Using RNA interference to identify genes required for RNA interference

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RNA interference (RNAi) is a phenomenon in which double-stranded RNA (dsRNA) silences endogenous gene expression. By injecting pools of dsRNAs into Caenorhabditis elegans, we identified a dsRNA that acts as a potent suppressor of the RNAi mechanism. We have used co-injection of dsRNAs to identify four additional candidates for genes involved in the RNAi mechanism in C. elegans. Three of the genes are C. elegans mes genes, some of which encode homologs of the Drosophila chromatin-binding Polycomb-group proteins. We have used loss-of-function mutants to confirm a role for mes-3, -4, and -6 in RNAi. Interestingly, introducing very low levels of dsRNA can bypass a requirement for these genes in RNAi. The finding that genes predicted to encode proteins that associate with chromatin are involved in RNAi in C. elegans raises the possibility that chromatin may play a role in RNAi in animals, as it does in plants.

RNA interference (RNAi) works in a remarkable variety of organisms, including animals, plants, fungi, and protists (1–4). RNAi can be used as a tool to phenocopy the loss of function of one specific gene at a time (5), and it has been exploited in screens designed to identify developmental genes, by injecting pools of multiple double-stranded RNAs (dsRNAs) into animals and examining phenotypes in their progeny (6–10).

The process by which dsRNA silences gene activity is not completely understood, although the mechanism is known to involve cleavage of both the dsRNA and the corresponding endogenous mRNA into 21–25 nucleotide fragments (11–14). Cleavage of the dsRNA in animal cells requires Dicer, a member of the RNase III family of nucleases (15–17). To date, only a handful of other proteins have been implicated in RNAi in animal cells, and all of these are predicted to interact with RNA—an RNaseD-like protein, an RNA-dependent RNA polymerase-like protein, and an eIF2C-like protein (18–20). Some of the Caenorhabditis elegans smg genes, which participate in non-sense-mediated mRNA decay, are required for the RNAi effect to persist (21). In plants and fungi, RNAi has been proposed to additionally involve chromatin, because RNAi-like phenomena require proteins predicted to interact with chromatin—a SWI2/SNF2 component, a DNA methyltransferase, and a DNA helicase-like protein (22–25)—as well as proteins predicted to interact with RNA (22, 26). No such role for chromatin has yet been implicated in animal cells.

We have fortuitously identified a dsRNA that acts as a potent suppressor of the RNAi mechanism. Here, we describe the method we used and additional genes with potential roles in RNAi that we have identified by using this approach.

Materials and Methods

Strains. C. elegans was maintained as in ref. 27. Strains and alleles used were Bristol Strain N2 (wild type), SS186: mes-2(bn11) unc-4(e120)/mnc1 dpy-10(e128) unc-52(e444)II; SS262: mes-3(bn35) dpy-5(e61); SS268: dpy-11(e224) mes-4(bn3) unc-76(e911)V/nTi(V;V); JK2663: dpy-11(e224) mes-4(bn67) V/nTi(V;V); SS222: mes-3(bn21)I; SS282: mes-6(bn64); dpy-11(e224)/nTi(V;V); SS360: mes-6(bn66) dpy-20(e1282)V/nTi(V;V).

dsRNA Preparation and Injection. Templates for in vitro transcription were generated by a two-step PCR from wild-type genomic DNA. Primers for the first step included 20 bases specific to the target sequence and 15 bases of the T7 promoter sequence. The resulting PCR product was purified by using a Qiagen (Chatsworth, CA) PCR purification kit according to the manufacturer’s recommendations. This product was used as a template for a second PCR using oligonucleotides containing the full-length T7 promoter sequence. One to two micrograms of the product were then gel-purified and used as a template in an in vitro transcription reaction by using Ambion’s (Austin, TX) T7 Megascript Kit according to the manufacturer’s recommendations. The integrity of the dsRNA was assessed by gel electrophoresis, and the concentration was determined by spectrophotometry. For storage of dsRNAs, the resulting solution was mixed with 2 volumes of 100% ethanol and kept at −80°C. RNAi-to-RNAi assays were performed by coinjecting 30 ng/μl of target dsRNA (for example, mm-20) with 100 ng/μl of test dsRNA (for example, mut-7) into either the gut or gonad of adult wild-type hermaphrodites. For each experiment in Fig. 3C, 500 ng/μl of dsRNA was injected.

Soaking. Soaking experiments were performed by the method of Maeda et al. (10) with the following alterations: both wild-type and mutant worms were incubated together in 8 μl of soaking buffer with either 100 or 800 ng/μl of dsRNA for 24 h, and transfers to freshly seeded NGM plates were carried out daily.

Results and Discussion

During a pilot screen aimed at identifying genes with essential roles in embryogenesis, we found that one pool of eight dsRNAs that was expected to produce embryonic lethality did not (Fig. 1A): injecting C. elegans adult hermaphrodites with a pool containing dsRNA corresponding to the essential gene glp-1 (28, 29) and seven other dsRNAs resulted only in viable embryos. In contrast, injecting the glp-1 dsRNA alone resulted in a high degree of embryonic lethality (Fig. 1B). To determine whether the lethality produced by glp-1(RNAi) was being suppressed by a specific dsRNA in the pool, we co-injected glp-1 dsRNA with each other dsRNA from the pool. We found that only injection of a dsRNA corresponding to the M04B2.3 gene could suppress glp-1 dsRNA-mediated lethality (Fig. 1C). Additionally, removing the M04B2.3 dsRNA from the pool was sufficient to restore embryonic lethality on injection (Fig. 1D), indicating that the glp-1(RNAi) phenotype is not suppressed nonspecifically by coinjection of multiple other dsRNAs.

M04B2.3 dsRNA might suppress glp-1(RNAi) by suppressing glp-1 loss of function. Alternatively, it might suppress glp-1(RNAi) by suppressing the RNAi mechanism. To distinguish between these possibilities, we first tested whether M04B2.3...
Fig. 1. Using pools of dsRNAs to identify genes that may be required for RNAi. (A) Ten pools injected in the pilot screen and the embryonic lethality that resulted from each injection. A pyramid of bars under each of the 10 pool numbers represents the results from injecting a pool of eight dsRNAs (top row), two groups of four dsRNAs (second row), and then single dsRNAs (third row) into hermaphrodites. Each bar is filled to a degree representing the percent embryonic lethality resulting from that injection (i.e., completely filled bars represent 100% embryonic lethality). Lethality was scored in embryos that were laid the day after injection by at least 10–15 adults that survived injection. dsRNAs were reinjected in subgroups or as single dsRNAs for each pool that included a dsRNA expected to produce embryonic lethality (black dot) and/or was found to result in more than 20% embryonic lethality. All other subgroups or single dsRNAs, which were not injected, are represented as empty gray bars. As can be seen, 10 dsRNAs expected to produce embryonic lethality based on their published mutant and/or RNAi phenotypes were present in seven of the 10 pools. Of these seven pools, only pool 2 failed to produce embryonic lethality. An asterisk marks the M04B2.3 dsRNA, in pool 2. The black bar next to M04B2.3 represents the glp-1 dsRNA. dsRNAs injected were the following, (Left to Right): pool 1 (C03C10.3, C25A1.8, E00B2.3, F56G4.3, F31F6.3, T05F1.2, C04F12.9, F33G12.1), pool 2 (F14B6.3, F40G12.11, Y11D7A.13, C56E3.13, C7E4.1, F02A9.6, M04B2.3, C16C8.16), pool 3 (C9F5.6, C38D9.2, D109D.2, C27C12.1, K07A1.2, F22A12.4), pool 4 (E52D10.7, K07A1.2, F22A12.4, K08E4.2, F40F8.9, T02C12.1, C32E8.8, C17E7.9, ZK632.1), pool 5 (ZK484.3, C27D9.1, C04F12.9, F33G12.1), pool 6 (F14B6.3, F40G12.11, Y11D7A.13, C56E3.13, C7E4.1, F02A9.6, M04B2.3, C16C8.16, pool 7 (F38E1.7, D2024.5, F20B6.7, R04D3.3, C29A12.3, ZK632.1), pool 7 (F38E1.7, D2024.5, F20B6.7, R04D3.3, C29A12.3, ZK632.1), pool 8 (DY3.7, F17C11.10, F28D1.2, K08A8.1, C50B6.3, 2016).
dsRNA could suppress glp-1 loss-of-function mutations. Injection of M04B2.3 dsRNA failed to suppress the lethality produced by two glp-1 alleles, q231, and q224 (28) (data not shown). Next, we determined whether M04B2.3 dsRNA suppresses the RNAi mechanism by testing whether it could suppress the lethality produced by other dsRNAs. We found that M04B2.3 dsRNA was able to suppress the lethality produced by RNAi of several structurally unrelated, essential genes (Fig. 1E), suggesting that M04B2.3 dsRNA is a potent suppressor of the RNAi mechanism.

M04B2.3 appears to be relatively unique in its ability to suppress the phenotypes of co-injected dsRNAs, because none of the other genes tested in the pilot screen produced a similar effect. The pilot screen comprised 10 pools of eight dsRNAs, all of which correspond to transcripts that are enriched in *C. elegans* oocytes in comparison to somatic tissues (30). Seven of the 10 pools included dsRNAs corresponding to genes already known to be essential for embryogenesis, and all of these pools, except the one that included M04B2.3 and glp-1, resulted in embryonic lethality (Fig. 1A). Injections of subsets of dsRNAs from each of these pools revealed that every dsRNA expected to produce embryonic lethality did so, with the exception of glp-1 dsRNA. Because the pools included only one suppressor (M04B2.3) and 38 dsRNAs that neither produced lethality themselves nor suppressed the phenotypes of coinjected previously known lethal dsRNAs, we estimate that very few dsRNAs corresponding to germ-line-enriched transcripts can suppress RNAi in pools.

Suppression of RNAi by M04B2.3 dsRNA might be caused by loss of M04B2.3 mRNA. Alternatively, the M04B2.3 dsRNA might suppress RNAi, because it contains a specific dsRNA sequence that is a potent competitor for the RNAi machinery; to test this, we generated dsRNAs corresponding to the 5' and 3' halves of the M04B2.3 gene and coinjected either half with mom-2 or glp-1 dsRNAs. If a specific sequence in the M04B2.3 dsRNA is a strong competitor for the RNAi machinery, we would expect only the half that contained this sequence to suppress RNAi, whereas if M04B2.3 is required for RNAi, we would expect both halves to suppress RNAi. We found that both of these dsRNAs suppressed the lethality produced by mom-2 and glp-1 dsRNAs (data not shown); this and further results below suggest that suppression is produced by loss of M04B2.3 mRNA.

These results suggest that injecting multiple dsRNAs can be used as an efficient method to identify genes required for RNAi. Others have found recently that RNAi of *C. elegans* and fly versions of Dicer and fly Argonaute2 can reduce the effectiveness of RNAi of particular genes (15, 16, 31). We asked whether RNAi of additional known components of the RNAi machinery could also suppress phenotypes associated with these dsRNAs. dsRNAs corresponding to two genes required for RNAi, mut-7 and rde-1 (18, 20), were each coinjected with either mom-2 or hmp-2 dsRNA. Like M04B2.3, both rde-1 and mut-7 were able to suppress the embryonic lethality produced by RNAi of mom-2 or hmp-2, whereas control dsRNAs could not (Fig. 1F). On the basis of these results and of our results from the pool from which M04B2.3 was identified, we conclude that injecting as many as eight dsRNAs at a time can be used as an efficient method to identify candidates for genes involved in the RNAi mechanism in *C. elegans*. For convenience, we refer to the injection of multiple dsRNAs for this purpose as an RNAi-to-RNAi assay.

A BLAST search revealed that the M04B2.3 gene (Fig. 2A) has strong similarity to a human gene, GAS41, which was found as an amplified gene in glioblastomas (32, 33). We therefore refer to the M04B2.3 gene as glp-1, for GAS41-like. Both the human GAS41 and *C. elegans* glp-1 proteins are predicted to associate with DNA, based on the presence of an acidic domain and on sequence similarity to a conserved domain of the predicted chromatin-modifying protein TFIIF. GAS41 has been found to bind another human protein, the leukemia translocation protein AF10, by yeast two-hybrid, GST pulldown, and coimmunoprecipitation assays (34). Because the human AF10 gene has strong similarity to a *C. elegans* gene, zfp-1 (both share predicted LAP/PHD finger, zinc finger, and leucine-rich domains; Fig. 2B), we tested whether RNAi of zfp-1 also suppresses RNAi. We found that injection of zfp-1 dsRNA suppressed the lethality produced by mom-2 and hmp-2 dsRNAs (Fig. 3A), suggesting that like glp-1, the zfp-1 gene may also be required for RNAi.

Two other human genes with sequence similarity to GAS41, AF-9 and ENL, encode proteins that can bind human Polycomb (35). We therefore tested whether *C. elegans* polycomb-group genes are required for RNAi. Only two Drosophila polycomb-group genes, Enhancer of zeste and extra sex combs, have recognizable *C. elegans* homologs, named mes-2 and -6, respectively, for their maternal effect sterile loss-of-function phenotypes (36, 37). MES-2 and -6 proteins exist in a complex with a novel protein, MES-3 (38). mes-2, -3, -6, and a gene with some similarity to mes-2, called mes-4, are each required maternally for embryonic lethality.
survival of larval germ cells (39, 40). Loss of function of any of these four mes genes causes a defect in transgene silencing, a phenotype that has also been seen in mutants deficient in RNAi (20, 41). We tested each of these four mes genes by the RNAi-to-RNAi assay, and found that the lethality produced by mom-2 or pos-1 dsRNA was indeed suppressed by coinjection of mes-3, -4, or -6 dsRNA, but not by coinjection of mes-2 dsRNA (Fig. 3B), suggesting that mes-3, -4, and -6 may be required for RNAi, and that mes-2 is not.

As an important test of our proposal that certain of the mes genes play roles in RNAi, we determined whether worms bearing loss-of-function mutations in each of these genes are also RNAi-deficient. We injected mom-2, hmp-2, or par-2 dsRNAs into homozygous mes mutant hermaphrodites at a dsRNA concentration typical for C. elegans RNAi experiments (6, 19) (see Materials and Methods). Consistent with our RNAi-to-RNAi results, the lethality produced by each dsRNA was suppressed in mes-3, -4, and -6 null mutants, and it was not suppressed in mes-2 null mutants (Fig. 3C).

Our results appear to contradict some of the results of Tabara et al. (20), who reported that injecting pos-1 dsRNA, at a concentration just sufficient to produce a high degree of lethality in wild-type worms, produced high degrees of lethality in mes-2, -3, -4, and -6 loss-of-function backgrounds. Others have found previously that C. elegans dcr-1 is required for RNAi under some but not all conditions (17). We wished to determine whether this is also true for mes-3, -4, and -6, and if so, under what conditions these genes are required for RNAi. We tested whether the disparity between our results and Tabara et al. (20) could be attributed to differences in which mes alleles were tested, the specific dsRNAs assayed, or the concentration of dsRNA injected. For mes-6, our results revealed that a null allele (see Fig. 3 legend) was RNAi-deficient, but the weaker allele (37) that had been tested previously by Tabara et al. (20) was not (Figs. 3C and 4A). For mes-3 and -4, interestingly, an effect was found to depend on the concentration of dsRNA injected. Null alleles of mes-3 and -4 suppressed the effects of injecting typically used (6, 19) concentrations of dsRNA but, surprisingly, not the effects of injecting much lower concentrations of dsRNA (Fig. 4B). A similar, but weaker, concentration dependence was found in the null mes-6 allele as well (Fig. 4C). We note that this result is not consistent with, and in fact is the inverse of, what one might expect from a hypomorphic allele of a gene required for RNAi. This peculiar effect is also not specific to one mode of delivery of dsRNA, because introducing dsRNA by soaking worms in dsRNA, instead of injecting the dsRNA, produced similar results (Fig. 4C). These results suggest that low con-

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Fig. 3. Testing candidate genes for roles in RNAi. All axes are as in Fig. 1B; vertical ticks mark lethality in 25% intervals and horizontal ticks mark time at 12 h intervals. (A) Lethality produced by mom-2 dsRNA or hmp-2 dsRNA injected with or without zfp-1 dsRNA. (B) Lethality produced by mom-2 alone (top row) or coinjected with dsRNAs corresponding to mes-2, -3, -4, and -6, or a control (unc-54) dsRNA. (C) Lethality produced by injecting dsRNAs (mom-2, hmp-2, or par-2) in wild-type N2 worms or mes mutants (labeled along left side). Asterisk indicates that for par-2, bn21ts was tested at 25°C in place of bn35. ND, not determined.

Asterisk indicates that for pos-1, dsRNA injected with or without zfp-1 dsRNA.

B

dsRNA injected

alone

+ mes-2 dsRNA

+ mes-3 dsRNA

+ mes-4 dsRNA

+ mes-6 dsRNA

+ unc-54 dsRNA

C

dsRNA injected

wild-type

mes-2 (bn11)

mes-3 (bn35)

mes-4 (bn23)

mes-6 (bn61)

par-2

Figures 3B and 3C are published as supporting information, for complete results with additional replicates of these experiments.
Fig. 4. mes-3, -4, and -6 are required for RNAi under certain conditions. Effectiveness of RNAi is shown in (A) mes-6(bn64) and N2 tested by injection, (B) mes-3(bn3), mes-4(bn23), and N2 tested by injection (mes-3 and N2 control carried out simultaneously are shown in gray) (mom-2 dsRNA was also injected in mes-3(bn35), with similar results; not shown), and (C) mes-2(bn11), mes-3(bn35), mes-4(bn23), mes-6(bn64), and N2 tested by soaking worms in 100 or 800 ng/ml pos-1 of dsRNA. For injection experiments, numbers reflect lethality of embryos laid on the day after injection (from 15–24 to 44–59 h postinjection). For soaking experiments, numbers (average ± SD for a minimum of three trials) reflect lethality of embryos laid between 38 and 62 h after the end of soaking. All of the mes alleles used here appear to be null alleles by immunostaining (refs. 36, 37, 52; Y. Fong and S. Strome, personal communication).

centrations of dsRNA elicit a response that is uniquely able to bypass a requirement for mes-3, -4 and, to a lesser degree, mes-6, in RNAi. MES-2, -3, and -6 are associated in a complex in C. elegans embryos (38); our results indicate that not all members of this complex are involved in RNAi. Our results also suggest that a very low level of mes-6 activity may be sufficient to function in RNAi, because only a null allele of mes-6 was RNAi-deficient, and because loss of mes-2 function does not block RNAi (Figs. 3 B and C and 4C); loss of mes-2 function has been found previously to result in lower levels of MES-6 protein, levels similar to the weaker of the two mes-6 alleles we tested (37). We conclude from our results that mes-3, -4, and -6 are required for RNAi under some but not all conditions.

Our results have identified a total of five candidates for genes involved in the RNAi mechanism in C. elegans–gfl-1, zfp-1, mes-3, -4, and -6. For three of these (mes-3, -4, and -6), null mutants were also RNAi-deficient. Given the complete congruence of our RNAi-to-RNAi results with our results using mutants, we would predict that null mutants of the other two genes would also be RNAi-deficient; however, this remains to be determined. We have not found any phenotypes other than RNAi deficiency after RNAi of gfl-1 or zfp-1. Because the RNAi mechanism is silenced in these experiments, it is not clear whether a phenotype that would be seen in a loss-of-function mutant would result from RNAi of these genes; therefore, whether these genes have additional functions remains to be seen.

All of the genes we have identified encode proteins that are predicted to associate with chromatin (refs. 36, 37, and 42; S. Strome, personal communication). RNAi in animals has been found to work not at the level of DNA but by degrading mRNA, and mRNA degradation can occur even in the absence of DNA (13). How then might chromatin play a role in RNAi? Our results to date can suggest only speculative models. One possibility is that the role of chromatin is indirect—that inactivation of genes such as the polycomb-group genes results in transcriptional misregulation that may block RNAi by saturating the RNAi mechanism with excess RNA or by down-regulating genes that encode components of the RNAi mechanism. We consider this unlikely, because RNAi can in fact occur in strong mes-3 and -4 mutants when dsRNAs are introduced at low concentration. An alternative hypothesis that is consistent with current data is that introducing dsRNA in animal cells might result in both degrading mRNA and also recruiting a transcriptional silencing complex to the targeted locus. Such a mechanism might conceivably be required for a penetrant RNAi effect, by further reducing a pool of endogenous mRNA through repressing transcription of specific loci. In plants, introduction of dsRNA can cause both degradation of endogenous transcripts and silencing of the relevant locus (43), although silencing of the locus is thought to occur through DNA methylation (43, 44). If dsRNA does induce gene silencing in C. elegans, it is unlikely that it occurs through DNA methylation, because little to no DNA methylation can be detected in C. elegans (45). On the basis of our results, we have proposed that low concentrations of dsRNA might elicit a response that is uniquely able to bypass a requirement for mes-3, -4, and -6 in RNAi. One mechanism that might be active only in the presence of low concentrations of dsRNA is amplification; it has been reported recently that dsRNAs can be amplified by short interfering RNAs (siRNAs) priming the production of new dsRNAs (46, 47). It will be of interest to determine whether this amplification mechanism is active only when low concentrations of dsRNA are introduced, and if so, whether amplification of dsRNAs might bypass a requirement for mes-3, -4, and -6 in RNAi. If our model, that dsRNA in animal cells might recruit a transcriptional silencing complex to the targeted locus, is correct, one might expect that amplification of dsRNAs could result in RNAi sufficiently strong and lasting to bypass a requirement for transcriptional silencing.

As with a standard genetic screen, an RNAi-based screen would be expected to have the potential to find both direct and indirect players in the process being studied. Hence, like other proteins implicated in RNAi by genetics alone (18–21), how would be expected to have the potential to find both direct and indirect players in the process being studied. Hence, like other proteins implicated in RNAi by genetics alone (18–21), how
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Fig 5. Complete results for Figs. 1 and 3 with additional replicates of experiments. Average percent lethality +/– SD is shown for each experiment (blue). For experiments in which suppression was being assayed, the percent lethality of each experiment, and only the control that was carried out at the same time and with the same dsRNA preparation, were compared; the average difference between control and experimental percents lethality +/– SD is shown (pink). Each experiment is labeled with the dsRNAs that were injected, followed by the number of replicates of each experiment in parentheses. Each replicate consisted of scoring all of the progeny of 10–15 worms that survived injection for embryonic lethality. Lethality of embryos laid during a time window of approximately 20–30 h after injection was scored approximately 24 h later. Injections were carried out in N2 wild-type worms except where otherwise noted.