535) and human Pumilio mutants (gift from Traci Hall, NIEHS, NIH, NC; 828–1,176 aa) (21) were PCR-amplified and ligated into pCS2+3HA:GLD-2–GLD-2. FBF-2 and PUF8 were inserted into the XmaI and StuI restriction sites and the human Pumilio mutants were inserted into the Ncol site. The GLD-2 mutant D608A and the FBF-2 mutant R65-YE were introduced into the fusion proteins, as previously described (18, 26). All pCS2* constructs were linearized by NotI digestion.

Reporter plasmids. A 22-nucleotide oligo of FBEa from the GLD-1 3′ UTR with three cytosine/thymine repeats before and after it (ctgctctttagaagtcgctactatcctctctc) was inserted into the plg-M52 plasmid by removing the M52 stem loops between the SpeI and BamHI restriction sites (plg-FBE).

A UGO to ACA mutant was created using a similar oligo (ctgctctttagaagtcgctactatcctctctc). Constructs were amplified by PCR with the addition of a 50 residue poly(A) tail using oligos ljo424 (taatacgactcactatagggacgtatgtaaaggccaa-gaccctggactcactatagggacgtatgtaaaggccaa) and ljo426 (ttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
The PAT assay was performed as described with the following oocytes. C. elegans no. 38

RNA Extraction and Analysis. Oocyte RNA was prepared by using TRI reagent, following the manufacturer’s instructions (Sigma). RNAs were separated on a 6% polyacrylamide gel and analyzed by autoradiography.

Western Blotting. Western blotting was performed as previously described (33).

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