LETTERS

# RNA interference is an antiviral defence mechanism in *Caenorhabditis elegans*

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**RNA interference (RNAi) is an evolutionarily conserved sequence-specific post-transcriptional gene silencing mechanism that is well defined genetically in *Caenorhabditis elegans*[1–4]. RNAi has been postulated to function as an adaptive antiviral immune mechanism in the worm, but there is no experimental evidence for this. Part of the limitation is that there are no known natural viral pathogens of *C. elegans*. Here we describe an infection model in *C. elegans* using the mammalian pathogen vesicular stomatitis virus (VSV) to study the role of RNAi in antiviral immunity. VSV infection is potentiated in cells derived from RNAi-defective worm mutants (*rde-1*; *rde-4*), leading to the production of infectious progeny virus, and is inhibited in mutants with an enhanced RNAi response (*rrf-3*; *eri-1*). Because the RNAi response occurs in the absence of exogenously added VSV small interfering RNAs, these results show that RNAi is activated during VSV infection and that RNAi is a genuine antiviral immune defence mechanism in the worm.**

Vesicular stomatitis virus (VSV), an enveloped virus with a non-segmented, negative-sense RNA genome, was an attractive candidate to infect *C. elegans* cells. VSV is particularly notable for its broad host range, including both mammalian and insect hosts[5], and for its ability to infect and replicate at temperatures used in culturing *C. elegans* (18–25 °C). In addition, as the prototypical member of the Rhabdoviridae, it is among the best characterized of the negative-stranded RNA viruses, its life cycle has been extensively studied in mammalian cell culture[6] and VSV recombinant viruses have been used as vectors for gene expression, vaccines and cell targeting[7–10].

A replication-competent, recombinant VSV (Fig. 1a) expressing the enhanced gene encoding green fluorescent protein (VSV-GFP) was able to infect primary cell cultures isolated from wild-type (N2) worms (Fig. 1b, VSV). GFP expression requires infectious virus because no fluorescent signals were detected in cells infected with virus that had been irradiated with ultraviolet (Fig. 1b, UV-VSV). In addition, antibodies against the VSV nucleocapsid (N) and glycoprotein (G) recognized only VSV-infected cells and not mock-infected cells (Fig. 1c). By using primers specific to N gene coding sequences in a reverse transcriptase-mediated polymerase chain reaction (RT–PCR) assay specific for positive-sense viral RNAs, a product was detected in VSV-infected cells (Fig. 1d, lane 2), but not in uninfected cells or cells infected with UV-VSV (Fig. 1d, lanes 1 and 3). The N gene RT–PCR signal, coupled with the presence of GFP and viral proteins (Fig. 1b, c), confirmed that synthesis of the viral transcripts was occurring within infected worm cells. Strand-specific RT–PCR products were also obtained from infected but not UV-VSV infected cells with the use of a primer set (P–M) amplifying the intergenic region between the P and M genes from negative-sense (genomic) viral RNAs (Fig. 1d). During genome replication, the viral polymerase synthesizes a full-length, positive-sense RNA (anti-genome) species that is complementary to the viral genome and serves as a template for the synthesis of additional progeny RNA genomes. Because the intergenic regions are present in the full-length antigenome and progeny genome RNAs and in a small fraction of subgenomic, read-through messenger RNAs the P–M RT–PCR product from infected samples indicates that increased levels of viral genomes are present above that found in the initial inoculum of virus. Thus viral replication as well as viral gene expression was occurring in the infected *C. elegans* cells.

Establishment of a *C. elegans* model of VSV infection allowed us to test directly whether RNAi acts as an antiviral immune mechanism in the worm. RNAi is triggered by processing double-stranded RNAs

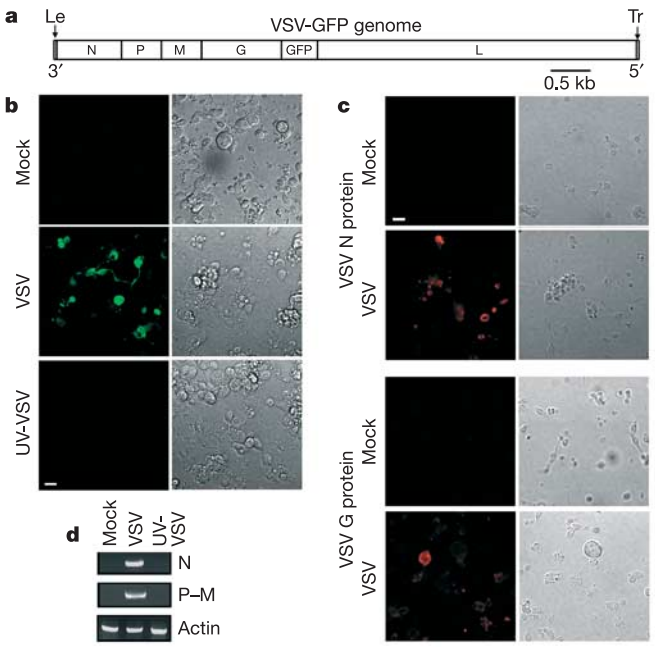


**Figure 1 | VSV infection of *C. elegans* cells in culture.** **a**, Genome organization of VSV-GFP recombinant virus. The structural proteins of the VSV virion are the nucleocapsid (N), phosphoprotein (P), matrix (M), glycoprotein (G) and viral replicase (L). **b–d**, Cells, mock infected or infected with VSV-GFP or UV-inactivated VSV-GFP (UV-VSV), were analysed at 48 h after infection. Scale bar, 7 μm. **b**, GFP fluorescence images (left) and differential interference contrast images (right) of cells. **c**, Immunofluorescent staining of cells for VSV-N or VSV-G proteins. **d**, RT–PCR analyses for N gene sequences, the P to M intergenic region of the VSV genome and *C. elegans* actin.

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(dsRNAs) into small interfering RNAs (siRNAs). These siRNAs associate with an RNA-induced silencing complex (RISC) to mediate the sequence-specific cleavage of target mRNAs and to induce post-transcriptional gene silencing (PTGS)[2–4]. Genetic screens in the worm have identified several genes involved in this process[11,12]; among them are *rde-1* and *rde-4* as potential modulators of viral immunity. RDE-1 is a member of the argonaute gene family[11] and RDE-4 is a dsRNA-binding protein that interacts with both the trigger dsRNA and RDE-1 (ref. 13). The RDE-4–RDE-1 complex functions at the initiation step of RNAi, where it is thought to recognize dsRNA and target it for cleavage into siRNAs by Dicer[13]. Null mutants in *rde-1* or *rde-4* are incapable of inducing RNAi but have no other apparent phenotypes[11]. VSV infection of *rde-1* and *rde-4* mutant cells resulted in a higher percentage of GFP-positive cells in the culture (Fig. 2a, b). In addition, the mean GFP fluorescence intensities within individual cells continued to increase over time in infected *rde* cells (Fig. 2c). Because GFP is expressed from the fourth cistron, it is one of the least abundantly expressed proteins in VSV-GFP-infected cells, so the percentages of GFP-positive cells may be an underestimate of the total percentage of infected cells within the culture. To increase the sensitivity and obtain a more accurate assessment, infected cells were also immunofluorescently stained for N protein (the most abundant viral protein in an infected cell) and analysed by flow cytometry. At 36 h after infection, the percentage of infected *rde-1* cells (61%) in the culture was again about double that for N2 cells (33%). Consistent with the increased numbers of infected cells, there were greater amounts of both N and G proteins immunoprecipitated from radiolabelled infected *rde* cell lysates than from N2 cell lysates (Fig. 2d).

To determine whether VSV infection is productive in *C. elegans*, N2 and *rde* cells were infected with VSV and the production of infectious virus was followed over time (Fig. 2e). A rapid and significant increase in viral titres was seen in infected *rde* cells between 24 and 30 h after infection, which reached a plateau by 36 h after infection (Fig. 2e). The kinetics of virus growth in the *rde* mutants is consistent with that normally observed during a synchronized single cycle of productive viral infection. In contrast, no significant increase in viral titres was detected at late times of infection in the culture supernatants of infected N2 cells. The small but continuous increase in virus titres observed over the entire infection period (0–48 h after infection) in N2 cells indicated that this titre might be due primarily to the elution of virus particles off

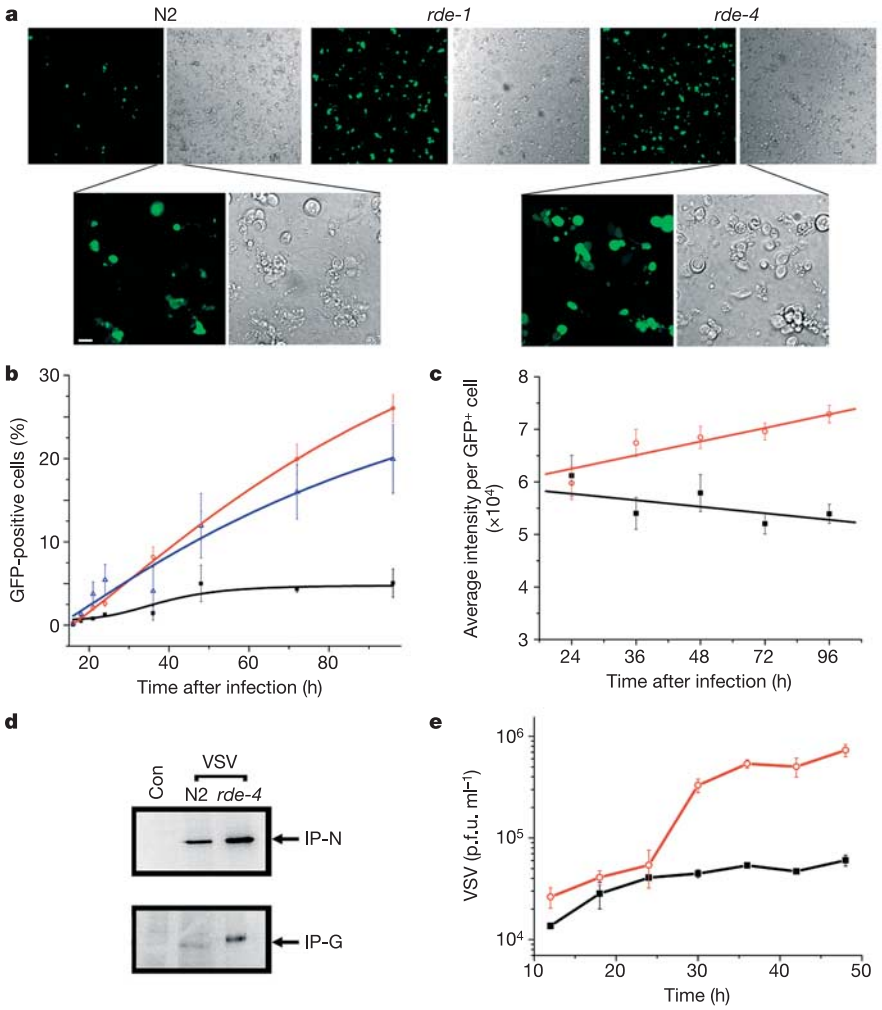


**Figure 2 | Increased infection of RNAi-deficient mutants. a–c**, GFP expression in VSV-GFP-infected N2, *rde-1 (ne219)* and *rde-4 (ne301)* cells. **a**, Confocal images at low magnification (top row) and high magnification (bottom row; scale bar, 7 μm) at 48 h after infection. **b**, **c**, Percentage of GFP-positive cells (**b**) and intensities (four to eight individual fields) of GFP fluorescence per cell (**c**) at each time after infection. Black squares, N2; red circles, *rde-4*; blue triangles, *rde-1*. Results are means ± s.e.m. **d**, Immunoprecipitation (IP) of VSV N and G proteins from equivalent numbers of infected N2 and *rde-4* cells. Con, control. **e**, Virus titres from infected N2 cells (black squares) and *rde-1* cells (red circles). Similar results were obtained with infected *rde-4* cells. Results are means ± s.e.m.

cells from the initial inoculum, and that no detectable amounts of new virus were produced in these cells.

Therefore, in an RNAi-null background, VSV infects a higher percentage of cells than in wild-type N2 cells, and both viral proteins and the GFP reporter accumulate to higher levels. Most significantly, VSV infections in *rde* mutants result in productive viral infections with significant increases in viral titres, which are absent from wild-type N2 cells. These data show that the RDE-1 and RDE-4 components of the RNAi pathway are important in inhibiting VSV infection, which limit the extent of viral gene expression and the production of infectious virus.

If an RNAi-null background potentiates viral infection, then mutations that enhance RNAi responses would be predicted to attenuate viral infection. Recently two worm mutants (*rrf-3* and *eri-1*) have been identified in which RNAi responses are enhanced by modulating intracellular siRNA levels[14,15]. RRF-3, a member of the RNA-dependent RNA polymerase (RdRP) gene family in *C. elegans*, seems to inhibit RdRP-directed siRNA amplification, and worms with mutations in *rrf-3* are more sensitive, especially in neurons, to RNAi responses induced by dsRNAs[14]. ERI-1, a member of the DEDDh nuclease family, preferentially cleaves siRNAs. Thus, siRNAs are more stable and accumulate in *eri-1* mutants, resulting in enhanced gene suppression[15]. Comparison of VSV infection in *rrf-3* and *eri-1* mutants with that in N2 cells revealed that the percentages of VSV-infected cells were indeed lower in both *rrf-3* and *eri-1* than in N2 cultures (Fig. 3a). However, a small subpopulation of *eri-1* and *rrf-3* cells (about 2%) remained permissive to VSV infection, and GFP intensities in these VSV-permissive *rrf-3* and *eri-1* cells increased slightly over time (Fig. 3b), in contrast to the decrease observed in N2 cells. This indicates that the RNAi response might remain negatively regulated in this subpopulation of *rrf-3* and *eri-1* cells, thus allowing the accumulation of viral proteins. A similar phenotype was observed in the whole worm in *eri-1* mutants, in which some neurons remain refractory to the RNAi response[15]. This virus-permissive phenotype could be explained either by the differential expression of the two known negative regulators of RNAi, *eri-1* and *rrf-3*, or perhaps by the presence of other as yet unidentified host suppressors of RNAi. Nevertheless, the decreased numbers of GFP-positive cells indicate that VSV infection is attenuated in host backgrounds displaying an enhanced RNAi response.

The phenotypes observed on infection of N2 and the RNAi-enhanced mutant cells indicate that the RNAi pathway might be induced on viral infection without the exogenous addition of synthetic siRNAs. RNase protection assays were performed to determine whether siRNAs were indeed generated as a consequence of viral infection (Fig. 4). Protected bands of about 20–30 nucleotides were observed when RNA from infected N2 cells was hybridized with radiolabelled VSV probes. In contrast, no protection was observed in reactions using RNA from mock-infected N2 or virus-infected *rde-4* cells. The presence of virus-specific siRNAs in infected N2 cells indicates that the RNAi response is induced on virus infection.

Collectively, the genetic and biochemical data in both RNAi-null and enhanced host backgrounds establish RNAi as a cellular antiviral defence mechanism in *C. elegans*. Pretreatment of mammalian cells with synthetic siRNAs against viral sequences, to initiate the RNAi pathway artificially, limits infection by several viruses including polio, influenza and HIV[16–18]. This, coupled with the recognized role of RNAi in antiviral immunity in plants[19–21] and insects[22,23], indicates that RNAi might serve a similar function throughout evolution. Moreover, we show here that, similarly to infections with plant or insect viruses, RNAi responses in the worm are induced as a consequence of VSV infection. These data, together with recent evidence that HIV infection can induce RNAi responses in human cells[24], indicate that activation of the RNAi pathway might be a routine physiological response of the host to viral infections. Indeed, many viruses, both plant and animal pathogens, have evolved suppressors of RNAi to bypass this cellular immunity and enable viral replication[24–26].

The relative genetic simplicity of *C. elegans* and the availability of an extensive collection of mutants makes the worm an attractive model host for identifying genetic determinants that affect the susceptibility or resistance of a host to viral infection. In addition to viral determinants, susceptibility to infection and disease is dependent on the genetic background and physiological status of

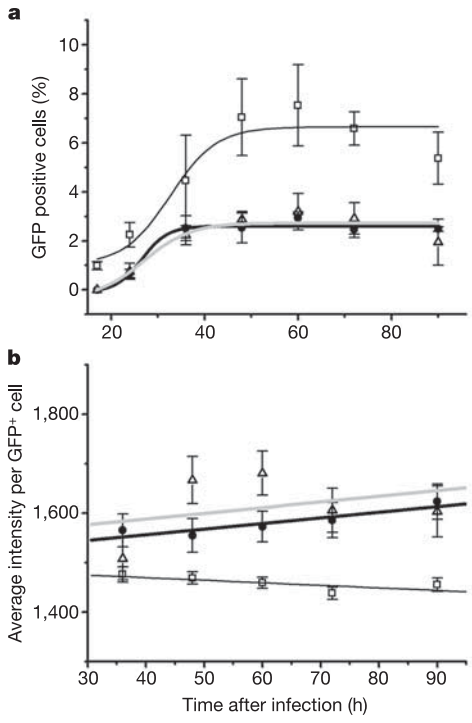


**Figure 3 | Increased resistance of RNAi-hypersensitive mutants to VSV infection.** Cells from N2 (squares), *eri-1(mg 336)* (filled circles) and *rrf-3(pk1426)* (triangles) strains were infected with VSV-GFP, and GFP expression over time were analysed by confocal microscopy. **a**, Percentages of GFP-positive cells. **b**, Average intensities of GFP fluorescence per GFP-positive cell. Results are means ± s.e.m. for two to four independent fields at each time after infection.

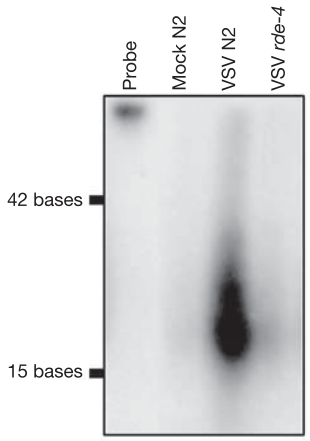


**Figure 4 | Virus-specific siRNAs in infected N2 cells.** RNAs from mock-infected N2, VSV-infected N2 and VSV-infected *rde-4* cells at 24 h after infection were analysed by RNase protection assays with the use of a radiolabelled probe specific for VSV N.

the host. The demonstration that VSV can infect *C. elegans* provides a new model system for identifying genes involved in regulating resistance or susceptibility not only to VSV but also to a wide range of viruses, using VSV-based vectors and pseudotyped viruses. The RNAi pathway is an example of such a set of host susceptibility determinants.

## METHODS

**Primary cell culture of *C. elegans*.** Cells were isolated as described in ref. 27, with slight modifications. Early embryos were isolated after bleach treatment of cultures of synchronized adult hermaphrodites on sucrose step gradients. The embryos were incubated with chitinase ($5\,U\,ml^{-1}$) and chymotrypsin ($10\,mg\,ml^{-1}$). Monolayer cultures of the resultant dissociated cells were grown at 25 °C in a modified L15 medium containing 10% fetal bovine serum, 30 mM sucrose and $100\,U\,ml^{-1}$ penicillin/streptomycin.

**VSV infection.** Virus stocks were grown in baby hamster kidney (BHK-21) cells at low multiplicity (multiplicity of infection (m.o.i.) 0.01 plaque-forming units per cell) and infectious titres were measured by plaque assay on HeLa cells at 37 °C. *C. elegans* cultures were infected at high multiplicity (m.o.i. 10) by adding virus to the culture medium for 2 h at 25 °C and the virus inoculum was replaced with fresh medium. For virus growth curves, aliquots of the culture medium were taken at intervals of 6 h and viral titres were determined by plaque assay on BHK-21 cells.

**Immunoprecipitation.** Cell monolayers ($4 \times 10^6$ cells) were labelled from 42 to 48 h after infection with [$^{35}$S]methionine ($100\,\mu Ci\,ml^{-1}$), then harvested and lysed in RIPA buffer. Samples were incubated with monoclonal antibodies recognizing VSV N or G proteins, and Staph-G-conjugated beads were used to collect the antibody complexes. Samples were analysed by 10% SDS–PAGE and autoradiography.

**RT–PCR analyses.** RNA was isolated from infected cells with the use of Trizol. Equivalent amounts of total RNA were used in strand-specific RT–PCR reactions with specific RT primers that hybridized to viral sequences of either positive sense (N gene coding) or negative sense (P–M intergenic region). For the N gene, the forward primer is complementary to nucleotides 730–747 and the reverse primer is identical to nucleotides 1331–1365 of the minus-sense viral genome. For the P–M intergenic region, the forward primer is complementary to nucleotides 1499–1526 and the reverse primer was identical to nucleotides 2548–2570 of the viral genome. For the actin gene of *C. elegans*, the primer sequences are identical to nucleotides 124–146 (forward primer) and complementary to nucleotides 820–849 (reverse primer) of the coding sequence.

**RNase protection.** Radiolabelled probes were generated by T7 transcription of cDNA plasmids of VSV N, P, M, G and L genes[28]. RNA fractions enriched for small RNAs (less than 200 bases) were isolated from cells by using the mirVana miRNA isolation kit (Ambion) and RNase protection assays used the mirVana miRNA detection kit (Ambion) in accordance with the manufacturer's protocols. Samples were analysed on a 15% denaturing polyacrylamide gel.

**Confocal analysis.** Cell monolayers were either examined for GFP fluorescence or processed for immunofluorescent staining by fixing them in 1% paraformaldehyde, permeabilizing them with 0.1% saponin plus 1% BSA and incubating them with primary antibodies against VSV-N or VSV-G proteins and phycoerythrin-conjugated secondary antibodies at appropriate dilutions.

Image analyses were performed with MetaMorph software. GFP images were thresholded and the percentage of GFP-positive cells and the intensity of GFP fluorescence in individual cells were measured.

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