Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes

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Double-stranded RNA interference (RNAi) is an effective method for disrupting expression of specific genes in *Caenorhabditis elegans* and other organisms[1-5]. Applications of this reverse-genetics tool, however, are somewhat restricted in nematodes because introduced dsRNA is not stably inherited[5]. Another difficulty is that RNAi disruption of late-acting genes has been generally less consistent than that of embryonically expressed genes, perhaps because the concentration of dsRNA becomes lower as cellular division proceeds or as developmental time advances[1]. In particular, some neuronally expressed genes appear refractory to dsRNA-mediated interference. We sought to extend the applicability of RNAi by *in vivo* expression of heritable inverted-repeat (IR) genes. We assayed the efficacy of *in vivo*-driven RNAi in three situations for which heritable, inducible RNAi would be advantageous: (i) production of large numbers of animals deficient for gene activities required for viability or reproduction; (ii) generation of large populations of phenocopy mutants for biochemical analysis; and (iii) effective gene inactivation in the nervous system. We report that heritable IR genes confer potent and specific gene inactivation for each of these applications. We suggest that a similar strategy might be used to test for dsRNA interference effects in higher organisms in which it is feasible to construct transgenic animals, but impossible to directly or transiently introduce high concentrations of dsRNA.

To test the feasibility of specific gene disruption via *in vivo* expression of dsRNA, we constructed transgenic nematodes that synthesized hairpin dsRNA (ref. 3) from IR genes under the control of the heat-shock–inducible promoter *hsp16-2* (Fig. 1; refs 6–8). We first compared effects of conventional RNAi via injection of dsRNA, expression of sense and antisense genes, and *in vivo* production of dsRNA using *C. elegans* predicted gene C37A2.5, which is essential for progression past the L2 larval stage (N.T., S.L.W. and M.D., unpublished data). Conventional RNAi through injection of C37A2.5 dsRNA (ref. 1) produced a high yield of L2-stage–arrested F1 progeny (Table 1). Expression of the antisense strand, which can be effective for specific gene inactivation[9], resulted in a modest percentage of phenocopy progeny, whereas expression of the sense stand was ineffective. To test *in vivo* RNAi, we heat-shocked young adults of transgenic lines harbouring extrachromosomal *hsp16-2*$_p$C37A2.5(IR). *In vivo* promoter-driven RNAi reproduced the ΔC37A2.5 null phenotype, with efficiencies approaching that of direct injection of dsRNA (Table 1). Likewise, promoter-driven RNAi disrupted the Mi-2 chromatin remodelling homologue F2612.7 (ref. 10) to phenocopy the sterile phenotype of a deletion of this gene (Table 1). We concluded that *in vivo*-driven RNAi is effective, and that this technique should enable generation of large populations of phenocopy mutants, even when development or reproduction is blocked.

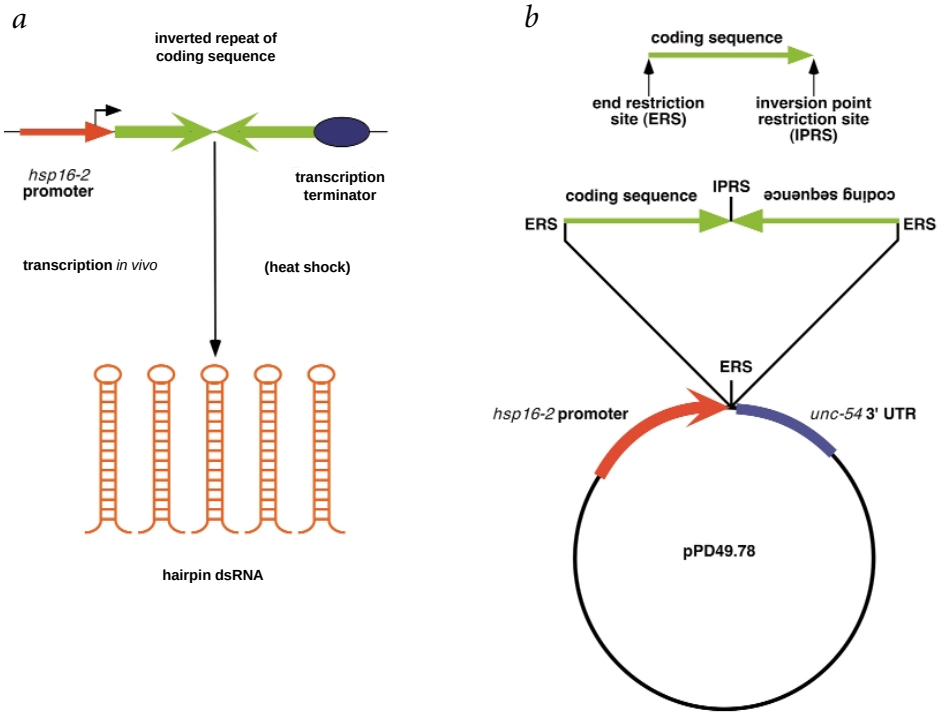

Fig. 1 Strategy for generation of heritable and inducible RNAi. ***a***, A strategy for *in vivo* RNAi. A strong heat shock-inducible promoter was fused to a direct IR gene. Upon heat shock of transgenic animals harbouring this gene, transcripts were generated which are predicted to fold back in a uni-molecular reaction to generate double-stranded RNA in all cells that express the heat-shock gene. The size of the single-stranded loop that occurs after foldback is not known. ***b***, Construction of inducible IR genes. Exon-rich genomic DNA (or cDNA) was amplified using two primers that introduced unique restriction sites at the fragment ends. One restriction site was used to generate the IR and was ultimately situated at the inversion point (IP). The other restriction site (designated as end) was used to join the IR to the vector. Amplified fragments were digested with the enzyme situated at the IP restriction site (IPRS) and ligated together. Digestion at the end restriction site (ERS) enabled the fragment to be cloned into a similarly digested, CIAP-treated *C. elegans* expression vector. We used vector pPD49.78 (ref. 22), which includes the *hsp16-2* promoter and the 3′ UTR of muscle myosin *unc-54*.

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Table 1 • *In vivo* dsRNA interference

Gene disruption approach	Trial/line 1	Trial/line 2	Trial/line 3	Trial/line4
dsRNA C37A2.5 injected	94±8	89±4	97±5	89±7
pPD49.78 (*hsp16-2*$_p$ alone) + heat shock	0	0	0	0
hsp16-2$_p$C37A2.5 sense + heat shock	0	0	0	0
hsp16-2$_p$C37A2.5 antisense + heat shock	9±4	9±4	11±6	–
hsp16-2$_p$C37A2.5(IR) – heat shock	0	0	0	0
hsp16-2$_p$C37A2.5(IR) + heat shock	67±3	79±6	84±5	56±7
hsp16-2$_p$F26F12.7(IR) – heat shock	1±0.9	2±1	1±0.9	3±1.3
hsp16-2$_p$F26F12.7(IR) + heat shock	58±4	59±5	75±8	82±6
ds *mec-4* RNA injected	12±7	19±5	15±6	–
hsp16-2$_p$*mec-4*(IR) – heat shock	0	0	0	–
hsp16-2$_p$*mec-4*(IR) + heat shock	58±4	60±7	61±8	–
ds *unc-8* RNA injected	0	0.8±.01	0	0
hsp16-2$_p$*unc-8*(IR) – heat shock	0	0	0	0
hsp16-2$_p$*unc-8*(IR) + heat shock	17±3	11±5	14±2	13±3

Results for four injection trials using conventional RNAi or heat-shock–induced *in vivo* RNAi in four transgenic lines (unless otherwise noted) are indicated. Numbers indicate the percentage of F1 progeny arrested at the L2 stage ±s.d.

C. elegans translation elongation factor 2 kinase eEF-2 (encoded by *efk-1*; ref. 11) phosphorylates eEF-2, an activity abolished by a Tc1 insertion into the active site (Fig. 2*a*, and A.R., C. Mendola, L. Zhang and J. Culotti, unpublished data). We found that kinase activity in the offspring of heat-shocked *hsp16-2*$_p$*efk-1(IR)* transgenic parents was reduced in four of six lines assayed. We were not able to perform an analogous assay on a population of phenocopy mutants induced by conventional RNAi, as several hundred animals were required. We concluded that inducible IR genes are effective in generating populations amenable to biochemical analysis.

Injected dsRNA is not uniformly effective in disrupting gene expression in the nervous system. For example, we found that only 6 of 210 progeny from three lines harbouring integrated *unc-119*$_p$*GFP* (expressed in all neurons) injected with double-stranded *GFP* RNA showed reduced fluorescence (Fig. 2*b*). We therefore examined the effects of endogenously expressed dsRNA species on gene inactivation in the differentiated nervous system. We constructed a plasmid that directed *in vivo* expression of double-stranded *GFP* RNA upon heat shock and tested for extinction of fluorescent signals generated by cell-specific *GFP* reporter fusions (Fig. 2*b*). We co-introduced the *hsp16-2*$_p$*GFP(IR)* construct and *unc-119*$_p$*GFP* (pIM175 (ref. 12); expressed at high levels throughout the nervous system[13]), selected lines exhibiting strong GFP fluorescence, heat shocked in the L4 stage and examined fluorescence in their progeny. Approximately 79% of roller progeny from 3 (of 5) lines harbouring *unc-119*$_p$*GFP* and *hsp16-2*$_p$*GFP(IR)* exhibited 'knock-down' effects, with fewer than 10 cells detectable in most (Fig. 2*b*). We did not detect any consistent pattern of cells that appeared refractory to fluorescence inactivation, suggesting that all cells in the nervous system are susceptible to the effects of *in vivo* RNAi.

We next tested effects of heat-shock induction of *hsp16-2*$_p$*GFP(IR)* on expression of an integrated *mec-4*$_p$*GFP* gene, which is specifically expressed in the six touch-receptor neurons[14]. On average, 85% of roller progeny of heat-shocked parents harbouring the extragenic *hsp-16*$_p$*GFP(IR)* transgene had GFP signals that were either eliminated or attenuated (2/4 lines; Fig. 2*b*). We observed similar effects in only 11 of 270 progeny of a line harbouring an integrated *mec-4*$_p$*GFP* reporter injected with dsGFP RNA.

We also tested for dsRNA-mediated inactivation of *C. elegans* neuronal genes. Conventional RNAi mediated by introduced *mec-4* dsRNA induced touch-insensitivity in 46 of 300 (15%) offspring of injected wild-type parents. On average, 60% progeny of heat-shocked lines harbouring *hsp16-2*$_p$*mec-4(IR)* were insensitive to touch (Table 1). Additionally we tested the effectiveness of *in vivo*-directed RNAi in the inactivation of *unc-8*, a neuronally expressed gene that we have found to be resistant to the effects of conventional RNAi. The *unc-8* dominant gain-of-function allele *n491* induces uncoordinated locomotion characterized by the inability to back up; the loss-of-function phenotype is nearly wild type[15]. Injection of *unc-8* dsRNA did not disrupt the gain-of-function phenotype (2 phenocopy mutants generated among 1,300 progeny of injected parents). By contrast, 13% of the progeny of heat shocked unc-8(n491) parents harbouring *hsp16-2*$_p$*unc-8(IR)* were effectively targeted (Table 1). Our results indicate that sequences expressed in terminally differentiated neurons can be targeted by *in vivo*-induced RNAi, and in some instances effects are more potent than those observed after injection of dsRNA.

For all nine cases, heat shock of control lines carrying the expression vector alone or low-temperature growth of lines carrying the *hsp16-2*$_p$*(IR)* genes did not produce any abnormal phenotypes (we assayed for the anticipated knockout phenotype, morphological and locomotion defects, and fertility and developmental abnormalities; >100 animals examined per line). Thus, effects of *in vivo* RNAi appear to be highly specific, consistent with reported tight regulation of the *hsp16-2* promoter[8] and the selective precision of RNAi (ref. 1). Moreover, *in vivo* RNAi was effective in many tissue types, including neurons (Fig. 2*b*). (C37A2.5 and *efk-1* are expressed early in development and later in a broad range of cells including body wall and pharyngeal muscles, neurons, hypodermis and intestine (N.T., A.R. and M.D., unpublished data); MI2 homologue F2612.7 is expressed in hypodermis; MI2 homologue T14G8.1 is expressed in hypodermis and pharynx (S.L.W., N.T. and M.D. unpublished data); and *myo-2* is expressed in pharyngeal tissue[16].)

Our analysis establishes that endogenous IR genes can be expressed to generate dsRNA species with biological effects similar to, and in some cases more effective than, those of directly injected dsRNA. There are several advantages of expressing heri-

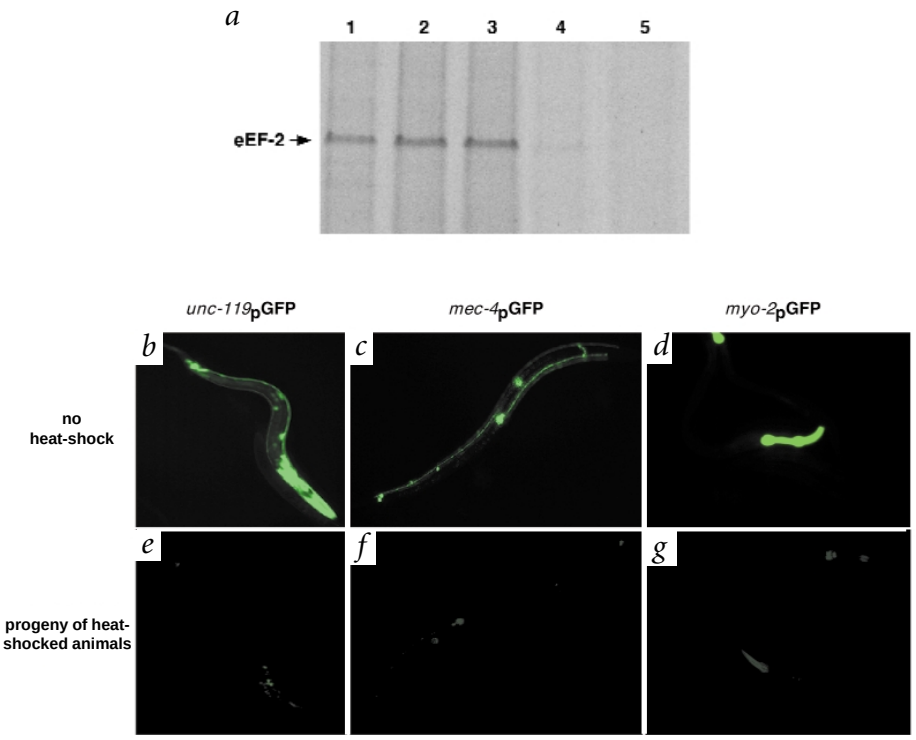

Fig. 2 Double-stranded RNA synthesized *in vivo* RNAi disrupts *C. elegans* gene expression. ***a***, Enzymatic assay for elongation factor-2 kinase activity (EFK-1). EFK-1 activity was assayed as described[11] in reactions in which 0.5 μg rabbit reticulocyte eEF-2 was added to worm protein extracts. The arrow indicates the eEF-2 protein position. Lane 1, wild type; lane 2, line harbouring extrachromosomal *hsp16-2*$_p$*Cefk-1(IR)*, non-heat shocked; lane 3, a transgenic line harbouring extrachromosomal parental vector pPD49.78, heat shocked; lane 4, line harbouring extrachromosomal *hsp16-2*$_p$*Cefk-1(IR)*, heat shocked; lane 5, Tc1 active site insertion *Cefk-1* mutant. *b–g*, *In vivo* RNAi disrupts GFP expression in neurons and pharyngeal muscle. Progeny of transgenic lines harbouring extrachromosomal *unc-119*$_p$*GFP* (***b***,***e***; *unc-119* is expressed in all neurons[13]), integrated *mec-4*$_p$*GFP* (***c***,***f***; *mec-4* is expressed in six touch sensory neurons[14]) or *myo-2*$_p$*GFP* (***d***,***g***; *myo-2* is expressed in pharyngeal muscle[16]) and *hsp16-2*$_p$*GFP(IR)* were compared at 20 °C or consequent to parental heat shock at the L4 stage (35 °C, 4 h). Progeny of similarly heat-shocked *unc119*$_p$*GFP*, *mec-4*$_p$*GFP* or *myo-2*$_p$*GFP* lines exhibited no apparent reduction in intensity of neuronal fluorescence (data not shown). In parallel conventional RNAi experiments, 6 of 210 progeny of an *unc-119*$_p$*GFP* parent, 11 of 270 progeny of a *mec-4*$_p$*GFP* parent, and 57 of 240 progeny of a *myo-2*$_p$*GFP* parent exhibited detectable reduction in GFP signal.

table IR genes: (i) stable lines harbouring the potential for gene inactivation can be easily maintained; (ii) assays requiring large numbers of mutant phenocopies are feasible; and (iii) inhibition can be inducible, and thus may be used for stage-specific gene inactivation. In some cases, the endogenous high level of dsRNA product presumed produced upon heat shock appears to make for more potent inhibition than germline-injected dsRNA. Although we have focused our initial studies on the use of the inducible *hsp16-2* promoter, our findings suggest that it may be possible to inactivate specific genes for the duration of their expression period by integrating a transgene in which the promoter of the gene of interest drives transcription of an IR segment of the same gene. In addition, because dsRNA can inactivate genes in flies, plants, trypanosomes and planaria[17–21], and gene-based delivery can be effective in trypanosomes and plants [18,21], *in vivo*-directed RNAi may be effective in other organisms. A similar strategy for *in vivo*-driven RNAi might be successfully applied to inactivate specific genes in organisms that can be genetically engineered, but are not readily amenable to direct injection of dsRNA, such as the mouse.

Methods

Nematode strains. We reared and maintained *C. elegans* strains as described[22]. We constructed transgenic lines by injecting plasmid DNA (100 ng/μl) using standard protocols[23]. In all experiments we used plasmid pRF4 (ref. 24), which harbours a dominant *rol-6* allele that causes a readily distinguished roller phenotype in transgenic animals, as a co-transformation marker.

Construction of IR genes. We amplified by PCR exon-rich genomic DNA (or cDNA) using two primers that introduced unique restriction sites at the fragment ends. We digested the amplified fragment with one of the enzymes and ligated to generate an IR. We then digested with the other enzyme, the restriction site for which was positioned at the IR fragment ends, and ligated into CIAP-treated vector pPD49.78 (ref. 23), which includes the *hsp16-2* promoter and the 3′ UTR of muscle myosin *unc-54*. The cDNA and genomic DNA amplified for RNAi ranged from 0.58 to 1.45 kb. Alternative cloning strategies included digestion at two naturally occurring restriction sites to excise the gene fragment of interest with subsequent two-step ligation as above, or direct tri-molecular ligation of the doubly digested fragment into CIAP-treated vector previously linearized with one of the endonucleases at the fragment end. We found the efficiency of cloning inverted repeats to be low but acceptable in the *Escherichia coli* DH5α strain (in general, a few per hundred candidates screened) and relatively high in the *E. coli* SURE strain (Stratagene), a bacterial host tolerant of IRs (about 1/20 candidate constructs correct). The *hsp16-2*$_p$*unc-8(IR)* construct, however, was difficult to generate (1,000 candidates screened, 0.58 kb of cDNA sequence in the repeat) for reasons that are not clear. Slower-growing bacterial transformant colonies appear to have an enhanced chance of harbouring the IR gene. The yield of plasmid DNA from IR genes harboured in the *E. coli* DH5α strain was low (~3–5 μg per 50 ml culture); when the SURE strain was used as host, yields were improved (80–100 μg per 50 ml culture). Clone structures were verified using multiple restriction digests according to standard protocols.

RNA interference assays. For standard RNAi, we prepared dsRNA from cDNAs or coding sequence-rich genomic DNAs (0.58–1.2 kb) injected into N2 adults as described[1]. We scored progeny born to injected adults (10 adults per group) 12 h or more after injection (older progeny exhibit a lower phenocopy rate). For genetically directed RNAi mediated by expression of IR genes, we selected 50 transgenic roller L4s from lines harbouring various *hsp-16*$_p$*(IR)* constructs plus co-transformation plasmid pRF4 (array transmission frequency >60%; ref. 24) and reared continuously at 20 °C (non-heat shock), or heat shocked for 4 h at 35 °C, before returning to 20 °C. We scored progeny of these animals for phenotypes of interest at embryonic or larval stages as appropriate. In all experiments, at least 100 animals were scored per experimental trial. Co-expression of sense and antisense genes, which can be effective[25], was not tested. C37A2.5 is required for developmental progression past the L2 stage. Deletion of chromatin remodelling gene homologue F26F12.7 causes sterility (S.L.W. and M.D., unpublished data). Treated progeny of transgenic lines harbouring *hsp16-2*$_p$F26F12.7(IR) were scored for the percentage that failed to develop into fertile adults. A similar strategy for *in vivo* disruption of a second MI-2 homologue, T14G8.1, yielded 59% and 72% sterile in progeny of two lines scored after heat shock (data not shown). *mec-4* is expressed in six mechanosensory neurons and is required for touch sensi-

tivity. Double-stranded *mec-4* RNA or plasmid *hsp16-2*$_p$*mec-4(IR)* was introduced into wild-type animals and progeny were scored for touch insensitivity. *unc-8(n491)* is a dominant gain-of-function mutation that causes coiling and backward paralysis; locomotion in a loss-of-function mutant is nearly normal[15]. Double-stranded *unc-8* RNA or plasmid *hsp16-2*$_p$*unc-8(IR)* was introduced into the *n491* background and progeny were assayed for backing proficiency. Note that to regain backing ability, gene expression must be knocked down in most *unc-8*–expressing cells, ~60 neurons. On average at least half of lines for a given gene assayed conferred potent interference on heat activation.

Acknowledgements

We thank G. Patterson for critical reading of the manuscript and K. Pavur for eEF-2 kinase assays. This work was supported by grants from the National Institutes of Health (M.D. NS37955, NS344435; A.R. GM57300). N.T. is supported by a Human Frontiers in Science Program Organization Research Fellowship.

Received 2 September; accepted 23 November 1999.

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