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SCIENCE DIRECT
International Journal for Parasitology 35 (2005) 935–940
www.elsevier.com/locate/ijpara

Rapid communication

Development of methods for RNA interference in the sheep gastrointestinal parasite, *Trichostrongylus colubriformis*

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Received 18 May 2005; received in revised form 3 June 2005; accepted 3 June 2005

Abstract

The efficiency of RNA interference (RNAi) delivery to L1 through L3 stage worms of the sheep parasitic nematode *Trichostrongylus colubriformis* was investigated using several techniques. These were: (i) feeding of *Escherichia coli* expressing double stranded RNA (dsRNA); (ii) soaking of short interfering (synthetic) RNA oligonucleotides (siRNA) or in vitro transcribed dsRNA molecules; and (iii) electroporation of siRNA or in vitro transcribed dsRNA molecules. Ubiquitin and tropomyosin were used as a target gene because they are well conserved genes whose DNA sequences are available for several nematode parasite species. Ubiquitin siRNA or dsRNA delivered by soaking or electroporation inhibited development in *T. colubriformis* but with feeding as a delivery method, RNAi of ubiquitin was not successful. Feeding was, however, successful with tropomyosin as a target, suggesting that mode of delivery is an important parameter of RNAi. Electroporation is a particularly efficient means of inducing RNA in nematodes with either short dsRNA oligonucleotides or with long in vitro transcribed dsRNA molecules. These methods permit routine delivery of dsRNA for RNAi in *T. colubriformis* larval stage parasites and should be applicable to moderate to high-throughput screening.

Keywords: RNAi; Parasitic nematode; Gene expression; Ubiquitin; *Trichostrongylus colubriformis*; Electroporation

RNA interference (RNAi) was first described in the nematode *Caenorhabditis elegans* in 1998 (Fire et al., 1998) and later shown to be essentially the same process first described as post-transcriptional gene silencing in plants (Boutla et al., 2002; Ketting and Plasterk, 2000). The initial report in nematodes described the use of microinjection to deliver in vitro transcribed double stranded RNA (dsRNA) to fourth stage larvae (L4) or young adult *C. elegans* hermaphrodites leading to an RNAi phenotype in the progeny of the injected worms. In general, the phenotype was expressed most strongly in the F1 generation and was usually not heritable (Fire et al., 1998). Later work has shown that the dsRNA can be delivered by a variety of means, including feeding of worms with genetically modified *Escherichia coli* that express the dsRNA (feeding RNAi; Kamath et al., 2001; Timmons et al., 2001; Timmons and Fire, 1998) and also expression from a transgene in genetically modified worms (Tavernarakis et al., 2000). The availability of feeding RNAi has enabled large-scale RNAi screens of the *C. elegans* genome to be carried out, several of which have been published (Fraser et al., 2000; Gonczy et al., 2000; Piano et al., 2000).

Although RNAi is conserved through all metazoan eukaryotes so far examined and can be elicited efficiently using several delivery methods in *C. elegans*, to date there have been only four reports of RNAi in parasitic nematodes. Urwin et al. (2002), working with *Heterodera glycines* and *Globodera pallida* and Lustigman et al. (2004) with *Onchocerca volvulus*, soaked larvae at an early stage of development in relatively high concentrations of dsRNA and scored the resultant phenotype at a later but still pre-parasitic stage of development. Hussein et al. (2002) and Aboobaker and Blaxter (2003) treated parasitic adults of *Nippostrongylus brasiliensis* and *Brugia malayi* respectively, by soaking worms in dsRNA and subsequently detected altered

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0020-7519/$30.00
doi:10.1016/j.ijpara.2005.06.001

transcript levels. These authors also reported effects on viability and fertility (as well as microfilarial morphology) following maintenance of *B. malayi* adults with some dsRNAs in vitro. Although these reports have clearly demonstrated the susceptibility of at least five species of parasitic nematode to RNAi, there have been no reports of RNAi delivery by feeding or other methods more easily adapted to large-scale screening and the methods reported do not seem to be have been adopted widely. This is particularly true of the method described for *B. malayi*, where relatively large volumes and high RNA concentrations would most likely preclude large scale forward screening. The paucity of publications successfully utilising RNAi in parasitic nematodes suggests that this technology has not become routine in research on these worms.

We tested a variety of methods for delivering dsRNA molecules to the sheep gastrointestinal parasite *Trichostrongylus colubriformis* with the aim of developing an RNAi method that is reproducible and amenable to large scale screens (thousands of RNAi targets). For these initial studies, we chose *T. colubriformis* ubiquitin (*Tc ubq-1*) as the RNAi target. Ubiquitin had previously been identified as the target of an unusually potent dsRNA in a large scale, random RNAi screen in *C. elegans* that we have carried out (*Ce ubq-1*, data not shown; see also www.wormbase.org). Ubiquitin coding sequences are highly conserved (e.g. >99% amino acid sequence identity from *C. elegans* to *Homo sapiens*) and available in GenBank for many nematodes and the sequence from *T. colubriformis* was cloned by degenerate PCR using primers based on known ubiquitin sequences such as shown in Fig. 1. For feeding in *T. colubriformis* we also tested *T. colubriformis* tropomyosin (*Tc tmy*-1, GenBank accession number J04669; full length cDNA cloned into pL4440) because of the apparent failure of *Tc ubq-1* feeding. Our aim was to determine whether the failure of feeding with *Tc ubq-1* was an indication that feeding would not work for any gene target. Tropomyosin was chosen because *Ce tmy-1* has a strong RNAi phenotype and the parasite sequence is publicly available.

As expected, feeding *C. elegans* L1 s with *E. coli* strain HT115(DE3), referred to hereafter as HT115, transformed with a *Ce ubq-1* derived clone (following induction of dsRNA production with IPTG; Timmons and Fire, 1998) resulted in complete suppression of post-L1 development in *C. elegans* (Fig. 2A). Under the same conditions, virtually all *C. elegans* fed untransformed control *E. coli* HT115, or HT115 expressing dsRNA from two different *T. colubriformis* genes, *Tc ubq-1* or *Tc tmy-1*, matured to fertile adults (defined as adult worms containing eggs in utero after 80 h culture). *Caenorhabditis elegans* fed bacteria containing dsRNA directed against the *Ce unc-22* gene (Fire et al., 1998) showed the expected highly penetrant 'twitcher' phenotype but did not show a significant developmental delay.

Trichostrongylus colubriformis eggs (recovered by standard salt flotation from the faeces of sheep harbouring monospecific infections of *T. colubriformis*) were hatched and cultured to L3 stage infective larvae with untransformed HT115 or recombinant HT115 expressing various dsRNAs as a food source. Unlike the effect of *Ce ubq-1* on *C. elegans*, no effect on development was observed in *T. colubriformis* larvae fed *E. coli* expressing *Tc ubq-1* dsRNA. We also observed no effect on *T. colubriformis* larval development with HT115 expressing *Ce ubq-1* or *Ce unc-22* dsRNA (Fig. 2B), indicating that *E. coli* HT115 is a suitable food source that supports development of *T. colubriformis* larvae.

We next tested whether *Tc ubq-1* dsRNA would produce a developmental phenotype when delivered by soaking *T. colubriformis* L1 stage larvae in a solution containing purified in vitro transcribed dsRNA. For this, we used the same plasmid constructs used to express dsRNA in transformed *E. coli* for the feeding experiments above. In this case, the dsRNA was produced by in vitro transcription and subsequently purified. Soaking of *C. elegans* larvae with *Ce ubq-1* dsRNA, but not with control dsRNA, produced a significant reduction in the number of fertile adults that resulted following culture (Fig. 2C). In contrast, no significant developmental delay was observed in *T. colubriformis* larvae soaked with *Tc ubq-1* dsRNA or the controls (Fig. 2D).

As an alternative to soaking larvae with dsRNA produced by in vitro transcription, we also tested whether a developmental phenotype would occur following the soaking of larvae in the presence of 22 bp synthetic dsRNA (siRNA) derived from ubiquitin coding sequence. Such short dsRNA oligonucleotides, which mimic the active product of the RNAi processing machinery, have proven effective in provoking an RNAi response in several organisms where long dsRNA has proven ineffective or induced non-specific effects (e.g. *Drosophila melanogastor*, Elbashir et al., 2001a; fish species, Boonanuntanasarn et al., 2003; Dodd et al., 2004 and mammals, Elbashir et al., 2001b). Synthetic siRNAs were produced based on either *Ce ubq-1* or *Tc ubq-1*. The sequences were selected from a relatively poorly conserved region in which there are predicted to be two or more mismatches between the species so that the siRNAs should be species specific (see Fig. 1).

Ce ubq-1 derived siRNA, but not *Tc ubq-1* derived siRNA, caused a significant developmental delay in *C. elegans*. The effectiveness of siRNA in *C. elegans* in Fig. 2C was somewhat surprising because this species has been reported to be poorly susceptible to RNAi from siRNA. Systemic RNAi in *C. elegans* is thought to be dependent on the expression of SID-1, a specific transporter for dsRNA molecules that mediates the spread of the dsRNA to most non-neuronal tissues in the worm (Winston et al., 2002; Feinberg and Hunter, 2003; Tijsterman et al., 2004). SID-1 is inefficient at transporting small dsRNAs such as siRNAs, so the strong phenotype observed following incubation of *C. elegans* with *Ce ubq-1* siRNA may reflect the high potency of RNAi targeting ubiquitin expression or perhaps the existence of a transport mechanism for small RNAs that mediates spreading.