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The Role of RNA Editing by ADARs in RNAi

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Summary

Adenosine deaminases that act on RNA (ADARs) are RNA-editing enzymes that deaminate adenosines to create inosines in double-stranded RNA (dsRNA). Here we demonstrate that ADARs are not required for RNA interference (RNAi) and that they do not antagonize the pathway to a detectable level when RNAi is initiated by injecting dsRNA. We find, however, that transgenes expressed in the somatic tissues of wild-type animals are silenced in strains with deletions in the two genes encoding ADARs, *adr-1* and *adr-2*. Transgene-induced gene silencing in *adr-1;adr-2* mutants depends on genes required for RNAi, suggesting that a dsRNA intermediate is involved. In wild-type animals we detect edited dsRNA corresponding to transgenes, and we propose that editing of this dsRNA prevents somatic transgenes from initiating RNAi in wild-type animals.

Introduction

ADARs are a class of RNA editing enzymes that deaminate adenosines to create inosines in dsRNA (reviewed in Bass, 2002). Inosine is read as guanosine during translation, and thus, one function of editing is to generate multiple protein isoforms from the same gene. ADARs bind dsRNA substrates without sequence specificity, and the structure of the dsRNA determines how extensively it will be edited. Short, imperfectly base-paired dsRNAs are selectively edited, with only about 10% of the adenosines being deaminated at complete reaction, whereas long, highly base-paired dsRNAs are hyperedited, with up to 50% of the adenosines converted to inosines (Nishikura et al., 1991; Polson and Bass, 1994). Editing disrupts a stable A:U base pair to create a less stable I:U mismatch. Therefore, editing can alter both the sequence of the dsRNA and its structure, producing a molecule with increased single-stranded character.

In addition to being targeted by ADARs, dsRNA triggers a number of potent cellular responses, including the posttranscriptional gene silencing pathway known as RNA interference (RNAi). In an early step in RNAi, the initiating dsRNA is cleaved into small interfering RNAs (siRNAs), 21–23 nucleotides long, by the enzyme Dicer (Bernstein et al., 2001; Elbashir et al., 2001; Zamore et al., 2000). After processing by Dicer, siRNAs associate with a multicomponent complex called the RNA-induced silencing complex (RISC) that recognizes and cleaves the cognate message (Hammond et al., 2000; Zamore et al., 2000). siRNAs may also serve as primers for RNA-dependent RNA polymerases (RdRPs) that may be involved in perpetuating the RNAi response (Lipardi et al., 2001; Sijen et al., 2001).

Due to the ability of ADARs to create sequence and structural changes in dsRNA, ADARs could potentially antagonize RNAi by several mechanisms. For example, editing could prevent the dsRNA from being recognized and cleaved by Dicer (Bass, 2000). Consistent with this hypothesis, hyperedited dsRNA added to *Drosophila* cell-free extracts is inefficiently processed by Dicer and is a poor trigger of RNAi (Scadden and Smith, 2001). Additionally, if A to I changes occurred in siRNAs, they could potentially prevent the siRNAs from base pairing sufficiently with the cognate message to allow cleavage of the mRNA by the RISC. Likewise, editing in siRNAs could also interfere with the ability of an RdRP to recognize the siRNA as a primer. Although it seems plausible that ADARs could antagonize RNAi by one or more of these mechanisms, RNAi functions effectively in wild-type animals, suggesting that ADARs may be prevented from antagonizing the pathway in vivo.

In *C. elegans* a growing number of genes are known to be involved in the RNAi pathway (Domeier et al., 2000; Grishok et al., 2001; Ketting et al., 1999, 2001; Knight and Bass, 2001; Sijen et al., 2001; Tabara et al., 1999; Tijsterman et al., 2002; Winston et al., 2002). Several of the genes required for effective RNAi, including *rde-2*, *mut-2*, and *mut-7*, are also required for gene silencing induced by transgenes or cosuppression, indicating that these two pathways overlap. However, these two pathways are not identical. Not all of the genes required for RNAi are necessary for cosuppression, and likewise, not all of the genes involved in cosuppression are essential for RNAi (Dernburg et al., 2000; Ketting and Plasterk, 2000). Additionally, RNAi silences genes expressed in both the *C. elegans* germline and soma, whereas cosuppression silences genes expressed in the germline but rarely those expressed in somatic tissues.

Here we investigate the role of ADARs in RNAi using *C. elegans* strains containing deletions in the two genes encoding ADARs, *adr-1* and *adr-2*. We observe that *adr-1;adr-2* double mutants respond identically to wild-type animals when RNAi is initiated by injecting dsRNA. However, we find that *adr-1:adr-2* mutants differ from wild-type animals in that transgenes can induce gene silencing in somatic tissues of *adr-1;adr-2* mutants via the RNAi pathway. Transgene-induced silencing in *adr-1;adr-2* mutants is similar but not identical to cosuppression in the germline. We also present evidence that, in wild-type animals, dsRNA is generated from transgenes by an unknown mechanism but that editing prevents the dsRNA from initiating RNAi.

Results

adr-1 and *adr-2* Are Not Required for RNAi

Two genes that encode ADARs are found in the *C. elegans* genome: *adr-1* and *adr-2* (Hough et al., 1999; Ton-

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Table 1. ADARs Are Not Required for Efficient RNAi

dsRNA	Wild-Type	*adr-1;adr-2*
pos-1	100% *emb*, n = 789	100% *emb*, n = 691
unc-22	100% *unc*, n = 689	100% *unc*, n = 296
unc-54	100% *unc*, n = 792	100% *unc*, n = 521
unc-47	0% *unc*, n = 584	0% *unc*, n = 751

dsRNA corresponding to the genes listed was injected into wild-type and *adr-1;adr-2* double mutants at 0.5 mg ml^{-1}. The percentage of the F1 generation exhibiting the resulting RNAi phenotype for each dsRNA is shown.

kin et al., submitted). We have obtained animals with deletions in both *adr-1* and *adr-2*, and molecular and biochemical evidence indicates that *adr-1;adr-2* double mutants completely lack editing activity (Tonkin et al., submitted). To investigate the role of ADARs in RNAi, we injected dsRNA corresponding to several genes into *adr-1;adr-2* mutants. The resulting RNAi phenotype in the F1 generation was compared to that of progeny from wild-type animals injected with the same dsRNA (Table 1 and Figure 1). When animals were injected with *pos-1* dsRNA at 0.5 mg ml^{-1}, 100% of the progeny from both wild-type and *adr-1;adr-2* mutants displayed the *pos-1* embryonic lethal phenotype (Table 1). Similarly, dsRNA targeting *unc-22*, *unc-54*, and a GFP transgene produced an identical RNAi response (twitching, paralysis, and decreased fluorescence, respectively) in the progeny of wild-type and *adr-1;adr-2* mutants. Progeny of *adr-1;adr-2* mutants fed bacteria expressing dsRNA corresponding to *unc-22* also responded similarly to the progeny of wild-type animals grown under identical conditions (data not shown). These results indicated that ADARs are not required for an RNAi response.

We investigated whether ADARs were capable of antagonizing RNAi by injecting dsRNA at concentrations that resulted in an RNAi phenotype in only a fraction of the progeny. The percentage of wild-type animals that exhibit an RNAi phenotype is dependent on the concentration of dsRNA used to trigger the response (Parrish et al., 2000). If ADARs were capable of antagonizing RNAi, then the minimum dsRNA concentration needed to induce an RNAi response might be less in *adr-1;adr-2* mutants than in wild-type animals. When animals were injected with dsRNA corresponding to *unc-22* at concentrations that resulted in only a fraction of the progeny exhibiting the RNAi response, we did not observe any significant difference between *adr-1;adr-2* mutants and wild-type animals in the efficiency of the dsRNA to trigger RNAi (Figure 1G). Similar results were obtained when *pos-1* dsRNA was injected at lower concentrations (data not shown). To determine if the site of injection was a determinant in the ability of ADARs to antagonize RNAi, dsRNA was injected into the body cavity of wild-type and *adr-1;adr-2* mutants at 1.25 ng μl^{-1}, and the injected animals were examined for the *unc-22* phenotype on subsequent days. Wild-type and *adr-1;adr-2* mutants exhibited similar sensitivity to RNAi under these conditions (data not shown). These data indicate that ADARs do not substantially antagonize RNAi under the conditions of our assays.

Neuronal Resistance to RNAi Is Not Due to ADAR Activity

Neuronally expressed genes in *C. elegans* are resistant to RNAi when dsRNA is delivered by injection (e.g., Figure 1E), and effective gene silencing in neurons occurs only when the initiating dsRNA is expressed from a transgene (Tavernarakis et al., 2000). In *C. elegans*, *adr-1* is expressed at the highest levels in neurons and the developing vulva (Tonkin et al., submitted), and in mammals, ADAR1 is expressed at the highest levels in the brain (Paul and Bass, 1998). Taken together, these data suggested that the partial ineffectiveness of RNAi in the neurons of *C. elegans* might be due to ADARs antagonizing the pathway.

We examined whether the partial resistance of neurons to RNAi was due to the activity of ADARs by injecting GFP dsRNA into *adr-1;adr-2* mutants expressing GFP in the neurons and most other cells. Following injection, GFP was effectively silenced in the majority of cell types, except neurons in the F1 generation of both wild-type and *adr-1;adr-2* mutants (Figures 1A–1F). We also targeted *unc-47*, which is an endogenous gene that is expressed in the neurons. Progeny from *adr-1;adr-2* mutants and wild-type animals injected with *unc-47* dsRNA failed to exhibit the *unc-47* phenotype (Table 1). These data imply that ADARs do not contribute to the partial resistance of neuronally expressed genes to RNAi.

Transgenes Induce RNAi in the Somatic Tissues of *adr-1;adr-2* Mutants

We also examined the effectiveness of RNAi in *adr-1;adr-2* mutants when the pathway was triggered from dsRNA produced from transgenes. The *adr-1;adr-2* homozygous deletions were introduced into a previously described transgenic strain (Knight and Bass, 2001) that contains a *sur5::GFP* transgene, a *rol-6* marker, and a heat shock-inducible RNA hairpin (inverted repeat) construct corresponding to the GFP sequence (*hsp16-2$_p$GFP[IR]*) (Tavernarakis et al., 2000). Wild-type animals carrying these transgenes exhibited strong GFP fluorescence that was silenced following the induction of RNAi by heat shock (Figures 2A and 2B). Surprisingly, when this transgenic line was crossed into an *adr-1;adr-2* mutant, we observed strong GFP silencing even without inducing synthesis of dsRNA by heat shock (Figures 2C and 2F). GFP was silenced in all cell types in the *adr-1;adr-2* mutants, including the neurons. To confirm that the loss of GFP fluorescence in the *adr-1;adr-2* mutants was not due to a loss of the *sur5::GFP* transgene, we crossed the *adr-1;adr-2* transgenic hermaphrodites with wild-type males. The resulting progeny, which were heterozygous at each *adr* locus, all exhibited robust GFP fluorescence (data not shown), indicating that the transgene was present in the *adr-1;adr-2* double mutant but was being silenced.

Strong silencing was also observed in both the *adr-1* and *adr-2* single mutants, although not to the level observed in the double mutant (Figures 2D–2F). In *adr-2* (−/−) animals GFP was silenced slightly less than in the *adr-1;adr-2* double mutant, and in *adr-1* (−/−) animals GFP was silenced less than in *adr-2* mutants (Figure 2D and 2E). These data are consistent with editing and chemotaxis defects seen in *adr* mutants, which indicate

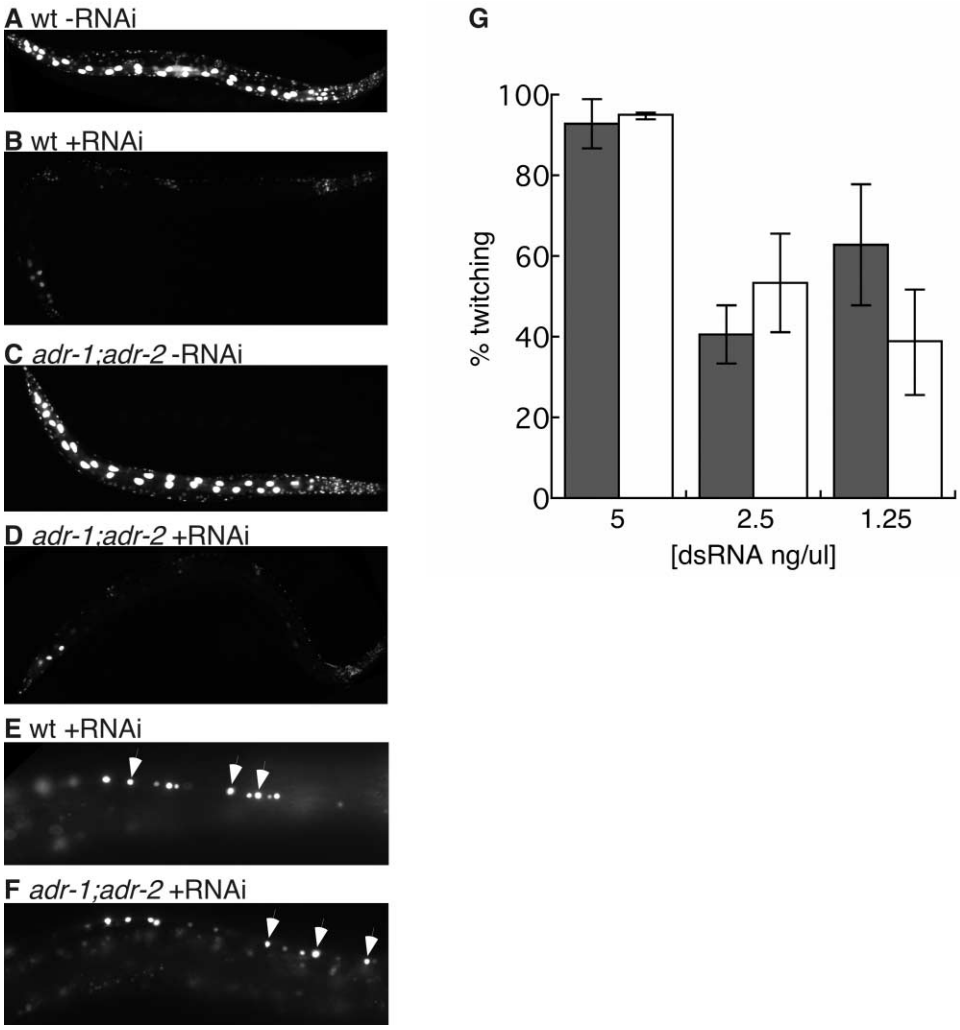


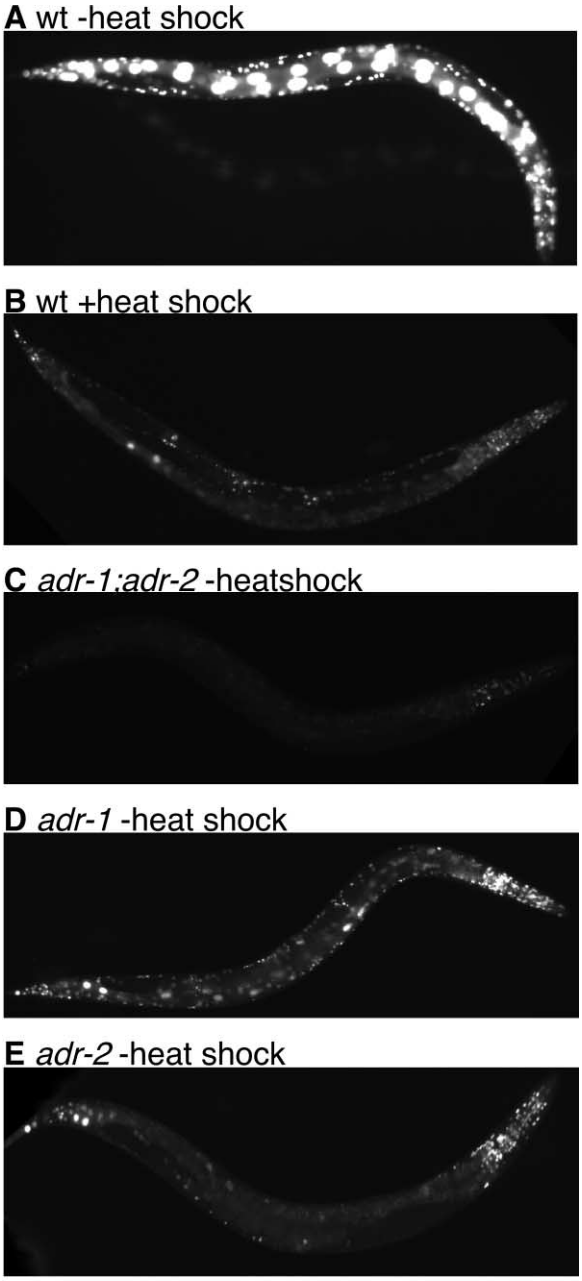
Figure 1. RNAi Initiated by Injecting dsRNA Is Effective in Wild-Type and *adr-1;adr-2* Mutants

Animals shown in (A)–(D) contain an integrated array consisting of complex DNA and the *sur5::GFP* construct. GFP is expressed from the *sur5::GFP* transgene in the nucleus of several different cell types. Wild-type (A) and *adr-1;adr-2* double mutants (C) exhibit strong GFP fluorescence. After injecting GFP dsRNA, GFP is silenced in most cells of wild-type (B) and *adr-1;adr-2* animals (D). A close-up of the neuronal cells (arrows) reveals that they remain resistant to RNAi in wild-type (E) and *adr-1;adr-2* mutants (F). (G) *unc-22* dsRNA was injected at 5, 2.5, and 1.25 ng ul^{-1} into wild-type (shaded) and *adr-1;adr-2* (white) animals. The RNAi phenotype was scored in the F1 generation. Error bars indicate the standard error of the mean.

that *adr-1* (−/−) animals have less severe phenotypes than *adr-2* (−/−) animals (Tonkin et al., submitted). GFP silencing has not been lost in the *adr-1;adr-2* double mutant or in either of the single mutants through several generations.

To determine if GFP expression was being silenced in *adr-1;adr-2* mutants through the RNAi pathway, we introduced mutations in genes required for RNAi into the *adr-1;adr-2* animals containing the GFP transgenes. *rde-1* is required for RNAi under all conditions in all tissues, and its gene product is thought to act early in the pathway, perhaps by stabilizing siRNAs (Grishok et al., 2000; Parrish and Fire, 2001; Tabara et al., 1999). When the *rde-1* homozygous mutation was introduced into *adr-1;adr-2* animals containing the transgenes described above, GFP silencing was suppressed (Figure 3). We also observed suppression of GFP silencing in *adr-1;adr-2* mutants when we targeted the gene encoding Dicer (*dcr-1*) by RNAi (data not shown).

Our data indicated that either the *sur5::GFP* transgene or the *hsp16-2$_p$GFP(IR)* transgene was triggering RNAi in the *adr-1;adr-2* double mutant. To determine which of the two GFP-containing transgenes was responsible for inducing RNAi in the *adr-1;adr-2* double mutant, we used another strain containing the *sur5::GFP* transgene and a heat shock-inducible *unc-22* inverted repeat (*hsp16-2$_p$unc-22[IR]*). When grown at 20°C, wild-type worms bearing this array had strong GFP fluorescence but did not display the characteristic twitching phenotype of an *unc-22* null mutant (Figure 4A). Upon heat shock, wild-type animals retained their GFP fluorescence but now exhibited the *unc-22* phenotype (Figures 4A and 4B). When this transgenic strain was crossed into the *adr-1;adr-2* mutant, animals displayed the *unc-*



F

Genotype	% GFP silencing
adr-1(+/+);*adr-2*(+/+)	0 (215)
adr-1(+/+);*adr-2*(-/-)	100 (241)
adr-1(-/-);*adr-2*(+/+)	100 (114)
adr-1(-/-);*adr-2*(-/-)	100 (133)

Figure 2. Transgenes Induce Gene Silencing in *adr-1;adr-2* Mutants

Animals in each panel contain an extrachromosomal array consisting of *sur5::GFP*, *hsp16-2$_p$GFP(IR)*, and *rol-6*.

(A) Wild-type animals exhibit strong GFP fluorescence when grown at 20°C.

(B) Following induction of RNAi by heat shock, GFP is substantially silenced in wild-type animals.

(C) *adr-1;adr-2* double mutants exhibit strong GFP silencing even without inducing RNAi by heat shock.

(D and E) GFP silencing is also observed without heat shock in both the *adr-1* (D) and *adr-2* (E) single mutants.

(F) L4 and young adult progeny from wild-type, *adr-1*(−/−), *adr-2*(−/−), and *adr-1*(−/−);*adr-2*(−/−) animals were examined for GFP silencing without exposure to heat shock. The number of animals examined for each genotype is shown in parentheses.

22 phenotype even without heat shock and also had substantially reduced GFP fluorescence (Figure 4C). These data indicate that transgenes do not need to be designed to express a dsRNA in order to trigger RNAi in *adr-1;adr-2* mutants and demonstrate that endogenous genes can also be silenced by transgenes in the *adr-1;adr-2* mutants.

To further confirm that the *sur5::GFP* transgene and transgenes with synthetic inverted repeats were capable of inducing RNAi in *adr-1;adr-2* mutants, we created transgenic lines consisting of the *sur5::GFP* transgene alone or the *hsp16-2$_p$unc-22(IR)* transgene together with the *rol-6* construct. Wild-type animals harboring the *sur5::GFP* construct exhibited strong GFP fluorescence, but in *adr-1;adr-2* mutants GFP was substantially silenced (data not shown). Likewise, wild-type animals with the *hsp16-2$_p$unc-22(IR)* transgene appeared normal at 20°C, but *adr-2* (−/−) animals with the same transgene exhibited the *unc-22* phenotype when grown at 20°C without heat shock (data not shown). These data confirm that transgenes do not require a synthetic inverted repeat to induce silencing in *adr-1;adr-2* mutants. Interestingly, we did not observe silencing of the *rol-6* marker in any of our transgenic lines containing this sequence.

Transgenic *C. elegans* strains are created by injecting DNA into the syncytial gonad. The injected DNA can have several fates, including assembly into an extrachromosomal array that is inherited in a fraction of the progeny (Mello and Fire, 1995). Extrachromosomal arrays typically consist of hundreds of copies of the transgene arranged in tandem repeats (Hope, 1991; Mello et al., 1991; Stinchcomb et al., 1985). The extrachromosomal arrays described above were created by injecting plasmids containing the genes of interest, in some cases with an additional plasmid containing a marker gene that most likely would be assembled into the same array. Extrachromosomal arrays created in this manner trigger RNAi in *adr-1;adr-2* mutants as described above and can also induce cosuppression in the germline of wild-type animals (Gaudet et al., 1996; Jones and Schedl, 1995). Cosuppression, however, can be reduced by injecting the transgene of interest along with more complex DNA (Kelly et al., 1997). Complex DNA may reduce the repetitiveness of arrays by introducing random DNA between copies of the gene of interest (Kelly et al., 1997). We observed a similar reduction in transgene-induced RNAi in *adr-1;adr-2* mutants when we crossed in an array containing complex DNA in addition to *sur5::GFP* (Figure 1C). Thus, as in cosuppression, the repetitiveness of a transgene in an array may be a critical determinant in its ability to induce RNAi in *adr-1;adr-2* double mutants. The complex array used for the experiments in Figure 1 had also been integrated into the genome; therefore, we cannot rule out the possi-

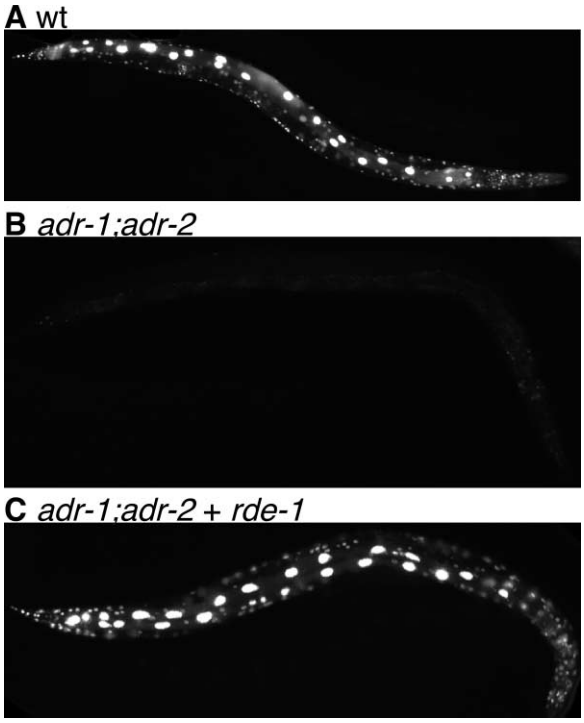


Figure 3. Transgene-Induced Gene Silencing in *adr-1;adr-2* Mutants Occurs through the RNAi Pathway

Animals shown in (A)–(C) contain an extrachromosomal array consisting of *sur5::GFP*, *rol-6*, and *hsp16-2$_p$GFP(IR)*. All animals were grown at 20°C.

(A) GFP is expressed in wild-type animals.

(B) GFP is silenced in *adr-1;adr-2* double mutants even without heat shock.

(C) When *rde-1*, which is an essential gene in RNAi, is crossed into the *adr-1;adr-2* mutants, GFP silencing is suppressed.

bility that integration of the array may also have contributed to its inability to induce silencing in an *adr-1;adr-2* double mutant.

Evidence of Edited dsRNA Corresponding to Transgenes Is Found in Wild-Type Animals

Our data indicated that transgenes were capable of triggering RNAi in the *adr-1;adr-2* double mutants, and the requirement of *dcr-1* and *rde-1* strongly suggested that a dsRNA intermediate was involved. We hypothesized that the silencing in the *adr-1;adr-2* animals was initiated by small amounts of dsRNA synthesized either from transgenes containing inverted repeats (even without heat shock) or from aberrant transcription from the *sur5::GFP* transgene. In wild-type animals this small amount of putative dsRNA may be deaminated, which would prevent it from triggering RNAi, but in *adr-1;adr-2* mutants, the dsRNA would retain its original sequence and structure and be capable of initiating RNAi (Figure 5).

To attempt to detect this putative dsRNA intermediate, we performed an RT-PCR under conditions that would only amplify cDNA reverse transcribed from antisense transcripts corresponding to the *sur5::GFP* transgene. Using this method, we amplified a reverse transcriptase-dependent product corresponding to the antisense strand of the *sur5::GFP* transgene in wild-type animals. I, like G, prefers to base pair with C, so A to I changes in an antisense RNA appear as T to C transitions when the genomic DNA is compared to the cDNA. Sequencing of the PCR product amplified from antisense RNA corresponding to GFP revealed T to C changes indicative of A to I editing (Figures 6A and 6B). Editing was detected throughout an approximately 250 nucleotide region corresponding to the 3′ end of the GFP sequence (Figure 6C). ADAR editing sites exhibit a 5′ neighbor preference (Polson and Bass, 1994), and in *C. elegans* a 5′ U is most preferred, followed by A and then C, and a 5′ G is the least preferred (Morse and Bass, 1999). We found that edited sites in the GFP sequence followed these preferences (Figure 6C), further indicating that the sequence changes were a result of editing by ADARs. Nearly identical editing patterns were observed in three separate experiments from two different populations of animals (data not shown). The presence of editing in the antisense strand indicates that it was base paired with the sense strand to create a dsRNA since ADARs only use dsRNA as substrates. In *adr-1;adr-2* mutants carrying the transgene, the dsRNA intermediate was not detected. We propose that in the absence of ADAR activity the dsRNA corresponding to GFP is rapidly cleaved by Dicer and enters the RNAi pathway.

Discussion

Here we present the first in vivo characterization of the role of ADARs in RNAi and demonstrate that the enzymes can prevent somatic transgenes from inducing gene silencing via the RNAi pathway. We find that dsRNA delivered by injection efficiently triggers RNAi in both wild-type and *adr-1;adr-2* double mutants, and we do not detect any evidence that RNA editing antagonizes RNAi under these conditions. dsRNA injected into wild-type worms is edited, however (Domeier et al., 2000), but our data suggest that the amount of editing that occurs in dsRNA injected into wild-type animals is not sufficient to inhibit the RNAi pathway.

ADARs also do not appear to be responsible for the partial resistance to RNAi seen in neurons. Here we demonstrate that neuronally expressed genes remain refractory to RNAi in the absence of ADARs if dsRNA is delivered by injection. Because RNAi can be triggered in neuronal tissues by directly expressing dsRNA in these cells, it seems likely that neurons are refractory for other reasons. Recent evidence indicates that a putative RdRP, encoded by *rrf-3*, is involved in preventing RNAi in the neurons (Simmer et al., 2002).

We find that distinct differences exist, however, between *adr-1;adr-2* mutants and wild-type animals in the ability of transgenes to induce RNAi. In wild-type animals, somatic transgenes are under most circumstances expressed and not subject to extensive silencing. However, in *adr-1;adr-2* mutants, somatic transgenes can induce robust gene silencing. The strongest silencing occurs in the double *adr-1;adr-2* mutant, but silencing is also detectable in both of the single homozygous deletions. Furthermore, transgene-induced gene silencing

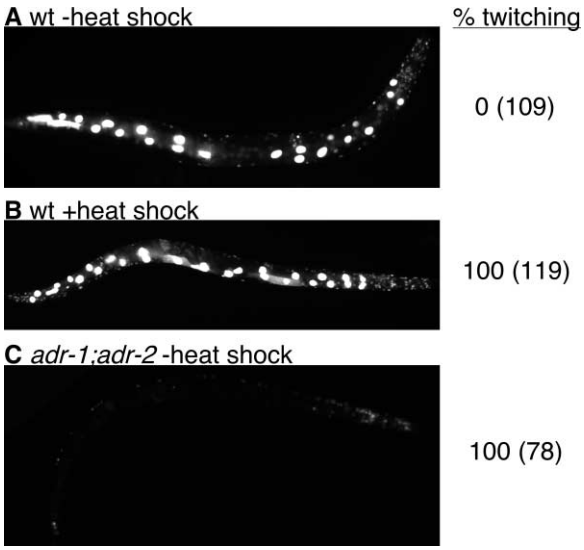


Figure 4. Transgenes Designed with or without Inverted Repeats Can Trigger RNAi in *adr-1;adr-2* Double Mutants

Animals shown contain a heat shock-inducible inverted repeat corresponding to *unc-22* (*hsp16-2*$_p$*unc-22[IR]*) as well as the *sur5::GFP* transgene.

(A) Wild-type animals grown at 20° C have strong GFP fluorescence and do not twitch.

(B) Following heat shock, wild-type animals retain GFP fluorescence but twitch due to the induction of RNAi targeting *unc-22*.

(C) *adr-1;adr-2* double mutants without heat shock have reduced GFP fluorescence and twitch, indicating that both transgenes induce RNAi.

The number of animals examined is shown in parentheses.

in *adr-1;adr-2* mutants requires molecular components essential for RNAi. Our data indicate that dsRNA is synthesized from transgenes in the somatic tissues of wild-type animals and suggest that editing of the dsRNA by both ADR-1 and ADR-2 prevents it from initiating RNAi (Figure 5).

The mechanism responsible for generating dsRNA corresponding to transgenes in *C. elegans* is unknown. For extrachromosomal arrays that contain inverted repeats under the control of a heat shock promoter, it is easy to imagine that, although the promoter is tightly regulated (Stringham et al., 1992), a hairpin dsRNA may be transcribed by leaky expression. In our studies, all of the strains that contained transgenes under the control of heat shock promoters also had either the *rol-6* or *sur5::GFP* transgenes as markers. In an extrachromosomal array, multiple copies of these markers are likely interspersed with the transgene containing the inverted repeat. It is possible that a promoter from one of these markers, if located next to the inverted repeat in the extrachromosomal array, may be driving the expression of the sequence by transcriptional readthrough. A similar mechanism could be responsible for generating antisense RNA from extrachromosomal arrays without inverted repeats. Transcriptional units may be arranged in these arrays in opposing orientations, where transcriptional readthrough from one promoter into the same gene on the opposite strand would generate a dsRNA. This seems a likely scenario since a less repetitive array created by injecting complex DNA along with the *sur5::GFP* transgene failed to induce silencing in the *adr-1;adr-2* double mutant. It is also possible that one of the four RdRPs found in *C. elegans* (Sijen et al., 2001; Smardon et al., 2000) may be involved in generating antisense RNA corresponding to the transgenes. Not all transgenes induce silencing in *adr-1;adr-2* mutants, however (e.g., *rol-6*), suggesting that the temporal and spatial expression of the transgene may also be an important determinant in whether a transgene can induce RNAi.

Transgene-induced RNAi in the *adr-1;adr-2* mutants appears remarkably similar to cosuppression. One mystery of cosuppression in *C. elegans* has been that it occurs almost exclusively in the germline. There has been only one previously reported instance of a transgene silencing an endogenous gene in the somatic tis-

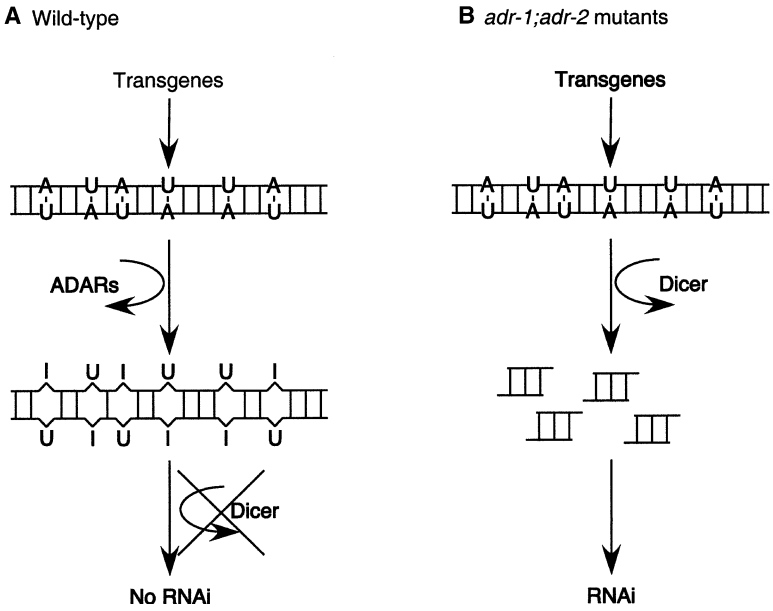
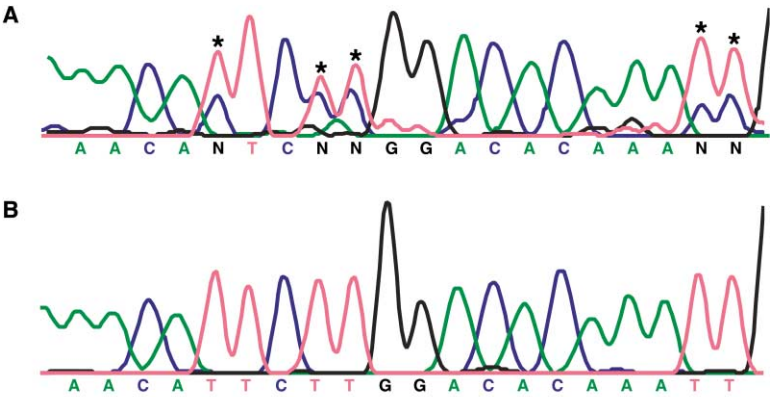


Figure 5. A Model for the Role of ADARs in RNAi

dsRNA is produced by an unknown mechanism from transgenes in both wild-type animals and *adr-1;adr-2* mutants.

(A) In wild-type animals, the dsRNA originating from transgenes is deaminated by ADARs. Editing of the dsRNA prevents it from being cleaved by Dicer, and the dsRNA fails to trigger RNAi.

(B) In *adr-1;adr-2* mutants, which completely lack A to I editing activity, the dsRNA generated from transgenes retains its original sequence and double-stranded structure, allowing cleavage into siRNAs by Dicer and initiation of RNAi.



C

5' neighbor	Total	Edited	% Edited
U	17	11	65
A	20	11	55
C	13	6	46
G	10	2	20

Figure 6. Edited Antisense RNA Is Found in Wild-Type Animals

(A) cDNA sequence of antisense RNA corresponding to GFP. The cDNA was amplified from approximately 175 worms, and the sequence represents the entire population sequence. A to I editing is indicated by T to C changes in the antisense sequence (A) compared to the genomic DNA sequence from the same animals (B). Five out of six T residues in the 20 nucleotide sequence shown in (A) show evidence of editing as indicated by an increase in the C trace (asterisks). (C) Editing of the adenosines throughout a 232 base pair region of GFP, from which a section is shown in (A), was tabulated. Adenosines are edited in a pattern consistent with known sequence preferences for *C. elegans* ADARs.

sues of a wild-type animal (Fire et al., 1991) despite the thousands of transgenic strains that have been studied. Here we demonstrate that the rarity of transgene-induced gene silencing in somatic tissues is in some instances attributable to ADAR activity. A distinct difference between cosuppression in the *C. elegans* germline and transgene-induced RNAi in the *adr-1;adr-2* double mutants is their dependence on *rde-1*. Cosuppression is *rde-1* independent (Dernburg et al., 2000; Ketting and Plasterk, 2000), but transgene-induced RNAi in the somatic tissues of *adr-1;adr-2* double mutants requires *rde-1*, indicating that although these two phenomenon appear related, subtle differences exist. *rde-1* belongs to a large gene family in *C. elegans* (Tabara et al., 1999), and therefore, it remains a possibility that one of the other gene family members may function in place of *rde-1* in cosuppression (Grishok et al., 2001).

It is somewhat surprising that cosuppression functions in the *C. elegans* germline since there is evidence that *adr-2* is expressed in this tissue (Y. Kohara, personal communication). Perhaps the level of ADAR activity in the germline is insufficient to prevent cosuppression, or cosuppression may not be mediated by dsRNA. If cosuppression involves a dsRNA intermediate, it may be protected from editing by localization to the cytoplasm, where ADARs are rarely found, or rapidly cleaved to siRNAs that may be too short to serve as ADAR substrates. In plants the correlation between the effectiveness of cosuppression and the absence of ADARs is strong. Plants are highly susceptible to cosuppression (Napoli et al., 1990; Smith et al., 1990; van der Krol et al., 1990) and also lack genes encoding ADARs.

Perhaps the simplest interpretation of our data is that the activities of ADARs and Dicer are physically separated. ADARs are primarily localized to the nucleus, but Dicer is found in the cytoplasm of embryonal carcinoma and HeLa cells (Billy et al., 2001). If the two activities are separated, dsRNA delivered by injection may trigger RNAi before ever being exposed to ADARs in the nucleus. When dsRNA is produced in the nucleus at low concentrations from transgenes, however, ADARs may first edit the dsRNA before the duplex is exposed to Dicer. In other organisms, highly edited RNAs are retained in the nucleus (Zhang and Carmichael, 2001). If a similar retention mechanism exists in *C. elegans*, it could further prevent edited dsRNAs from entering the cytoplasm and being cleaved by Dicer. At high concentrations of dsRNA, however, ADARs are subject to substrate inhibition (Hough and Bass, 1994), and this may explain why RNAi that is triggered in wild-type animals by inducing synthesis of dsRNA is not inhibited by ADARs.

Alternatively, it is possible that both ADARs and Dicer are present in the nucleus. In *C. elegans* the intracellular localization of Dicer remains unknown. *dcr-1* is required for RNAi in the *C. elegans* germline (Ketting et al., 2001; Knight and Bass, 2001), and reducing *dcr-1* levels by RNAi results in a decreased sensitivity to RNAi in the somatic tissues (Grishok et al., 2001). *C. elegans dcr-1* homozygous strains, however, only have defects in somatic RNAi when the triggering dsRNA is provided from a transgene (Knight and Bass, 2001). Although the reason *dcr-1* homozygotes only have defects in somatic RNAi triggered from transgenes remains unclear, it is intriguing that deletions in both *dcr-1* and the two genes encoding ADARs only effect somatic RNAi initiated in the nucleus. If ADARs and Dicer are nuclear, then at low concentrations of dsRNA, ADARs may simply have a higher affinity for dsRNA than Dicer.

In *C. elegans*, several ADAR substrates have been identified (Morse et al., 2002; Morse and Bass, 1999), but it is unclear exactly how editing of these substrates leads to the phenotypes of animals lacking ADARs (Tonkin et al., submitted). Many ADAR substrates in *C. elegans* and humans are edited in noncoding regions that are predicted to form long double-stranded regions, suggesting that, in addition to changing the coding potential of mRNAs, editing may also play other roles (Morse et al., 2002; Morse and Bass, 1999). It is intriguing to speculate that editing of naturally occurring dsRNAs may prevent these dsRNAs from triggering gene silencing as we observe with dsRNAs originating from transgenes.

Experimental Procedures

Strains and Culture Conditions

The following strains were maintained using standard culture conditions (Brenner, 1974): *LG1, adr-1(gv6)* (Tonkin et al., submitted); *LGIII*, *adr-2(gv42)* (Tonkin et al., submitted); *LGV, rde-1(ne219)* (Tabara et al., 1999); *LG?, pxIs6(sur-5::GFP,pRF4)* (Domeier et al., 2000), *bbEx1(sur5::GFP, hsp16-2$_p$GFP[IR], pRF-4)* (Knight and Bass, 2001), *bbEx2(sur5::GFP, hsp16-2$_p$unc-22[IR])*, *bbEx3(sur5::GFP), bbEx4 (hsp16-2$_p$unc-22[IR], pRF4)*.

Transgenics

The heat shock-inducible *unc-22* hairpin, *hsp16-2$_p$unc-22(IR)*, was constructed as previously described (Tavernarakis et al., 2000). In brief, 908 base pairs of *unc-22* sequence were amplified from N2 total DNA using primers *Kpn unc-22* 17509F (CGGGGTACCCGAA AGTGATTTGGCAACATTGG), which introduces a 5′ KpnI restriction site, and EcoRI *unc-22* 18416R (CCGGAATTCCTTTAACTGGAG CTCCCTCATCTGG), which introduces a 3′ EcoRI restriction site. PCR products were digested with EcoRI and ligated to form an inverted repeat. The ligation product was gel purified, digested with KpnI, and ligated into KpnI digested *hsp16-2$_p$GFP(IR)*. Transgenic lines were created by injecting plasmids as previously described (Mello and Fire, 1995).

RNAi

Sense and antisense RNA was prepared using standard T3 and T7 RNA polymerase (Invitrogen) reactions from PCR templates containing the appropriate phage promoter. dsRNA was prepared by combining equal concentrations of sense and antisense transcripts in 50 mM PIPES (pH 6.7). Sense and antisense strands were annealed by heating the solution containing both RNAs to 95°C followed by slow cooling for at least 30 min. The double-stranded nature of each duplex was confirmed by comparing its mobility in agarose gels to single-stranded transcripts. dsRNA used in determining the concentration dependence of RNAi was gel purified by electroelution from agarose gels.

dsRNA was injected into both gonad arms of young adult animals at 0.5 mg ml^{-1} unless otherwise specified. Animals recovered overnight on NGM agar and then were transferred to new plates and allowed to lay eggs for 16–24 hr. Progeny of injected animals were scored for the appropriate phenotype on subsequent days.

Heat shock experiments were preformed as previously described (Knight and Bass, 2001). In brief, L2 stage worms were heat shocked on 2 consecutive days at 33°C for 90 min. Worms were scored for the appropriate RNAi phenotype on the third day.

RT-PCR

One hundred seventy-five to two hundred wild-type worms harboring the *bbEx2* extrachromosomal array were collected in water and lysed by three freeze thaw cycles in liquid nitrogen. Two hundred microliters of denaturing solution (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% *N*-lauroylsarcosine, and 0.1 M 2-mercaptoethanol) was added to each worm pellet followed by incubation at 22°C for 15 min. RNA was isolated by two sequential phenol:chloroform extractions and collected by ethanol precipitation. Contaminating DNA was removed by three consecutive incubations with DNase I for 1 hr followed by phenol:chloroform extractions and ethanol precipitations. Total RNA was resuspended in 100 μl of water.

cDNA was synthesized using 10 μl of total RNA and 2 pmol of first strand primer in a 20 μl reaction using Superscript II according to the manufacturer's instructions. The first strand primer (GFP436FY: GYCAAGYYYGAAGGYGAYACCC, Y denotes C or T) was degenerate and could anneal to both edited and unedited antisense RNA. The RT reaction was treated with proteinase K at 65°C for 1 hr, phenol:chloroform was extracted, and cDNA was ethanol precipitated. Pelleted nucleic acid was resuspended in 20 μl of water, and 2 μl was used for PCR amplification. Two rounds of PCR using degenerate primers GFP436FY and GFP724RP (GTCTGGT RRRRGGRCRGGGCC, R denotes A or G) were required to detect the antisense transcript. The second round of PCR was performed using 1 μl of a 1:100 dilution of the first round reaction. PCR products were gel purified and sequenced using primer GFP436FY.

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