Transcriptional silencing of a transgene by RNAi in the soma of *C. elegans*

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The silencing of transgene expression at the level of transcription in the soma of *Caenorhabditis elegans* through an RNAi-dependent pathway has not been previously characterized. Most gene silencing due to RNAi in *C. elegans* occurs at the post-transcriptional level. We observed transcriptional silencing when worms containing the *elt-2∷gfp/LacZ* transgene were fed RNA produced from the commonly used L4440 vector. The transgene and the vector share plasmid backbone sequences. This transgene silencing depends on multiple RNAi pathway genes, including *dcr-1*, *rde-1*, *rde-4*, and *rrf-1*. Unlike post-transcriptional gene silencing in worms, *elt-2∷gfp/LacZ* silencing is dependent on the PAZ-PIWI protein Alg-1 and on the HP1 homolog Hpl-2. The latter is a chromatin silencing factor, and expression of the transgene is inhibited at the level of intron-containing precursor mRNA. This inhibition is accompanied by a decrease in the acetylation of histones associated with the transgene. This transcriptional silencing in the soma can be distinguished from transgene silencing in the germline by its inability to be transmitted across generations and its dependence on the *rde-1* gene. We therefore define this type of silencing as RNAi-induced Transcriptional Gene Silencing (RNAi-TGS). Additional chromatin-modifying components affecting RNAi-TGS were identified in a candidate RNAi screen.

In many organisms, gene silencing induced by homologous RNA plays an important role in protection against viruses and mobile genetic elements while also serving a regulatory function during development. The discoveries of RNA interference (RNAi) (Fire et al. 1998), short interfering RNAs (siRNAs) (Hamilton and Baulcombe 1999; Hammond et al. 2000; Zamore et al. 2000; Elbashir et al. 2001) and Dicer (Bernstein et al. 2001), the conserved RNase III-type protein required for production of siRNA from double-stranded RNA (dsRNA), brought together observations in different organisms to establish RNA-mediated silencing as an essential biological phenomenon. These discoveries also stimulated the recognition of a connection between siRNAs and microRNAs (for review, see Bartel 2004) in addition to implicating siRNAs and RNAi in chromatin silencing (Hall et al. 2002; Volpe et al. 2002).

The connection between RNAi and transcriptional silencing has been best studied in fission yeast, where siRNAs corresponding to centromere repeats have been found (Reinhart and Bartel 2002) and the RNAi pathway genes *dicer*, *argonaute*, and RNA-dependent RNA polymerase (RdRP) have been implicated in silencing at centromeres (Hall et al. 2002; Volpe et al. 2002). The hallmark of silenced chromatin is Histone 3 Lys 9 (H3-K9) methylation by the methyltransferase Clr-4 and association of H3-K9 with Swi6, a chromodomain protein homologous to Heterochromatin Protein 1 (HP1) (Hall et al. 2002; Volpe et al. 2002). The complex containing siRNAs associated with transcriptional silencing in *Schizosaccharomyces pombe*, RITS, has recently been purified (Verdel et al. 2004). Ago1, the only PAZ-PIWI protein in *S. pombe*, was found in the complex in addition to a chromodomain protein, Chp1, and a novel protein, Tas3.

A connection between RNAi, histone methylation, and DNA methylation in some cases of transposon and transgene silencing in plants has recently been established. It was first demonstrated by Wassenegger that RNA-induced silencing in plants often results in DNA methylation (Wasseneger et al. 1994). Later, dsRNA targeting the promoter region of transgenes in *Arabidopsis* and tobacco was shown to induce transcriptional silencing and DNA methylation (Mette et al. 2000). Members of the conserved gene families implicated in RNAi, *argonaute4* (*ago4*), *dicer-like3* (*dcl3*), and *RNA-dependent RNA polymerase2* (*rdr2*), have been shown to be respon-

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sible for initiation of silencing on the transcriptional level in plants (Zilberman et al. 2003; Chan et al. 2004; Xie et al. 2004).

The importance of the RNAi pathway for silencing of transgenes at the level of chromatin in *Drosophila* was recently demonstrated. Mutations in two genes encoding PAZ-PIWI proteins *piwi* and *aubergine*, as well as in the *spindle-E* gene encoding a DEAD-motif RNA helicase, resulted in a reduction in H3-K9 methylation and delocalization of HP1 and HP2 (Pal-Bhadra et al. 2004).

It is likely that transcriptional mechanisms contribute to the various silencing events involving repetitive elements in *Caenorhabditis elegans*. It was first recognized in *C. elegans* that RNAi is required for silencing of transposons (Ketting et al. 1999; Tabara et al. 1999). Additional examples of silencing of repetitive elements in *C. elegans* include cosuppression (Dernburg et al. 2000; Ketting and Plasterk 2000) and transgene silencing (Kelly et al. 1997). These silencing processes occur predominantly in the germline and are distinguished from other RNAi silencing by the lack of requirement for *rde-1* (Dernburg et al. 2000; Ketting and Plasterk 2000). The PAZ-PIWI protein Rde-1 was first shown to play an essential role in RNA silencing in *C. elegans* (Tabara et al. 1999). There are 24 PAZ-PIWI domain proteins and four RdRPs (Ego-1, Rrf-1, Rrf-2, and Rrf-3) in *C. elegans*, but thus far, none have been confirmed to be involved in the initiation of transcriptional silencing.

Silencing of repetitive transgenic arrays in the germline of *C. elegans* is stable and is inherited in an epigenetic fashion due to maintenance of the chromatin state (Kelly et al. 1997; Strome et al. 2001). This process has also been shown to be temperature dependent with stronger silencing observed at lower temperatures (16°C and 20°C) as compared with 25°C (Strome et al. 2001). Homologs of Polycomb Group genes, *mes-2* and *mes-6*, play a role in this process (Kelly and Fire 1998). Hpl-2, one of two *C. elegans* HP1 homologs, has been recently implicated in germline transgene silencing (Couteau et al. 2002). Also, inactive transgene arrays have been as-

sociated with H3-K9 methylation (Kelly et al. 2002). All of this is consistent with silencing at the level of transcription.

There are different responses to repetitive arrays in the soma and in the germline of *C. elegans*. For several transgenes with ubiquitous expression patterns, strong expression has been observed in all somatic lineages, while expression in the germline is stably silenced (Kelly et al. 1997). Knight and Bass have recently demonstrated that dsRNA is produced from the repetitive arrays of somatic transgenes and that this RNA is edited by adenosine deaminases (ADARs) (Knight and Bass 2002). Surprisingly, they found that somatic transgenes are silenced in worms with mutations in the ADAR-encoding genes, *adr-1* and *adr-2*, and that this silencing is dependent on *rde-1*. Evidence suggests that ADAR proteins modify dsRNA, suppressing its ability to generate RNAi activities. Thus, *adr-1* and *adr-2* mutant backgrounds represent conditions hypersensitive for RNAi silencing. Other conditions, which are known to enhance RNAi-related processes, include mutations in *rrf-3* gene (Simmer et al. 2002), probably by reducing competition for substrates for Rrf-1, and, for silencing of transgenes in the germline, culturing animals at lower as opposed to higher temperatures.

Here, we describe silencing of a somatic transgene in *C. elegans* induced by feeding of *Escherichia coli* expressing RNAs homologous to a portion of the transgene and the backbone of the repetitive array. This silencing targets pre-mRNA and corresponds to a decrease in the association of RNA polymerase II and acetylated histones with the transgenic array. We refer to this phenomenon as RNAi-induced Transcriptional Gene Silencing, or RNAi-TGS. RNAi-TGS is dependent on the RNAi pathway genes, *rde-1*, *rde-4*, *rrf-1*, and also on the *hpl-2* and *alg-1* genes, and is not heritable. Using a candidate RNAi screen, we identified additional genes that affect RNAi-TGS, including genes encoding RNA-binding proteins, Polycomb Group proteins, chromodomain proteins, and potential histone methyltransferases. We also show that transcriptional silencing of the transgenic ar-
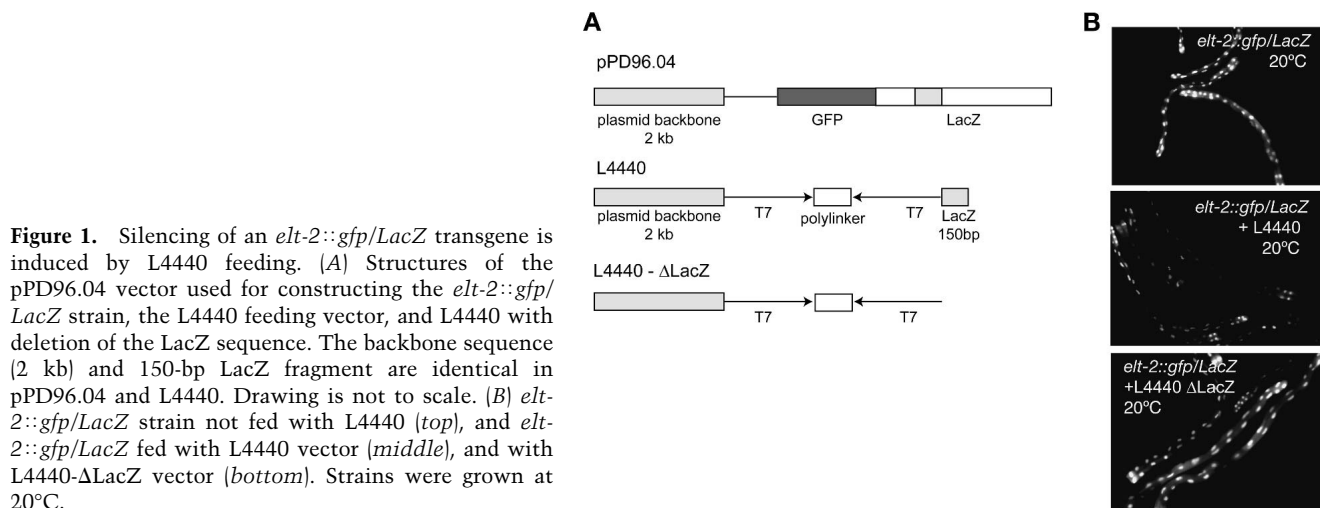

Figure 1. Silencing of an *elt-2::gfp/LacZ* transgene is induced by L4440 feeding. (*A*) Structures of the pPD96.04 vector used for constructing the *elt-2::gfp/LacZ* strain, the L4440 feeding vector, and L4440 with deletion of the LacZ sequence. The backbone sequence (2 kb) and 150-bp LacZ fragment are identical in pPD96.04 and L4440. Drawing is not to scale. (*B*) *elt-2::gfp/LacZ* strain not fed with L4440 (*top*), and *elt-2::gfp/LacZ* fed with L4440 vector (*middle*), and with L4440-ΔLacZ vector (*bottom*). Strains were grown at 20°C.

ray occurs spontaneously in the *rrf-3* mutant at 16°C and is dependent on *rde-1*. These data provide evidence for a connection between RNAi and transcriptional gene silencing at the chromatin level in *C. elegans*.

Results

Feeding induced homology-dependent silencing of the elt-2::gfp/LacZ transgene

Initiation of RNAi by feeding *C. elegans* dsRNA-expressing bacteria is a common technique (Timmons and Fire 1998). This method uses a KS+-based vector, L4440, containing two T7 promoters flanking a polylinker (Fig. 1A). Any sequence cloned between the T7 promoters will be transcribed into dsRNA upon induction of T7 polymerase production in bacteria (Timmons and Fire 1998).

In order to study cell division control in *C. elegans*, we used an *elt-2::gfp/LacZ* transgenic strain expressing GFP in intestinal nuclei (Fukushige et al. 1998). Our studies included extensive utilization of RNAi by feeding, and we serendipitously discovered that *elt-2::gfp/LacZ* expression in intestinal nuclei is silenced upon feeding of dsRNA-expressing bacteria regardless of the sequence inserted in the L4440 vector (Figs. 1B, 2A, 3). This silencing was observed in L4 and adult worms fed on L4440 containing bacteria from the time of hatching. Normally, adult worms were put on bacterial plates and their progeny evaluated for silencing. Typically, 80%–100% of 50–200 progeny worms exhibited silencing, with GFP expression reduced to an almost undetectable level in the middle portion of the intestine (Figs. 1B, 3). Surpris-

ingly, this silencing was not dependent on a particular gene sequence cloned into L4440 vector. In fact, the empty vector also successfully induced silencing (Figs. 1B, 2A).

We suspected that sequence homology between the transgene and the L4440 vector was responsible for the observed silencing. In *C. elegans*, transgenic strains are made by injection of plasmid DNA into the germline of adult hermaphrodites (Mello et al. 1991). This plasmid DNA is spontaneously arranged into extrachromosomal multicopy arrays or can be integrated into the genome after irradiation. The *elt-2::gfp/LacZ* transgenic strain was made using a construct based on the pPD96.04 plasmid from the Fire lab (Fig. 1A) and integrated into the X chromosome (Fukushige et al. 1998). We looked for possible sequence homology in the backbones of pPD96.04 and L4440 and found that they share a 2-kb identity in the region located immediately adjacent to the L4440 polylinker (Fig. 1A). Also, L4440 contains a 150-bp portion of LacZ sequence adjacent to the other side of the polylinker (Fig. 1A). All plasmids used for making transgenic strains are highly likely to contain sequences identical to those of the L4440 backbone, such as that of the ampicillin resistance gene; however, nonspecific silencing of transgenes by the L4440 feeding vector was not observed in most transgenic lines tested (Table 1).

Since the *elt-2::gfp/LacZ* transgene is expressing a GFP/LacZ in-frame fusion and its mRNA can be targeted for degradation by a small amount of LacZ dsRNA introduced with L4440, we hypothesized that the 150-bp LacZ gene portion in the L4440 vector might play a crucial role in silencing through the RNAi mechanism. We
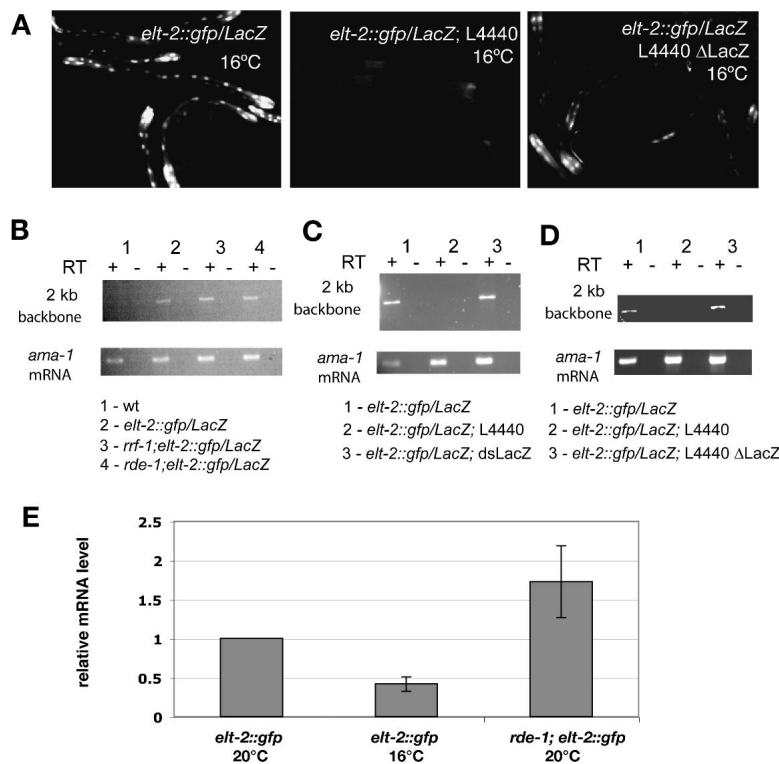

Figure 2. The common vector backbone sequence plays a role in L4440-dependent silencing of *elt-2::gfp/LacZ* transgene. (*A*, *left*) *elt-2::gfp/LacZ* strain not exposed to L4440 at 16°C; 100% of the observed worms express GFP. (*Middle*) *elt-2::gfp/LacZ* worms on L4440 at 16°C; 100% of the observed worms have no GFP expression or very weak expression. (*Right*) 80%–100% of *elt-2::gfp/LacZ* worms on L4440-ΔLacZ at 16°C have weak GFP expression. (*B–D*) RT–PCR-detecting transcript from 2-kb backbone sequence in *elt-2::gfp/LacZ* strains; 2-kb backbone RNA is not detected in worms silenced by L4440 (*C,D*, lane *2*). *ama-1* mRNA is shown as a control for RNA samples. (*E*) Quantification of the relative levels of *gfp/LacZ* mRNA in wild-type transgenic worms at 20°C and 16°C, and *rde-1(ne300)* worms by real-time RT–PCR; *ama-1* expression was used for internal reference. Mean values and ranges of the relative *LacZ/ama-1* ratios based on three real-time RT–PCR trials are shown.
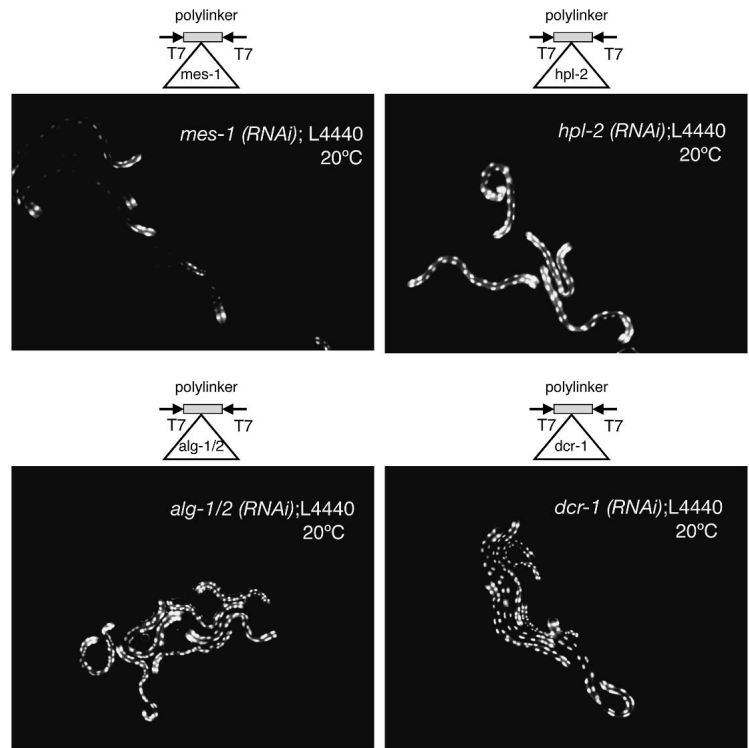
Figure 3. L4440-induced silencing of *elt-2::gfp/LacZ* is dependent on *dcr-1*, *alg-1/2*, and *hpl-2*. The *elt-2::gfp/LacZ* strain was fed on L4440 with cloned fragments of genes indicated *above* each image. Strains were grown at 20°C.

deleted the LacZ sequence from the feeding vector (L4440, ΔLacZ; Fig. 1A) and observed that efficient silencing was dependent on this portion of the vector (Fig. 1B, cf. middle and lower panels). Among the other GFP transgenic strains tested, the only strain silenced upon L4440 feeding, *myo-3::gfp/LacZ*, also contained an GFP/LacZ fusion (Table 1). Another LacZ transgenic strain, *unc-54::LacZ*, was silenced upon L4440 feeding (Table 1), supporting the correlation between the presence of a LacZ sequence in the transgene and its susceptibility to silencing by L4440.

Table 1. *Strains tested for silencing induced by L4440 feeding*

Strain	Repetitive array or single copy	Silencing by L4440	Strain reference
elt-2::gfp/LacZ	Repetitive, integrated	Yes	Fukushige et al. (1998)
pes-10::gfp	Repetitive, integrated	No	Montgomery et al. (1998)
unc-119::gfp	Repetitive	No	DP 132
myo-3::gfp/LacZ	Repetitive	Yes	PD 4251
pie-1::H2B/gfp	Single copy	No	AZ 212
pie-1::tubulin/gfp	Single copy	No	AZ 244
cye-1::gfp	Repetitive	No	KM 32
cki-1::gfp	Repetitive, integrated	No	VT 825
unc-54::LacZ	Repetitive	Yes	PJ 1044
myo-2::LacZ	Repetitive	No	PD 8251

The role of the plasmid backbone sequence in L4440-induced silencing

Transgenic arrays are known to be prone to silencing in the *C. elegans* germline. When worms are maintained at 16°C–20°C, the transgenes are mostly silenced during the third generation after transformation, while worms cultured at 25°C maintain strong expression of the transgenes for many generations (>100) (Strome et al. 2001).

In order to test whether feeding-induced silencing was temperature dependent, we performed experiments at 16°C. Interestingly, the vector construct lacking the LacZ sequence was able to induce weak silencing of the *elt-2::gfp/LacZ* strain at 16°C (Fig. 2A, cf. left and right panels). We predicted that this silencing might be due to the 2-kb sequence homology between the backbone of the transgene incorporated into the worm's genomic DNA and the backbone of the feeding plasmid. We tested whether the plasmid backbone sequence was transcribed in transgenic worms by RT–PCR, and indeed found that the whole 2-kb region of identity between pPD96.04 and L4440 was transcribed (Fig. 2B–D). This RNA was undetectable when the transgene was silenced by L4440 feeding, but still present in worms where GFP/LacZ expression was silenced by soaking in LacZ dsRNA (Fig. 2C). Silencing of the backbone RNA by feeding correlated with the level of silencing of GFP/LacZ transgene. For example, feeding the L4440 vector containing the LacZ fragment induced stronger silencing of both GFP/LacZ transgene (Fig. 2A) and the backbone RNA (Fig. 2D) as compared with feeding the vector with the LacZ portion deleted. Therefore, the presence of the LacZ fragment in

L4440 increased the degree of silencing of the transgenic array over that induced through transcription of the plasmid backbones.

We next investigated possible background silencing of the transgenic array in the *elt-2::gfp/LacZ* strain. Transgenes in the somatic tissue are not typically silenced to the same extent as transgenes in the germline. However, they could still be subject to some repression by RNAi or/and chromatin-related mechanisms. We used real-time RT–PCR to determine relative mRNA expression levels of *gfp/LacZ* transgene in the wild-type background and in the RNAi-deficient background of an *rde-1* mutant and found that expression was elevated by twofold in *rde-1* (Fig. 2E). This suggests that somatic transgene expression in this line is suppressed by an RNAi-related process. Furthermore, transgene mRNA levels were reduced in worms cultured at 16°C (Fig. 2E). Again, this is consistent with an endogenous process limiting expression of the transgene. Temperature dependence of L4440-induced silencing therefore probably reflects a stimulation of this endogenous process.

Silencing depends on RNAi pathway, Alg-1, and Hpl-2

We tested whether RNA feeding-induced silencing of *elt-2::gfp/LacZ* in this system is dependent on the RNAi pathway. The *elt-2::gfp/LacZ* transgenes were completely resistant to silencing when crossed into RNAi-deficient backgrounds, *rde-1(ne300)*, *rde-4(ne299)*, and *rrf-1(pk1417)* (Table 3, below).

We next tested whether the L4440 vector could prevent silencing of *elt-2::gfp/LacZ* transgene if the vector was designed to silence a gene in the RNAi pathway, i.e., contained a portion of the gene cloned between the T7 promoters (Fig. 3). In this case, the vector simultaneously initiates *elt-2::gfp/LacZ* silencing (vector induced) and also initiates RNAi silencing of the gene corresponding to the segment between the T7 promoters. If this latter gene is critical for the pathway leading to the transgene silencing, the vector-induced silencing will be suppressed. Using this technique, we observed that *dcr-1* is required for the silencing process (Fig. 3), which is consistent with involvement of the RNAi pathway. Many L4440 vectors containing portions of randomly chosen genes, like *mes-1* (Fig. 3), induced the same level of *elt-2::gfp/LacZ* silencing as an empty L4440 vector, and thus, these genes are probably not required for silencing of the transgenic array. More than 50 constructs, which supported silencing induced by feeding, are listed in Supplementary Table 1.

We also observed that *alg-1* and *alg-2* genes, which are not required in the classical RNAi pathway, are required for *elt-2::gfp/LacZ* silencing process (Fig. 3). Alg-1 is a PAZ-PIWI family gene shown to be essential for miRNA pathways (Grishok et al. 2001). The requirement for *alg-1* in *elt-2::gfp/LacZ* silencing was confirmed with *alg-1(gk214)* deletion mutant generated by the *C. elegans* Gene Knock-out Consortium (Supplementary Fig. 1A).

Since silencing of *elt-2::gfp/LacZ* has features of re-peat-induced silencing (such as temperature sensitivity), we investigated whether silencing in this system might occur on a transcriptional level. If silencing of *elt-2::gfp/LacZ* by L4440 occurs at the level of chromatin, then it might be sensitive to mutations in proteins known to suppress transcription at this level. We next tested the requirement for Hpl-2, a homolog of the Heterochromatin protein 1. RNAi of *hpl-2* by feeding prevented silencing of *elt-2::gfp/LacZ* in the presence of the feeding vector (Fig. 3). This result was confirmed using a deletion mutant in *hpl-2* generated by the *C. elegans* Gene Knock-out Consortium (Supplementary Fig. 1A). For both *alg-1* and *hpl-2* deletion mutants, desilencing of the *elt-2::gfp/LacZ* transgene in mutant backgrounds was more apparent at 16°C, where feeding-induced silencing is more stringent.

Pre-mRNA is targeted in the feeding-induced elt-2::gfp/LacZ silencing

Early studies of RNAi in *C. elegans* determined that mature mRNA was targeted during the silencing process (Montgomery et al. 1998). Specifically, the injection of dsRNA resulted in decreases in the levels of mRNA, but levels of the nuclear pre-mRNAs were not significantly repressed (Montgomery et al. 1998). This was confirmed in a Trypanosome system (Ngo et al. 1998). Importantly, an in vitro RNAi system revealed that mature mRNA is degraded during RNAi (Tuschl et al. 1999).

If silencing of the *elt-2::gfp/LacZ* transgene by L4440 vector is indeed at the stage of transcription (RNAi-Transcriptional Gene Silencing) and is distinct from traditional RNAi (RNAi - Post-Transcriptional Gene Silencing), it should lead to a decrease in the level of target pre-mRNA, while RNAi-PTGS should have no effect on the level of pre-mRNA. To test this, we soaked worms in a LacZ dsRNA solution, a technique similar to feeding (Tabara et al. 1998), to induce RNAi-PTGS.

The *gfp/LacZ* fusion transgene in the pPD96.04 construct contains numerous small introns, which allows detection of both pre-mRNA and mRNA by RT–PCR. Two pairs of primers were designed; one pair, specific for exons spanning two introns, produced a 357-bp product from the mature mRNA and a 458-bp product from pre-mRNA (Fig. 4A, top); the second pair, specific for the two introns and spanning an exon, detected only an 373-bp product from the pre-mRNA (Fig. 4A, middle). RT–PCRs were performed on RNA obtained from a nonsilenced *elt-2::gfp/LacZ* strain, a strain silenced by the feeding vector (L4440) and a strain silenced by soaking in LacZ dsRNA (dsLacZ). Using exon-specific primers, we observed that the level of *gfp/LacZ* pre-mRNA decreased along with the level of mature mRNA in worms silenced by feeding (Fig. 4A, top, lane 5). In soaked worms, pre-mRNA was detected while mRNA levels decreased (Fig. 4A, top, lane 7). Using intron-specific primers, we could not detect *gfp/LacZ* pre-mRNA in the worms silenced by feeding (RNAi-TGS) (Fig. 4A, middle, lane 5), but in worms silenced by soaking (RNAi-PTGS) pre-mRNA was still detected (Fig. 4A, middle, lane 7).
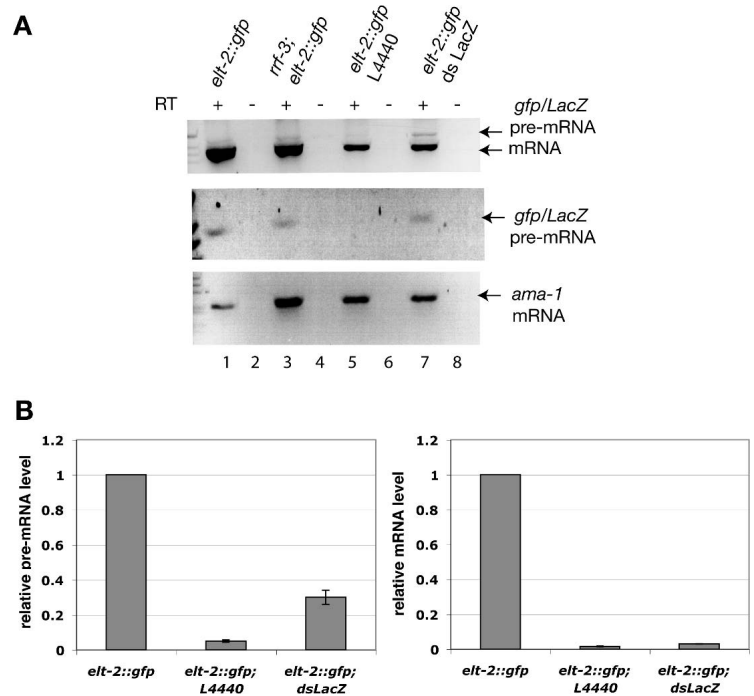
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Figure 4. RT–PCR analysis of pre-mRNA and mRNA levels during L4440-induced silencing of *elt-2::gfp/LacZ*. (*A*) RT–PCR of *gfp/LacZ* transgene; amplification with exon-specific primers (*top*) detects products from the mature mRNA (357 bp) and pre-mRNA (458 bp); amplification with intron-specific primers (*middle*) detects a product of 373-bp; *ama-1* mRNA RT–PCR detecting a 236-bp product is shown as control (*bottom*). (*B*) Quantification of the relative levels of *gfp/LacZ* mRNA and *gfp/LacZ* pre-mRNA shown in *A* by real-time RT–PCR; *ama-1* expression was used for internal reference. Mean values and ranges of the relative *LacZ/ama-1* ratios based on two real-time RT–PCR trials are shown.

To quantify the observed effects, we used additional sets of *gfp/LacZ* exon-specific and intron-specific primers in real-time RT–PCR (Fig. 4B). While the levels of both pre-mRNA and mRNA were dramatically reduced (by 20- and 50-fold, respectively) during RNAi-feeding, soaking in LacZ dsRNA led to reduction in mRNA level by 30-fold and reduction in pre-mRNA by only threefold. This slight reduction in pre-mRNA level during soaking in LacZ dsRNA might indicate that these conditions also weakly stimulate transcriptional silencing. These data indicate that *elt-2::gfp/LacZ* silencing induced by feeding vector occurs at the pre-mRNA stage, and thus, is distinct from RNAi-PTGS. Additional experiments illustrating a decrease in *elt-2::gfp/LacZ* pre-mRNA levels during silencing induced by the L4440 vector are presented in Supplementary Figure 2.

RNAi-TGS silencing in rrf-3 background

RdRPs have been implicated in homology-dependent silencing in plants, fungi, and nematodes. In *C. elegans*, *ego-1* and *rrf-1* are required for RNAi in the germline and soma, respectively (Smardon et al. 2000; Sijen et al. 2001). Another RdRP gene, *rrf-3*, has been reported to antagonize RNAi (Simmer et al. 2002). Therefore, RNAi-related processes are more efficient in the *rrf-3* mutant. Consistent with this, we found that feeding-induced silencing of *elt-2::gfp/LacZ* transgene was stronger in an *rrf-3* mutant background as compared with the wild-type background. For example, L4440 vectors containing the LacZ fragment and those lacking this homology to the *gfp/LacZ* transgene significantly reduced both the *gfp/LacZ* mRNA and pre-mRNA levels in this genetic background (Fig. 5C).

Interestingly, we found that expression of the *elt-2::gfp/LacZ* transgene was almost completely silenced in an *rrf-3* background at 16°C even without feeding on the L4440 vector (Fig. 5A). While the level of transgene expression is reduced at 16°C in wild-type worms (Fig. 2D), the effect is much more dramatic in *rrf-3* mutant. These results indicate that Rrf-3, similarly to Adr-1 and Adr-2, prevents silencing of the repetitive transgenes in somatic tissues of the wild-type worms. It is likely that temperature-dependent silencing of the *elt-2::gfp/LacZ* transgene in *rrf-3* background is mechanistically similar to the silencing induced by feeding discussed above. During this temperature-dependent silencing, not only was the level of *gfp/LacZ* pre-mRNA significantly reduced (Fig. 5B, lane 3), but also the 2-kb backbone-specific RNA was undetectable by RT–PCR (Fig. 5B). Silencing in *rrf-3 (pk1426)* background also depended on the RNAi pathway gene *rde-1(ne300)*, (Fig. 5A, cf. middle and bottom panels). Silencing at the level of transcription is frequently maintained by localization of histone deacetylase activities. Thus, we determined whether culturing worms in the presence of histone deacetylase (HDAC) inhibitors would result in suppression of this silencing. This was indeed the case (Table 2). All of this is consistent with RNA-induced transcriptional silencing (RNAi-TGS).

Silencing of elt-2::gfp/LacZ is accompanied by a decrease in histone H4 acetylation

To further confirm silencing of *elt-2::gfp/LacZ* at the level of transcription, we performed chromatin immunoprecipitation (ChIP) experiments using antibodies against acetylated histone H4 and antibodies against the
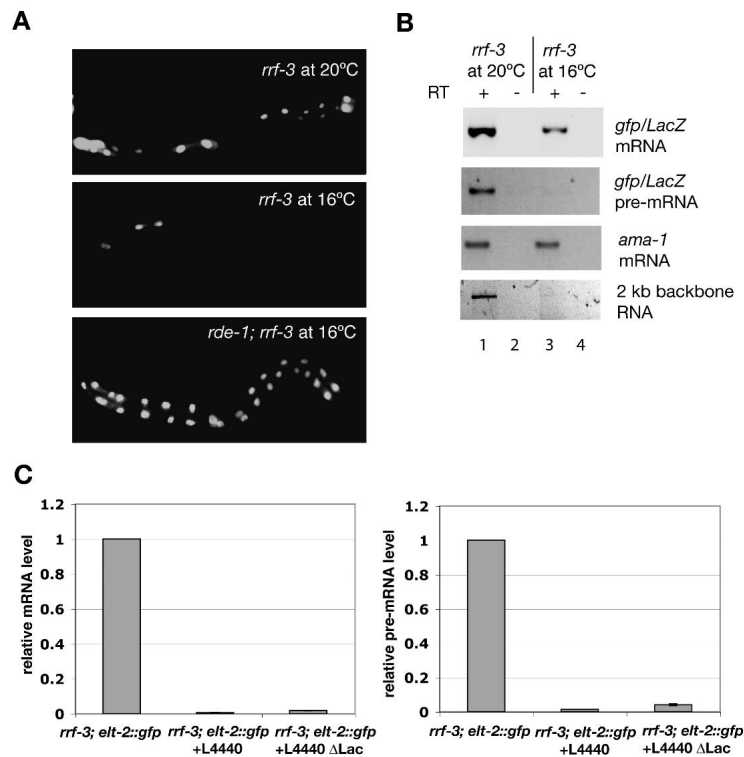
A

rrf-3 at 20°C

rrf-3 at 16°C

rde-1; rrf-3 at 16°C

B

Figure 5. Silencing of *elt-2::gfp/LacZ* in an *rrf-3* background. (*A*) *rrf-3;elt-2::gfp/LacZ* at 20°C (*top*); *rrf-3;elt-2::gfp/LacZ* at 16°C (*middle*); *rde-1; rrf-3;elt-2::gfp/LacZ* at 16°C (*bottom*). (*B*) RT–PCR of *gfp/LacZ* transgene; amplification with exon-specific primers (*top*), intron-specific primers (*middle*); *ama-1* control (*lower middle*), and primers specific for the 2-kb backbone sequence (*bottom*). (*C*) Real-time RT–PCR quantification of the relative levels of *gfp/LacZ* mRNA and *gfp/LacZ* pre-mRNA during silencing induced by feeding the L4440 or L4440 ΔLacZ vectors; *ama-1* expression was used for internal reference. Mean values and ranges of the relative *LacZ/ama-1* ratios based on two real-time RT–PCR trials are shown.

C

C-terminal domain (CTD) of polymerase II. Acetylated histones H3 and H4 typically associate with actively transcribed genes, while histone deacetylation correlates with transcriptional silencing.

We compared the level of histone H4 acetylation at the *elt-2::gfp/LacZ* locus in *rrf-3;elt-2::gfp/LacZ* strain cultured at 20°C with or without feeding with the L4440 silencing vector, and detected a decrease in histone acetylation under the silenced conditions (Fig. 6A,B). We tested the association of acetylated histone H4 with *elt-2::gfp/LacZ* DNA at two different regions of the transgene using two different sets of primers (Fig. 6A, top and middle). The set of primers used in the middle panel of Figure 6A is the same as in the middle panel of Figure 4A. We also tested the association of acetylated histone H4 with the 2-kb fragment of the backbone DNA and detected a decrease upon silencing. As an internal control, we assayed the acetylation levels of histone H4 at the *act-3* locus, a transcriptionally active actin gene. As expected, *act-3* association with acetylated H4 did not change upon feeding (Fig. 6A). We did not detect DNA precipitation by the agarose beads with-

out antibody (Fig. 6A) or with several nonspecific antibodies in independent ChIP experiments (data not shown).

We quantified the ChIP results presented in Figure 6A and several additional experiments by real-time PCR using a set of primers amplifying a short DNA segment (Fig. 6A, top). Real-time PCR analysis indicated an average 2.5-fold decrease in the relative ratios of *elt-2::gfp/LacZ* transgene DNA to *act-3* DNA when chromatin was precipitated with antibody to acetylated histone H4 under nonsilenced and silenced conditions, respectively (Fig. 6B, top). We detected a similar decrease in histone acetylation when an L4440 vector lacking LacZ fragment was used for the induction of silencing (Fig. 6B, top), indicating a common underlying mechanism. ChIP experiments with polymerase II CTD antibodies also detected less polymerase associated with the *elt-2::gfp/LacZ* under silenced conditions (Fig. 6B, bottom). These results strongly indicate that transcription is reduced at the *elt-2::gfp/LacZ* locus.

We next tested whether histone H4 acetylation is altered in RNAi pathway mutant backgrounds affecting

Table 2. *Effect of histone deacetylase inhibitors on* rrf-3; elt-2::gfp *silencing at 16°C*

GFP expression	No HDAC inhibitor	TSA (4 μM)	Apicidin (2 μM)	n-butyrate (6.7 mM)
Expression in more than 5 nuclei	2/40[a] (5%)	26/41 (63%)	21/42 (50%)	32/40 (80%)
Complete silencing	15/40 (38%)	0/41 (0%)	1/42 (2%)	0/40 (0%)

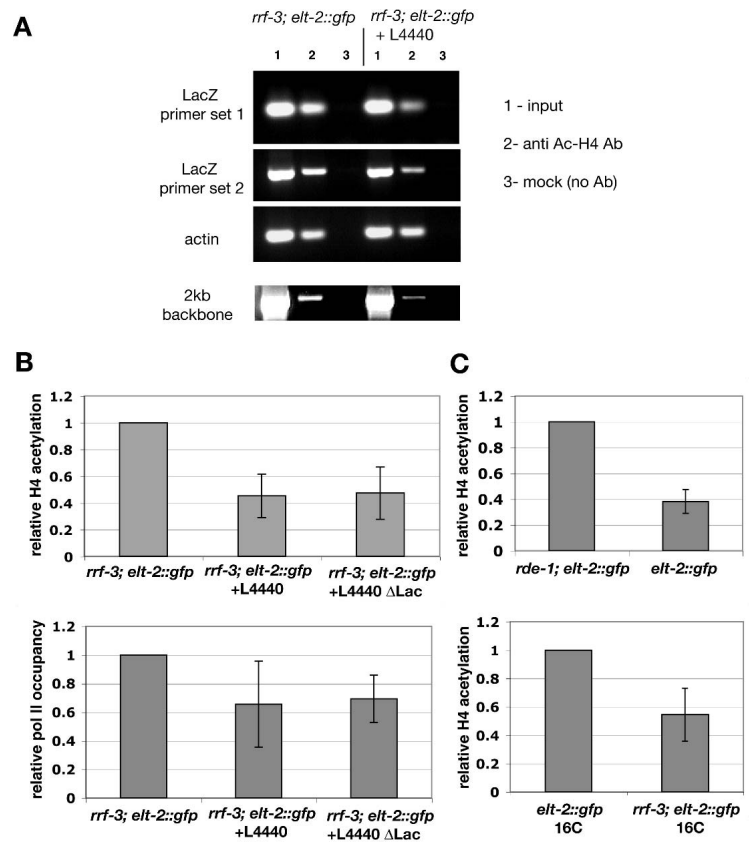[a]Fraction of worms showing phenotype of a total number scored.

Figure 6. *elt-2*::*gfp*/*LacZ* silencing is associated with decrease in histone H4 acetylation and polymerase II association. (*A*) ChIP with anti-acetyl-H4 antibodies was performed on extracts from *rrf-3*; *elt-2*::*gfp* strain with (*right*) and without (*left*) L4440 feeding. PCR products were amplified with primers specific for the *gfp*/*LacZ* transgene (*top*), 2-kb backbone sequence (*bottom*) or *act-3* gene (*middle*) as an internal control. (Lane *1*) DNA purified from 1/40 of input material was used. (Lane *2*) DNA bound to agarose A beads with anti-acetyl-H4 antibodies. (Lane *3*) DNA bound to agarose A beads without antibody. (*B,C*) Real-time PCR quantification of the relative amounts of *gfp*/*LacZ* DNA precipitated by anti-acetyl-H4 antibodies or 8WG16 Pol II antibodies at indicated conditions, *act-3* was used as internal reference. Mean values and ranges of the relative *LacZ*/*actin* ratios from two to three independent experiments are shown.

elt-2::*gfp*/*LacZ* expression. Indeed, the level of H4 acetylation at *elt-2*::*gfp*/*LacZ* locus was higher in the *rde-1* mutant as compared with wild-type transgenic strain (Fig. 6C, top), and it was lower in the *rrf-3* mutant at 16°C as compared with wild-type worms cultured under the same conditions (Fig. 6C, bottom). These results indicate that RNAi-related processes affects background *elt-2*::*gfp*/*LacZ* expression at the chromatin level.

Candidate screen for effectors of RNAi-TGS

Induction of *elt-2*::*gfp*/*LacZ* transcriptional silencing by feeding of the L4440 vector represents a convenient system, where the connection between RNAi and subsequent chromatin-based silencing can be studied. Since fragments of nearly all *C. elegans* genes have been cloned into the L4440 vector for the purpose of large-scale phenotypic analysis (Kamath and Ahringer 2003), this resource represents a useful tool for finding genes that affect silencing induced by the L4440 vector using the strategy described earlier in this work.

Approximately 100 genes known to be involved in RNAi or transgene silencing by previous studies, as well as candidate genes with the potential to be involved in RNAi-TGS, including histone deacetylases, potential histone methyltransferases, chromodomain-containing genes, and genes involved in chromatin remodeling (Supplementary Table 1) were simultaneously tested for

an effect on GFP silencing induced by the L4440 vector in a wild-type and *rrf-3 (pk1426)* background. Silencing was stronger in *rrf-3* mutants than in wild-type worms (Supplementary Fig. 3), and it was generally difficult to relieve *elt-2*::*gfp*/*lacZ* silencing in the *rrf-3* background. Thirty-seven percent of the candidates affected L4440-induced silencing in a wild-type background. A subset of those, comprising 12% of the candidate genes, affected L4440-induced silencing in *rrf-3* mutant worms.

This screen confirmed that known RNAi pathway genes were involved in silencing; see *rsd-3*, *rsd-6*, *tsn-1*, *mut-16*, and *smg-5* in Table 3. Some of the genes affecting silencing in this system overlapped with a set of genes involved in transposon silencing, most notably the F55F8.3 gene, which encodes a protein with WD domain and G-β repeats (Vastenhouw et al. 2003). The *tsn-1* gene implicated in *Drosophila* RNAi and miRNA pathways (Caudy et al. 2003) also affected silencing in this system. In agreement with the possible implication of histone modifications in the silencing process, we found that histone deacetylase *hda-2*, and genes encoding potential SET-domain methyltransferases, *set-1*, *set-2*, and T21B10.5 are also involved. We identified the HP1 homolog, *hpl-2*, to be important for RNAi-TGS earlier in this work; we found that another HP1 homolog, *hpl-1*, also affected silencing, but to a lesser degree. Beside Hpl-1 and Hpl-2, two other chromodomain proteins, Mrg-1 (Fujita et al. 2002) and T09A5.8 are also involved

Table 3. *Genes affecting RNAi-TGS of* elt-2::gfp/LacZ

Genes involved in RNAi

Gene	Name	Domain/function	Desilencing in WT	Desilencing in *rrf-3* mutant
B0379.3	*mut-16*	Chromatin organization (Vastenhouw et al. 2003)	++	++
W02D3.8	*smg-5*	Nucleotide binding protein (Domeier et al. 2000)	++	−−
F16D3.2	*rsd-6*	Tudor domain (Tijsterman et al. 2004)	++	++
F10G7.2	*tsn-1*	Tudor domain (Caudy et al. 2003)	++	−−
K12H4.8	*dcr-1*	dsRNa binding, RNAse III (Grishok et al. 2001; Knight and Bass 2001)	++	++
C34E11.1	*rsd-3*	ENTH domain (Tijsterman et al. 2004)	++	−−
K08H10.7	*rde-1 (ne300)*	PAZ/PIWI domain (Tabara et al. 1999)	+++	ND
T20G5.11	*rde-4 (ne299)*	dsRNA binding (Tabara et al. 2002)	+++	ND
F26A3.8	*rrf-1 (pk1417)*	RdRP (Sijen et al. 2001)	+++	ND
		Genes implicated in transposon silencing (Vastenhouw et al. 2003)		
K07A12.3	*asg-1*	mitochondrial ATP synthase g subunit	++	−−
F55F8.3		G-protein beta WD-40 repeat, similar to U4/U6 SNP	++	++
W01B11.3		snoRNA binding	++	++
ZK858.7		Translation initiation factor	++	−−
Y54E5A.4	*npp-4*	Nucleoporin FG repeat	++	+
Y106G6H.2	*pab-1*	Poly(A) binding protein	++	−−
F35G12.10	*asb-1*	Mitochondrial ATP synthase	++	−−
M01F1.3		Lipoic acid synthase, radical SAM	++	++
F37C12.4	*rpl-36*	Ribosomal protein L36	++	++
C08F8.2		ATP dependent helicase activity	++	−−
D2096.8		Nucleosome assembly protein	++	−−
		Genes with a chromatin-related function		
C08B11.2	*hda-2*	Histone deacetylase (Pothof et al. 2003)	++	−−
C04A2.3	*egl-27*	BAH/ELM2 domain (Solari et al. 1999)	++	−−
T09A5.8		Chromo domain	++	−−
K01G5.2	*hpl-2*	Chromo domain (Couteau et al. 2002)	++	−−
T26A5.7	*set-1*	SET domain (Terranova et al. 2002)	+	−−
C26E6.9	*set-2*	SET domain (Terranova et al. 2002)	++	−−
T21B10.5		Contains Zn-finger, SET domains	+	−−
Y43C5A.6	*rad-51*	DNA binding (Takanami et al. 1998)	++	−−
M04B2.3	*gfl-1*	Chromatin binding (Dudley et al. 2002)	++	−−
K08H2.6	*hpl-1*	Chromo domain (Couteau et al. 2002)	+	−−
Y37D8A.9	*mrg-1*	Chromo domain (Fujita et al. 2002)	++	++
F54F2.2	*zfp-1*	Chromatin/DNA binding (Dudley et al. 2002)	++	−−
C50E10.4	*sop-2*	SAM domain, Hox gene regulation (Zhang et al. 2004a,b)	++	+
C25A1.10	*dao-5*	Nuclear phosphoprotein, similar to sop-2 (BLAST)	++	−−
F55F8.4		Related to Tas3 (Verdel et al. 2004)	+	−−
		Genes involved in RNA binding and/or translational regulation		
F18A11.1	*puf-6*	Pumilio/Puf RNA binding (Wickens et al. 2002)	++	−−
B0273.2	*puf-7*	Pumilio/Puf RNA binding (Wickens et al. 2002)	++	−−
F48F7.1	*alg-1*	PAZ/PIWI domain (Grishok et al. 2001)	+	−−
B0035.12		RNA binding region, poly(A) binding	++	++
D1037.1		dsRNA binding	++	−−
		Genes involved in sumoylation		
K12C11.2	*smo-1*	Ubiquitin-like protein	+	−−
W02A11.4	*uba-2*	SUMO-activating complex	++	+

(+++) 100% of worms desilenced; (−) 80–100% of worms completely silenced; (++) 80–100% of worms desilenced; (−−) 100% of worms completely silenced; (+) >50% of worms desilenced.

(Table 3). We identified two PAZ-PIWI domain proteins, Rde-1 and Alg-1, as effectors in the transcriptional silencing pathway. The other 10 PAZ-PIWI genes tested, including *piwi*-related genes, *prg-1* and *prg-2*, did not have an effect on silencing (Supplementary Table 1). Finally, we found that the F55F8.4 gene, which encodes a protein related to RITS component Tas3 (Verdel et al. 2004; Supplementary Fig. 4) also affected silencing.

The recent discovery of RNA binding by *C. elegans* Polycomb Group gene *sop-2* (Zhang et al. 2004a) prompted us to test it in this system. Down-regulation of *sop-2* by RNAi prevented L4440-induced silencing in

wild-type and *rrf-3* worms (Supplementary Fig. 3, Table 3). Sop-2 might play an important role in connecting the RNAi pathway and chromatin-repressing components in homology-dependent silencing.

We also found that genes important for sumoylation, *smo-1* and *uba-2*, have a role in RNAi-TGS of the transgenic array. Many chromatin repressor proteins, including Sop-2 (Zhang et al. 2004b), require sumoylation for their function.

Discussion

In this study, we describe a system in *C. elegans* that leads to somatic transcriptional gene silencing via the RNAi pathway. Prior to this study, a role for RNAi in the initiation of TGS had not been established in *C. elegans*. Repetitive transgenes in the germline of *C. elegans* are silenced at the transcriptional level (Kelly and Fire 1998). This silencing is maintained across generations and is very stable, making it difficult to study the initiation of the process.

In the silencing of *elt-2::gfp/LacZ* transgene in the soma by feeding bacteria containing dsRNA, we assume that dsRNA adds to the level of dsRNA produced from the transgenic array and stimulates transcriptional silencing (Fig. 7). An increase in the level of secondary siRNAs produced by RdRp also is likely to play a role in this process (Fig. 7). There are numerous lines of evidence that *elt-2::gfp/LacZ* silencing in the soma is different from RNAi-PTGS and occurs predominantly at the level of transcription. First, this soma transgene silencing depends upon Alg-1, whereas RNAi mediated post-transcriptional silencing does not (Grishok et al. 2001). Second, generation of nuclear intron containing pre-mRNA is inhibited during silencing of the *elt-2::gfp/LacZ* transgene, which contrasts with previous results where post-transcriptional gene silencing by RNAi reduced the levels of mRNAs, but not nuclear pre-mRNAs (Montgomery et al. 1998). Third, inhibition of *gfp/LacZ* pre-mRNA production is caused by a plasmid containing no homology to the coding region of the *gfp/LacZ* transgene. Fourth, the soma transgene silencing was dependent upon heterochromatin factors such as HP1 type proteins. Fifth, treatment of worms with drugs that inhibit histone deacetylases suppressed the silencing of the transgene. Sixth, ChIP assays showed that the silenced soma transgene contained less acetylated histone H4 protein and less polymerase II than the nonsilenced state. This ensemble of observations strongly indicates that genes can be silenced in the soma of *C. elegans* at
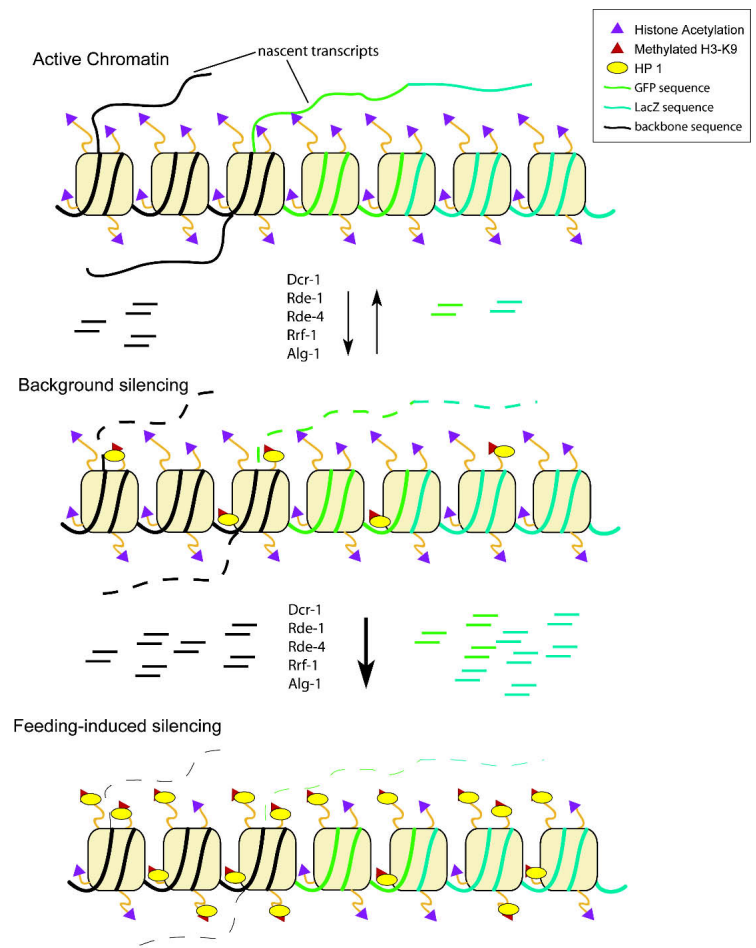

Figure 7. Schematic model showing a possible mechanism of RNAi-TGS at the *elt-2::gfp/LacZ* repetitive array. Transcription of the *gfp/LacZ* pre-mRNA (green/blue line) and bidirectional transcription in the repetitive array (black lines) occurs in the transgenic worms. dsRNA produced from the locus would be processed by Dicer, generating siRNAs, which might induce a low level of background silencing of the array. However, silencing of the array is not efficient without additional stimuli. Feeding worms with dsRNA produced from the L4440 vector brings the level of LacZ and backbone-specific siRNA to the threshold level, stimulating chromatin modifications, HP1 localization, and TGS at the locus.

the level of transcription through an RNAi-related process.

The RNA-dependent RNA polymerase *rrf-3* has been shown to antagonize RNAi processes (Simmer et al. 2002). Since other *C. elegans* RdRps, Rrf-1, and Ego-1, are essential for RNAi (Smardon et al. 2000; Sijen et al. 2001), Rrf-3 is thought to compete with them for a limited amount of shared components. We demonstrate that in the *rrf-3* mutant, the *elt-2∷gfp/LacZ* transgene becomes completely silenced at 16°C, and this effect depends on Rde-1. Under these conditions, the levels of transgene pre-mRNA and mRNA are greatly reduced in the silenced worms, and this inhibition is reversed if histone deacetylation is suppressed by drug treatment. These results suggest that dsRNA produced from the repetitive transgene can induce transcriptional silencing, and that RdRp activity and the PAZ-PIWI protein Rde-1 are essential for this process.

The RNAi-TGS pathway in the soma of *C. elegans* is probably similar to the recently described process in *S. pombe* (Noma et al. 2004), where siRNAs guide one or more types of RITS (RNA-induced initiation of transcriptional gene silencing) complexes to modify histones and produce heterochromatin. The requirement for feeding dsRNA in the silencing of the transgene in the soma could reflect the need to generate and amplify siRNAs, perhaps through the action of RNAi-PTGS and RdRp activities on the mRNA from the transgene. However, this is likely to be transient, as the mRNA would disappear as transcriptional silencing proceeds. The active PTGS process in *C. elegans* is accompanied by the constant production of secondary antisense siRNAs (Sijen et al. 2001), which are readily detectable by Northern blot (A. Grishok and C.C. Mello, unpub.) Using identical methods, we have not been able to detect siRNAs corresponding either to the *LacZ* or the backbone vector sequences (data not shown). This is likely due to a much lower abundance of siRNAs during TGS as compared with PTGS. For example, Birchler and colleagues detected siRNAs associated with PTGS of a transgene in *Drosophila*, but were unable to detect siRNAs when a similar transgene was silenced by TGS (Pal-Bhadra et al. 2002).

Dudley and colleagues (Dudley at al. 2002) have previously suggested that transcriptional regulation occurs during RNAi in *C. elegans*. They used RNAi to knock down genes known to be important for transcriptional regulation, *zfp-1* (Zinc Finger Protein 1) and *gfl-1* (GAS41- like), as well as genes with chromatin function, *mes-3*, *mes-4*, *mes-6*, and found that at some dsRNA concentrations this led to a decrease in RNAi efficiency when additional genes were targeted. We found using an RNAi screen to investigate RNAi-TGS, that targeting *zfp-1* and *gfl-1* reduced the efficiency of RNAi-TGS (Table 3). These genes are probably not important for RNAi-PTGS. For example, a *zfp-1* deletion mutant *zfp-1(ok554)* was completely sensitive to RNAi when we used bacteria expressing dsRNA specific to either *pos-1*, *unc-22*, or *alg-1/2* genes. It is possible that under conditions used by Dudley et al. (2002) for dsRNA injec-

tions, the RNAi pathway initiates transcriptional silencing.

We find it very intriguing that Polycomb Group gene *sop-2* is important for the RNAi-TGS pathway. Sop-2 has been recently shown to bind single stranded and double stranded RNA, and RNA binding by this protein is important for its function in Hox gene repression (Zhang et al. 2004a,b).

The requirement for the *alg-1* gene in RNAi-TGS clearly separates this process from RNAi-PTGS where *alg-1* is not essential (Grishok et al. 2001). Alg-1 and Alg-2 are PAZ-PIWI proteins that play a critical role in the production and function of miRNAs in *C. elegans* (Grishok et al. 2001). miRNAs primarily negatively regulate their targets at the level of translation, but can also target mRNA cleavage (for review, see Bartel 2004). It is not clear whether Alg-1 plays a role in transcriptional silencing directly, similarly to the *S. pombe* protein Ago1 (Noma et al. 2004; Verdel et al. 2004), or whether some miRNAs regulated by Alg-1 have targets involved in RNAi-TGS.

RNAi-TGS might have a role in transposon silencing in *C. elegans*. We found that 11 genes, identified in a screen for transposon silencing factors in the germline by Vastenhouw and colleagues, also affected RNAi-TGS of the *elt-2∷gfp/LacZ* (Table 3). Transposon silencing in *C. elegans* is thought to occur mostly post-transcriptionally, based on the lack of significant reduction of the pre-mRNA (Sijen and Plasterk 2003). However, some genes with potential function in the nucleus, such as the D2096.8 gene (encoding a potential nucleosome assembly protein) and *npp-4* (encoding nucleoporin), affect both transposon silencing (Vastenhouw et al. 2003) and the RNAi-TGS described here (Table 3).

A dsRNA feeding library containing bacteria targeting almost all *C. elegans* genes is a useful tool for large-scale phenotypic screens (Kamath et al. 2003). However, the finding that the vector used for construction of the library could elicit homology-dependent silencing of certain transgenes should be taken into account when transgenic worm strains are used for large-scale screens.

Materials and methods

C. elegans *strains*

Worms were maintained on NGM plates seeded with OP50 bacteria. The following strains were used: LGI, *rrf-1(pk1417)*; LGII, *rrf-3 (pk1426)*; LGIII, *hpl-2 (ok1061)*, *rde-4 (ne299)*, *zfp-1 (ok554)*; LGV, *rde-1 (ne300)*; LGX, *alg-1 (gk214)*, *elt-2∷gfp/ LacZ*. Additional transgenic strains used are listed in Table 1.

elt-2∷gfp/LacZ *silencing by feeding worms dsRNA*

RNAi by feeding was performed essentially as described in Conte and Mello (2003). Briefly, HT115 (DE3) bacteria were transformed with L4440 feeding vector and cultured on LB plates with 50 µg/mL ampicillin and 15 µg/mL tetracycline. A single colony was inoculated into 10–20 mL bacterial cultures in LB/Amp overnight; these cultures were used for seeding NGM/Amp/IPTG plates. HDAC inhibitors were added to the

bacterial cultures to the final concentrations indicated in Table 2 before seeding the plates. Single adult *elt-2*::*gfp*/*LacZ* or *rrf-3*;*elt-2*::*gfp*/*LacZ* hermaphrodites were put on each plate and their L4 or adult progeny were scored for GFP expression in the intestine. Experiments were performed in triplicate for each bacterial strain tested for silencing. Worms grown on plates were directly viewed with Zeiss Axoplan 2 microscope at 100× magnification using the appropriate filter. OpenLab 3.1.7 software (Improvision) was used for capturing images (100×) of live worms put on 2% agarose pads; the same exposure time (typically ~500 msec) was used for capturing images to be compared in the same experiment.

L4440 clones

The L4440 cloning vector, as well as *mes-1*, *let-2*, *pos-1*, and *unc-22* clones were obtained from the Mello lab. Fragments of *dcr-1*, *hpl-2*, and *alg-1* genes were amplified by PCR from *C. elegans* genomic DNA and cloned into L4440. The following primers were used: *dcr-1* (forward, GGTAAGAGCTGATTTACAATG; reverse, GAATCTTTAATCGGTCTACGA); *hpl-2* (forward, ATGTCGAGCAAATCAACAAAGC; reverse, TTAAAGCTCGTCGGCTTTTGGGT); *alg-1* (forward, ATGTCCGGCGGGCCGCAATATTTGCC; reverse, TCAAATAGAAACTAAATTAAATGCC). Additional bacterial clones containing L4440 with inserts of *C. elegans* genes constructed by Ahringer's group were obtained from MRC Geneservice.

Soaking *C. elegans* in dsRNA solution

LacZ dsRNA (1 kb) was prepared by in vitro transcription with T7 polymerase using PCR product as a template (Ambion). A portion of a LacZ gene (1 kb) was amplified by PCR from genomic DNA of *elt-2*::*gfp*/*LacZ* strain. PCR product was flanked with T7 promoter sequences (GCGTAATACGACTCACTATA) on both 5′ ends. Primers used for PCR amplification of LacZ sequence are as follows: forward, ATGACCATGATTACGGATTC; reverse, CAGCAGACCATTTTCAATC. A total of 10–15 adult *elt-2*::*gfp*/*LacZ* worms were soaked in 20 µL of 1 mg/mL LacZ dsRNA or water for 24 h, and their adult progeny was examined for GFP expression. Typically, progeny of LacZ dsRNA-soaked worms developed complete silencing of GFP in the intestine. Total RNA was prepared from the silenced progeny of soaked worms.

RT–PCR

Tri Reagent (MRC) was used for total RNA preparation from *C. elegans*, RNA was DNase treated with DNA-*free* reagent (Ambion). Of the total *C. elegans* RNA, 0.25 µg was used in 20 µL RT reaction with random hexamer primers (Ambion), and 1 µL of RT reaction was used to template a 50-µL PCR reaction. Primers used for amplification of 2-kb plasmid sequence are as follows: forward, AGGTGGCACTTTTCGGGGA; reverse, GCTTCCTCGCTCACTGACTCGC. Primers specific for *elt-2*::*gfp*/*LacZ* exon sequences are as follows: forward, exon 14, CTGCCATTGTCAGACATGT; reverse, exon 16, CAGAGGCACGGGCGCGAGATG. Primers specific for *elt-2*::*gfp*/*LacZ* intron sequences are as follows: forward, intron 14, CTAACTAACATGTTTCATTTAAATTTTCAG; reverse, intron 15, TAGTTAGTAGAACTCAGTTTAAACTTAC. Primers specific for *ama-1* exon sequences are as follows: forward, exon 3, GATCTACGATCTGTGCAAAGC; reverse, exon 4, CGAGTACTCGTTCGGCGGT.

Quantitative real-time PCR

The TaqMan system (Applied Biosystems) was used for cDNA amplification and SYBR Green dye (Qiagen) for the amplifica-

tion of DNA in ChIP assays. Reactions were run in triplicate on an ABI Prism 7000 Real-time PCR machine (Applied Biosystems). Relative fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. TaqMan probe and primers sets were designed using Primer Express Software (Applied Biosystems), probes for mature mRNAs were designed to span exon–exon junction, and the probe for *gfp*/*LacZ* pre-mRNA was intron specific. The sequences used were as follows: for *gfp*/*LacZ* mRNA, forward, exon 8, CAGGTCATGGATGAGCAGACG; reverse, exon 9, ACGGCGTTAAAGTTGTTCTGCTT; probe, TGCAGGATATCCTGCTGAT; for *gfp*/*LacZ* pre-mRNA, forward, exon 8, CAGGTCATGGATGAGCAGACG; reverse, intron 8, AAAATTTAAATGTTTAAAGTTAGTTAGTAACGAATAGTTT; probe, TGGTGCAGGATGTAAGTT; for *ama-1* mRNA, forward, GATGATCCGATGAATGATGGAAAG; reverse, CGGTATGATGGTTGATAGCGACC; probe, AGGTCGCAGGTGGATG; for *act-3*, forward, GCAGAAGGAAATCACCGCTCTTG; reverse, GCGATGATCTTGATCTTCATGG; for *gfp*/*LacZ* DNA amplification, primers were the same as for pre-mRNA.

ChIP

For preparation of protein lysates ~300–500 µL of packed L4 or young adult animals were used for each immunoprecipitation. Formaldehyde fixing, lysate preparation, and sonication was done according to Chu et al. (2002). ChIP was performed with Anti-acetyl-Histone H4 antiserum (#06-866 Upstate Biotechnology) according to the manufacturer's protocol; the same conditions were used for ChIP with 8WG16 Polymerase II antibodies (Covance).

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Transcriptional silencing of a transgene by RNAi in the soma of *C. elegans*

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