7. R. Rieger, F. Edenhofer, C. I. Lasmezas, S. Weiss, *Nature Med.* **3**, 1383 (1997).
8. I. Štefanova, V. Hořejší, I. J. Ansotegui, W. Knapp, H. Stockinger, *Science* **254**, 1016 (1991).
9. S. Mouillet-Richard *et al.*, *J. Biol. Chem.* **275**, 9186 (2000).
10. S. Mouillet-Richard, I. Laurendeau, M. Vidaud, O. Kellermann, J. L. Laplanche, *Microbes Infect.* **1**, 969 (1999).
11. S. Mouillet-Richard, data not shown.
12. S. M. Thomas and J. S. Brugge, *Annu. Rev. Cell Dev. Biol.* **13**, 513 (1997).
13. P. E. Scherer *et al.*, *J. Biol. Chem.* **270**, 16395 (1995).
14. T. M. Klein, E. D. Wolf, R. Wu, J. C. Sanford, *Nature* **327**, 70 (1987).
15. N. Nishida *et al.*, unpublished data.
16. E. M. Kramer, C. Klein, T. Koch, M. Boytinck, J. Trotter, *J. Biol. Chem.* **274**, 29042 (1999).
17. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
18. A. L. Gainer, G. S. Korbutt, R. V. Rajotte, G. L. Warnock, J. F. Elliott, *Transplantation* **61**, 1567 (1996).
19. K. Sobue and K. Kanda, *Neuron* **3**, 311 (1989).
20. Supported by grants from the Programme de recherche sur les ESST et les prions. We are greatly indebted to Z. Lam, V. Mutel, and J. G. Richards for outstanding experimental support and fruitful discussion. We are grateful to M. Buhler for technical assistance. C. Farquhar kindly provided 1A8 polyclonal antibodies, and SAF61 antibodies were raised at the Service de Pharmacologie et d'Immunologie (CEA, Saclay, France). S.M.R. is a member of the Direction générale de l'alimentation, Ministère de l'Agriculture et de la Pêche.

20 April 2000; accepted 26 July 2000

A Link Between RNA Interference and Nonsense-Mediated Decay in *Caenorhabditis elegans*

Mary Ellen Domeier,[1] Daniel P. Morse,[2] Scott W. Knight,[2] Michael Portereiko,[1] Brenda L. Bass,[2] Susan E. Mango[1]*

Double-stranded RNA (dsRNA) inhibits expression of homologous genes by a process involving messenger RNA degradation. To gain insight into the mechanism of degradation, we examined how RNA interference is affected by mutations in the *smg* genes, which are required for nonsense-mediated decay. For three of six *smg* genes tested, mutations resulted in animals that were initially silenced by dsRNA but then recovered; wild-type animals remained silenced. The levels of target messenger RNAs were restored during recovery, and RNA editing and degradation of the dsRNA were identical to those of the wild type. We suggest that persistence of RNA interference relies on a subset of *smg* genes.

Epigenetic silencing by dsRNA is a widespread phenomenon for regulating gene expression (*1*, *2*). This process, termed RNA interference, or RNAi, is thought to involve targeted degradation of homologous mRNAs (*3*–*7*). In *C. elegans*, seven genes have been shown to be important for RNAi: the *RNA-directed RNA polymerase* homolog *ego-1* (*8*), *mut-7* (*9*, *10*), *rde-2*, *rde-3*, *rde-4*, *mut-2*, and *rde-1*, which encodes a member of the *eIF2c/zwille* family (*10*). At present, it is unclear how the products of these genes function in RNAi, why some of the genes are required for silencing in the germ line but not the soma, or what roles the genes play in other processes such as transposition (*8*–*11*).

Based on the observation that both RNAi and nonsense-mediated decay involve RNA degradation, we examined whether proteins required for nonsense-mediated decay also functioned during RNAi. Seven *smg* genes have been identified, each of which is involved in nonsense-mediated decay (*12*, *13*). Mutations in five of these genes produce identical phenotypes, emphasizing that the SMG proteins act in a common pathway [*smg-1* through *smg-5* (*12*, *14*)].

To compare the effects of RNAi in wild-type (WT) and *smg* animals, we injected dsRNA corresponding to the *unc-54* gene, which encodes myosin heavy chain B and is expressed in body wall muscles (*15*). We chose *unc-54* because it generates a robust RNAi phenotype in which animals are paralyzed (*16*) and also because the severity of paralysis correlates with mRNA levels (*14*, *17*).

We observed that mutant *smg-2* animals recovered rapidly from *unc-54* RNAi-induced paralysis, whereas WT worms did not (Fig. 1A) (*18*). Progeny of injected mothers were examined daily for 4 days after injection. On days 1 and 2, both WT and *smg-2* larvae were severely paralyzed. However, *smg-2* mutants showed increased motility as they aged and moved almost as well as uninjected controls by day 4. We also observed recovery from RNAi in *smg-2* mutants carrying a *sur-5::GFP* transgene (Fig. 1B) (*18*). Thus, recovery was not specific to *unc-54* RNA or to body wall muscles, but occurred in many cell types and for at least two transcripts. GFP expression also rebounded in the neurons of WT worms, indicating that neurons have an intrinsic recovery mechanism that is independent of the *smg* genes.

To rule out the possibility that *smg-2* mutants recovered from RNAi for nonspecific reasons, such as being poor injection hosts, we injected *smg-2*(+/−) heterozygous mothers with *unc-54* dsRNA, scored recovery of individual offspring on day 3, and then determined the genotype of each scored animal. We found that progeny that failed to recover were rarely *smg-2* homozygotes (12% of paralyzed animals, $n = 158$). Conversely, siblings that recovered from RNAi were often *smg-2* homozygotes (55% of moving animals, $n = 131$). If segregation were random, 25% of paralyzed or moving animals would be expected to be *smg-2* homozygotes. These experiments demonstrate that recovery from RNAi depends on *smg-2* activity in the zygote and therefore does not reflect the inability of *smg-2* mutants to function as good injection hosts.

To examine whether *smg-2* mutations affected RNAi-mediated mRNA degradation, we measured endogenous RNA levels using a real-time semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) assay (*19*, *20*). Controls with WT and mutant *unc-54* animals demonstrated that our assay accurately reflected transcript levels (*21*). Furthermore, *smg-2* mutations altered *unc-54* RNA levels in ways that paralleled the phenotypic recovery. In WT larvae, *unc-54* RNA levels were reduced about 10-fold compared with uninjected controls and remained low throughout the time course. In *smg-2* animals, *unc-54* levels were reduced 10-fold on day 1, but rebounded rapidly, eventually reaching levels close to those of uninjected controls (Fig. 2). The reduction seen on day 1 was comparable to that seen in WT worms, indicating that the initial response was robust. These data demonstrate that *smg-2* mutants attenuate RNAi-mediated mRNA degradation.

Our data predict that for the effects of the *smg* genes on RNAi to be observed, the targeted mRNA must be transcribed continuously and RNAi must not induce lethality, or the animals will not be able to recover. These requirements explain why recovery in *smg* mutants was not observed previously (*7*). Earlier studies targeted *mex-3*, which is maternally transcribed and essential for embryogenesis (*22*). In addition, these studies assayed *smg-3* mutants, which fail to recover well from RNAi (see below).

[1]Huntsman Cancer Institute Center for Children and Department of Oncological Sciences, University of Utah, Salt Lake City, UT 84112, USA. [2]Department of Biochemistry and Howard Hughes Medical Institute, University of Utah, Salt Lake City, UT 84132, USA.

*To whom correspondence should be addressed. E-mail: susan.mango@hci.utah.edu

Three experiments demonstrated that *smg-2* mutant animals did not affect RNAi by altering the stability of the dsRNA. First, a similar decay profile was observed for dsRNA injected into either WT or *smg-2* animals (Fig. 3, A and B). Second, the amount of dsRNA introduced into worms was not limiting for RNAi initiation. All of the self-progeny from mothers injected with *unc-54* dsRNA were paralyzed when born on days 1 to 4. To produce more offspring, we mated *smg-2* mothers 4 days after injection and found that all of the cross progeny were also completely paralyzed at birth (i.e., day 5; progeny of seven out of seven mothers mated with WT males). Third, injection of a 10-fold higher concentration of dsRNA could not suppress recovery for *smg-2*(−) mutants. Progeny from animals injected with dsRNA (5 mg/ml) recovered from RNAi and began to move on days 3 to 4, similar to those injected with our standard concentration of 0.5 mg/ml ($n \geq 24$ for each concentration and genotype) (*23*).

In another assay to analyze differences in dsRNA metabolism, we asked whether injected dsRNA was modified by adenosine deaminases that act on RNA (ADARs), and whether this process was altered in *smg-2* mutants. By sequencing cDNAs derived from injected dsRNA, we observed evidence of A to I changes, but found that the modifications were similar in cDNAs from WT or *smg-2* worms (*21*). Thus, SMG-2 does not seem to affect RNAi by altering the stability or editing of dsRNA.

We examined alleles of five other *smg* genes to determine whether they also play a role in RNAi. Animals mutant for either *smg-5* or *smg-6* behaved similarly to *smg-2*, recovering rapidly from the effects of RNAi and ultimately expressing nearly WT levels of *unc-54* RNA (Fig. 4). Unexpectedly, *smg-1* homozygotes behaved like WT worms and did not recover from RNAi at all. Mutant *smg-3* and *smg-4* animals gave a weak, variable response, suggesting that *smg-3* or *smg-4* may not be required for persistence of RNAi. To ensure that the *smg-1* strain was defective for nonsense-mediated decay, we repeated our experiments with *smg-1; pha-4*(*zu225*) animals (*23*). The *pha-4*(*zu225*) allele is a nonsense codon that leads to larval arrest (*24*, *25*), unless the nonsense-mediated decay pathway is inactivated by a *smg* mutation such as *smg-1* (*26*). Finally, we performed RNAi for a second target, *unc-22*, to demonstrate that the differential sensitivities to the *smg* loci were not specific to *unc-54* (*23*). From the results with *unc-22* and *unc-54*, we conclude that *smg-1* is critical for nonsense-mediated decay but not for persistence of RNAi, whereas *smg-2*, *smg-5*, and *smg-6* are involved in both processes.

There was no correlation between the strength of the RNAi phenotype associated with a particular *smg* allele and the effect of that allele on nonsense-mediated decay. Of

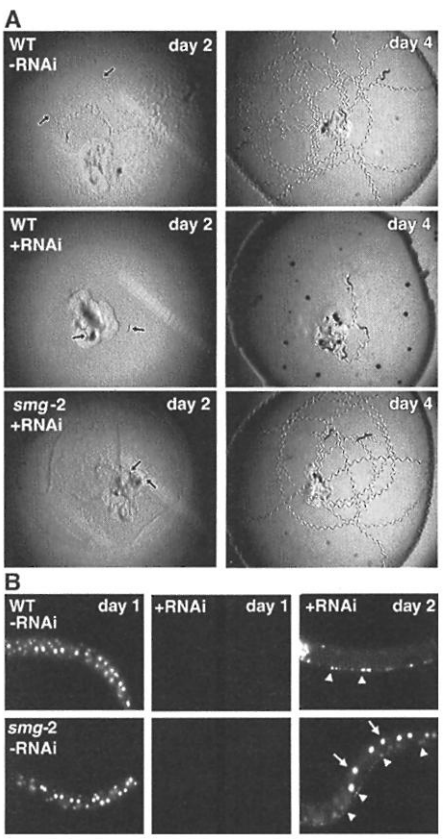


Fig. 1. (**A**) *smg-2* mutants recover from RNAi. The motility of WT and *smg-2* worms after injection with *unc-54* dsRNA (+RNAi) was compared with that of uninjected WT animals (−RNAi). Five animals were allowed to move freely for 10 min and photographed. *smg-2* animals were paralyzed on day 2 (left) but moved well by day 4 (right), whereas WT animals remained paralyzed throughout the time course. (**B**) Fluorescence imaging of transgenic *sur-5::gfp* animals (*35*) without (−RNAi) or with (+RNAi) injection of *gfp* dsRNA. Intestinal cells recovered from *gfp* RNAi in *smg-2* animals (arrows; bottom panels) but not in the WT (top panels). Neurons recovered from the effects of RNAi in both WT and *smg-2* worms (arrowheads). *smg*-mediated RNA degradation is active in neurons (*36*), suggesting that recovery from RNAi in neurons occurs by a distinct process.

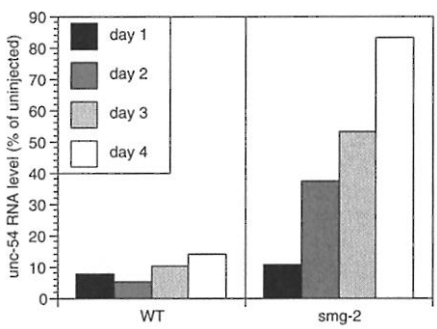
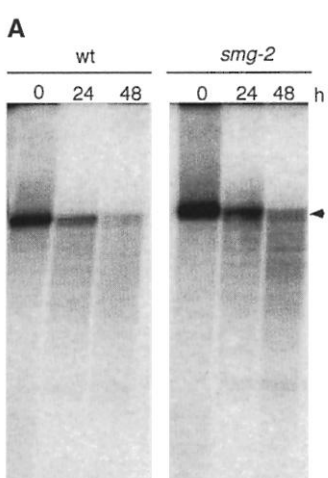


Fig. 2. *unc-54* mRNA levels correlate with motility. The amount of normalized *unc-54* RNA in injected animals relative to uninjected controls was monitored over 4 days (n = 3 to 4 experiments). Without injection, WT and *smg-2* animals contained about the same absolute level of *unc-54* RNA daily (*14*, *23*). The data set was considered inappropriate for evaluation by Student's t test, and instead was subjected to the Mann-Whitney U test. The difference in RNA levels from day 4 *smg-2* animals compared with WT worms was statistically significant (P = 0.032). The range of values on day 4 was 7 to 25% for WT and 50 to 100% for *smg-2*.



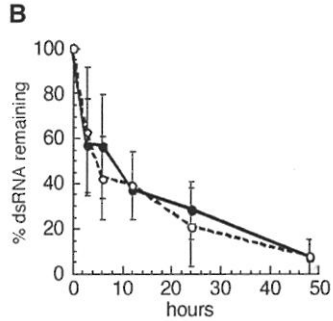


Fig. 3. (**A** and **B**) dsRNA is metabolized similarly in WT and *smg-2* animals. Sense and antisense RNA strands were ^{32}P-radiolabeled, annealed, and injected into gonads of WT or *smg-2* animals. RNA from three mothers and their offspring was isolated at the indicated times (h, hours) and analyzed by polyacrylamide gel electrophoresis. The arrowhead indicates the migration of full-length dsRNA. The smaller migrating species likely represent degradation products of the injected material or species generated by reincorporation of labeled nucleotide produced during degradation. The decay profile of dsRNA isolated from embryos was also similar between WT and *smg-2* animals (*23*). The graph compiles multiple experiments of the type shown in (A). The percentage of full-length dsRNA remaining in WT (●) or *smg-2* (○) animals was determined by quantifying bands on the PhosphorImager (0 hours = 100%). Each time point represents the average of ≥3 experiments except for the WT 12-hour time point (n = 1). Similar decay rates were observed for *unc-54* and *gfp* dsRNA, so the data were combined. Variability in the amount of labeled RNA introduced into individual animals by injection probably contributed to the standard deviation (error bars).

all the *smg* mutants, *smg-6* has the weakest nonsense-mediated decay phenotype (*27*, *28*). Yet, these mutants recovered well from RNAi. Conversely, two of the three *smg-1* alleles we assayed eliminate nonsense-mediated decay [*r861* and *e1228* (*12*, *29*)], but had no effect on RNAi. These findings suggest that the SMG proteins may each have a unique biochemical function, only some of which are required for RNAi. These data also demonstrate that recovery from RNAi is not due to a general inactivation of the nonsense-mediated decay pathway.

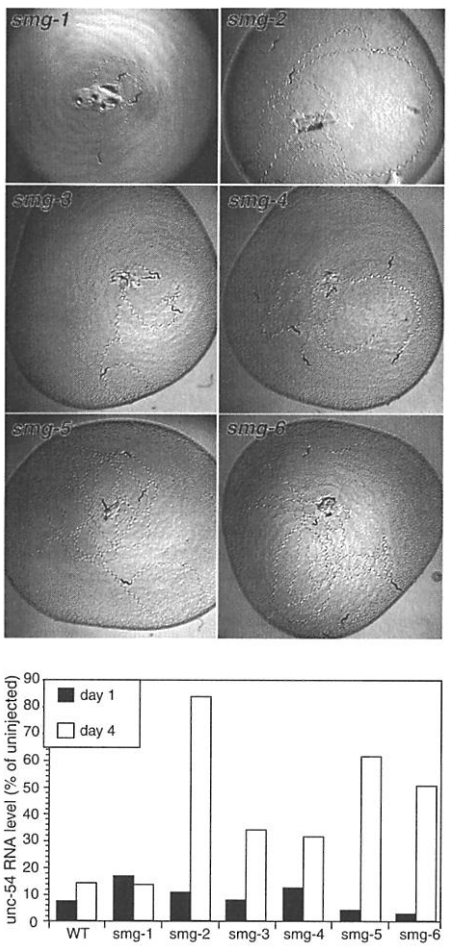


Fig. 4. Differential requirement for *smg* genes during RNAi. **(Upper panels)** Motility of RNAi-treated worms containing mutations in one of six *smg* genes was assayed on day 4 after injection. **(Lower panel)** Amount of *unc-54* RNA in injected animals relative to uninjected controls on day 1 and day 4, calculated and plotted as in Fig. 2B. The Mann-Whitney U test was used to determine whether the differences for day 1 versus day 4 were statistically significant. Differences were significant for *smg-2* ($P = 0.046$), *smg-3* ($P = 0.049$), *smg-5* ($P = 0.049$), and *smg-6* ($P = 0.032$); the difference between days 1 and 4 was not significant for *smg-4* ($P = 0.245$). There was also a statistically significant difference between *smg-2*, *smg-5*, and *smg-6* compared with *smg-3* and *smg-4* for day 4 RNA levels, suggesting that SMG proteins fall into two groups ($P = 0.036$). The range of values on day 4 was 13 to 14% for *smg-1*, 50 to 100% for *smg-2*, 20 to 50% for *smg-3*, 6 to 50% for *smg-4*, 35 to 100% for *smg-5*, and 25 to 100% for *smg-6*.

In summary, our experiments suggest that RNA interference occurs in two stages that can be distinguished genetically. Initial mRNA degradation occurs in the absence of *smg* function, whereas persistence of the silenced state depends on *smg-2*, *smg-5*, and *smg-6*. Page and co-workers have shown that SMG-5 and SMG-6 play a role in SMG-2 dephosphorylation, whereas SMG-1, SMG-3, and SMG-4 are necessary for SMG-2 phosphorylation (*30*). Because *smg-1* activity is not required for RNAi, the phosphorylated form of SMG-2 is either unnecessary for RNAi or can be produced by an alternate pathway. How might SMG-2, SMG-5, and SMG-6 function during RNAi? One idea is that the three SMG proteins might facilitate amplification of a signal required for silencing to last for the life of the animal. Evidence for amplification during RNAi has been observed previously (*16*, *31*), but the mechanism is unknown. SMG proteins could increase the number of dsRNA molecules by promoting endonucleolytic cleavage of existing dsRNA molecules, which has been observed in *Drosophila* (*4*, *5*). Alternatively, on the basis of the homology between *smg-2* and yeast *Upf1*, which encodes an adenosine triphosphatase with RNA-binding and helicase activities (*30*, *32*, *33*), the SMG proteins could unwind dsRNA to provide a template for RNA-directed RNA polymerase. RNA-directed RNA polymerase is critical for RNAi in *C. elegans* (*8*) and requires a single-stranded template in tomato plants (*34*). Finally, the sequence similarity between *smg-2* and *Upf1* suggests that SMG-2, and by extension RNAi, may be associated with ribosomes.

References and Notes

1. B. L. Bass, *Cell* **101**, 235 (2000).
2. A. Fire, *Trends Genet.* **15**, 358 (1999).
3. T. Tuschl, P. D. Zamore, R. Lehmann, D. P. Bartel, P. A. Sharp, *Genes Dev.* **13**, 3191 (1999).
4. S. M. Hammond, E. Bernstein, D. Beach, G. J. Hannon, *Nature* **404**, 293 (2000).
5. P. D. Zamore, T. Tuschl, P. A. Sharp, D. P. Bartel, *Cell* **101**, 25 (2000).
6. I. Korf, Y. Fan, S. Strome, *Development* **125**, 2469 (1998).
7. M. K. Montgomery, S. Xu, A. Fire, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 15502 (1998).
8. A. Smardon *et al.*, *Curr. Biol.* **10**, 169 (2000).
9. R. F. Ketting, T. H. Haverkamp, H. G. van Luenen, R. H. Plasterk, *Cell* **99**, 133 (1999).
10. H. Tabara *et al.*, *Cell* **99**, 123 (1999).
11. R. F. Ketting and R. H. Plasterk, *Nature* **404**, 296 (2000).
12. J. Hodgkin, A. Papp, R. Pulak, V. Ambros, P. Anderson, *Genetics* **123**, 301 (1989).
13. B. M. Cali, S. L. Kuchma, J. Latham, P. Anderson, *Genetics* **151**, 605 (1999).
14. R. Pulak and R. P. Anderson, *Genes Dev.* **7**, 1885 (1993).
15. S. Brenner, *Genetics* **77**, 71 (1974).
16. A. Fire *et al.*, *Nature* **391**, 806 (1998).
17. R. A. Pulak and P. Anderson, *Mol. Cell. Biol.* **8**, 3748 (1988).
18. Strains were maintained at 20°C [except for *smg-1(cc546ts)*, which was maintained at 25°C] as described (*15*) and were of the following genotypes: *smg-1(cc546ts) I*, *smg-1(r861) I*, *smg-1(e1228) him-2(e1065) I*, *smg-2(e2008) I*, *unc-54(r293) I*, *smg-5(r860) I*, *smg-6(r896) III*, *smg-3(r867) IV*, *him-5(e1490) V*, and *smg-4(ma116) V*. RNAi experiments were conducted according to standard procedures (*16*). Detailed protocols are given in (*21*).
19. T. B. Morrison, J. J. Weis, C. T. Wittwer, *Biotechniques* **24**, 954, 960, 962 (1998).
20. Worms were harvested by freezing in liquid nitrogen. We picked 36 animals on day 1 [i.e., first-stage larvae (L1s) from embryos laid on day 0], 24 on day 2, 18 on day 3, and 12 on day 4 (i.e., L4s from embryos laid on day 0). Total RNA was isolated by guanidinium-phenol extraction (*25*). First-strand cDNA was synthesized with random hexamers and mouse mammary leukemia virus reverse transcriptase according to specifications (Life Technologies). Semiquantitative PCR was performed with a LightCycler28 (Idaho Technologies) with actin for normalization. To ensure that only endogenous, spliced mRNA was detected, we used primers that spanned exon boundaries and were outside of the region of sequence identity shared with the dsRNA. A melting temperature profile and agarose gel analysis of the final PCR product demonstrated that the product did not contain primer dimers. No signal was detected with a genomic DNA control. In most experiments, *unc-54* RNA levels normalized to actin were compared with the levels in uninjected controls at the equivalent developmental stage (also normalized to actin). Each set of injection/RNA isolation/RT was performed at least three times, and individual cDNA samples were evaluated in triplicate for each experiment.
21. Supplemental Web material is available at *Science* Online at www.sciencemag.org/feature/data/1051686.shl.
22. B. W. Draper, C. C. Mello, B. Bowerman, J. Hardin, J. R. Priess, *Cell* **87**, 205 (1996).
23. M. E. Domeier *et al.*, data not shown.
24. S. E. Mango, E. J. Lambie, J. Kimble, *Development* **120**, 3019 (1994).
25. M. A. Horner *et al.*, *Genes Dev.* **12**, 1947 (1998).
26. L. Kaltenbach and S. E. Mango, unpublished data.
27. B. M. Cali and P. Anderson, *Mol. Gen. Genet.* **260**, 176 (1998).
28. M. Morrison, K. S. Harris, M. B. Roth, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 9782 (1997).
29. P. Anderson, personal communication.
30. M. F. Page, B. Carr, K. R. Anders, A. Grimson, P. Anderson, *Mol. Cell. Biol.* **19**, 5943 (1999).
31. A. Grishok, H. Tabara, C. C. Mello, *Science* **287**, 2494 (2000).
32. P. Leeds, J. M. Wood, B. S. Lee, M. R. Culbertson, *Mol. Cell. Biol.* **12**, 2165 (1992).
33. K. Czaplinski, Y. Weng, K. W. Hagan, S. W. Peltz, *RNA* **1**, 610 (1995).
34. W. Schiebel *et al.*, *Plant Cell* **10**, 2087 (1998).
35. T. Gu *et al.*, *Mol. Cell. Biol.* **18**, 4556 (1998).
36. L. Bloom and H. R. Horvitz, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3414 (1997).

37. We thank P. Anderson, A. Fire, and R. Parker for discussions and for providing strains; T. Morrison, J. Weis, and J. Weis for the LightCycler; K. Ullman for comments on the manuscript; and F. Adler and A. Tsodikov (Biostatistic Shared Resource) for statistical analysis. Some of the strains used were provided by the *Caenorhabditis elegans* Genetics Stock Center. Some oligonucleotides were synthesized by the Howard Hughes Medical Institute (HHMI) oligonucleotide synthesis facility at the University of Utah supported by the National Cancer Institute (grant 42014) and HHMI; DNA was sequenced by the University of Utah Health Sciences DNA Sequencing Facility supported in part by the National Cancer Institute (grant 5P30CA42014). B.L.B. is an HHMI Associate Investigator. S.E.M. is supported by the Huntsman Cancer Institute Center for Children.

27 April 2000; accepted 7 August 2000