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# DEPLETION OF Y ASSOCIATED PROTEINS BY RNAi DOWN-REGULATES THE Y RNA IN *CAENORHABDITIS ELEGANS*

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**Abstract**

Short non-coding RNAs (ncRNAs) perform their cellular functions in ribonucleoprotein (RNP) complexes, which are also essential for maintaining the stability of the ncRNAs. Depletion of individual protein components of Y ribonucleoprotein (Y RNP) particles by RNA interference (RNAi) in *Caenorhabditis elegans* produced specific phenotypes. Northern blot also confirmed the depletion of targeted Y RNP protein component. The depletion of specific Y RNP affected expression levels of the Y RNA. Depletion of candidate associated proteins may constitute an alternative strategy when investigating ncRNA-protein interactions and ncRNA functions. Depletion of both the protein components (Ro60 and La) of Y RNP complex indicated that these associated proteins are essential for the stability of the Y RNAs in *C. elegans*. RNAi technique when employed directly against Y RNA did not produced any phenotype and Northern blot showed no effect on the levels of Y RNA indicating that RNAi is not effective against Y RNA in *C. elegans*.

## Introduction

The non-coding Y RNAs were discovered in the early 1980s and was widely distributed among all forms of life. Non-vertebrate metazoans such as *Caenorhabditis elegans* and a few microbes including the eubacterium *Deinococcus radiodurans* have a single Y RNA (Debra *et al*., 1995), vertebrates encode up to four Y RNAs (Chen &Wolin, 2004; Perreault *et al*., 2007). Y RNA exists as a component of Ro ribonucleoprotein particles (Ro RNPs) (Lerner *et al*., 1981), which contain 60 kDa Ro60 protein (Chen &Wolin, 2004) and 50 kDa La protein (Bousquet &Deragon, 2009). These RNAs are ~100 nt in length and are transcribed by RNA polymerase III. Ro60 and La proteins have been implicated in protecting Y RNAs from exonucleolytic degradation, among other functions (Wolin &Cedervall, 2002; Chen & Wolin 2004). Recently it was shown that Y RNAs are essential for the initiation of chromosomal DNA replication, whereas Ro RNPs are implicated in RNA stability and RNA quality control (Alexander *et al*., 2010). The localization of Ro/La complexes is mainly cytoplasmic, but their assembly is performed in the nucleus (Soyeong *et al*., 2009; Simon *et al*., 1994). The Ro/La RNP particle has also been claimed to play an important role in the initiation of autoimmunity (Arbuckle *et al*., 2003).

RNAi was first discovered in *Caenorhabditis elegans* (Fire *et al*., 1998) and is a post-transcriptional sequence-specific gene silencing mechanism, occurring in a wide variety of eukaryotic organisms (Tijsterman *et al*., 2002; Ullu *et al*., 2004). It is triggered by the introduction of double-stranded RNA (dsRNA) and is homologous in sequence to the silenced gene (Jia &Sun, 2003).The dsRNAs are recognized by the evolutionary conserved Dicer enzyme (Bushman, 2003) which processes long dsRNA by an ATP-dependent reaction into 21-25 nucleotides double-stranded fragments, and is termed as termed siRNAs (Elbashir *et al*., 2001). The siRNA duplex contains 5´phosphate and 3´hydroxyl termini and single-stranded two-nucleotide overhangs at their 3´ends (Elbashir *et al*., 2001). These structural features are important for the entry of siRNA into the RNAipathway, as blunt ended siRNA or siRNA lacking a 5´phosphate group are inefficient triggers of RNAi both *In vivo* and *In vitro* ( Caplen *et al*., 2001). The siRNA duplexes are then incorporated into the multi-component RNA induced silencing complex (RISC) complex, a nuclease which can recognize and cleave a target mRNA (Ameres *et al*., 2007; Yoda *et al*., 2010). The depletion of Y-RNP proteins by RNAi, thus, corresponds to an opportunity to determine the role of these proteins in terms of stability and steadiness that they impart to Y RNA.

## Material and Method

**RNAi:** RNAi was carried out by feeding worms with *E. coli* HT115 carrying plasmid L4440, expressing a dsRNA fragment of the targeted gene, using *E. coli* HT115 carrying a plasmid without insert as negative control. Plasmid L4440 (Addgene) contains two T7 promoters in opposite orientation at each side of the MCS thereby yielding dsRNA when transformed into bacterial strains expressing T7 polymerase. Plasmids targeting specific genes were constructed by inserting a 1–2 kb genomic PCR fragment using primers pairs designed using PrimerPremier using the targeted gene sequences as template. The targeted gene sequences are cloned into the plasmid L4440. The resulting construct was transformed into HT115 (an RNase III-deficient strain of *E. coli* with an isopropyl-ß-D thiogalactopyranoside (IPTG)-inducible T7 polymerase (Timmons *et al*., 2001) using standard $CaCl_2$ transformation protocols and plated on 100 µg/ml ampiciline and 15 µg/ml tetracycline containing LB-agar plates and incubated at 37°C overnight. Selected colonies were minipreped (Promega) and the DNA subjected to restriction analysis. The bacterial cells were applied onto NGM plates (Brenner, 1974) supplemented with 100 µg/ml ampicillin, 15 µg/ml tetracycline and 1 mM IPTG and incubated at room temperature for 2–3 days before adding the worms to the plates. RNAi phenotypes were observed after 24–72 hours.

**Probes for northern blotting:** Digoxigenin-labeled RNA probes to detect depletion of mRNAs and Y RNA on Northern blots were produced by *In vitro* transcription. *In vitro* transcriptions reactions were set up with 1 mM each of ATP, CTP and GTP, 0.65 mM UTP, and 0.35 mM Digoxigenin-11-UTP (Roche) in a 10 µl reaction volume, using an enzymatically digested plasmid with the relevant insert as template. The *In vitro* transcription reactions

were incubated with T7 transcription polymerase (Invitrogen) at 37°C overnight and the reaction products were purified with Trizol (Invitrogen).

**Northern blotting:** Northern blotting was carried out to confirm the depletion of mRNAs and Y RNA. One µg of total RNA was denatured for 5 minutes at 70°C and loaded onto denaturing 6% polyacrylamide gels containing 7 M Urea. After electrophoresis for about 22 min at 250 V, RNA was transferred onto positively charged nylon membranes (Hybond-N+, Amersham Biosciences). After brief washing using 2 × SSC, the transferred blots were cross-linked to the membrane under short-wave UV light. After prehybridization at 68°C for 1 hour in Ultrahyb Ultrasensitive Hybridizaion Buffer (Ambion), the blots were subjected to hybridization with Digoxigenin-labeled RNA probes overnight at 68°C. Then the membranes were washed as follows: twice for 5 minutes at room temperature in 2 × SSC containing 0.1% SDS, 15 minutes at 68°C in 2 × SSC containing 0.1% SDS, 10 minutes at room temperature in 1 × Washing Buffer (DIG Wash and Blocking Buffer Set, Roche). After the above mentioned steps, the membranes were blocked for 30 min at room temperature in 1 × Blocking Buffer (DIG Wash and Blocking Buffer Set, Roche), then incubated with anti-Digoxigenin AP Fab fragments (Roche) diluted (1/10000) in 1 × Blocking Buffer at room temperature for 30 min. After that, the membranes were washed twice (15 minutes each time) at room temperature in 1 × Washing Buffer, and then equilibrated in 1 × detection buffer (DIG Wash and Blocking Buffer Set, Roche) at room temperature for 3 minutes, followed by incubation with several drops of CDP-star (Roche) at room temperature for 30 minutes. Chemiluminescent signals were recorded with a Chemi-Capt 3000 imaging system (Vilber).

**Primers pairs**

**unc-22**
Forward: 5′-AATGATCTCCCTTGTTGAGTGAA-3′
Reverse: 5′-CCACTCTTACTGCTACCAACGCTT-3′

**gpb-1**
Forward: 5′-CTCGGTGACTCCTAGACAAGAAA-3′
Reverse: 5′-AATCAGCAAATGACACAACACTG-3′

**Ro60**
Forward: 5′-CCTCGGTTCTGCATACACAGGAA-3′
Reverse: 5′-GAGCAAGTCGGATACTTAGATCG-3′

**La**
Forward: 5′-GACTGCACGGATGCCAACGTAC-3′
Reverse: 5′-GTTCAGGCACCAGATGTACACAT-3′

**Results and Discussion**

**RNAi system:** In order to substantiate the RNAi system, we chose as controls for RNAi method that were easy to assay in *C. elegans,* such as gpb-1, for which mutants are embryonic lethal, and unc-22, which results in a post-embryonic uncoordinated movements phenotype (Unc) (Timmons &Fire, 1998, Zwaal *et al*., 1996). In order to obtain RNAi effects, PCR fragments of the gene sequences (unc-22 {1151 bp} and gpb-1 {2098 bp}) were cloned in plasmid L4440 for synthesizing corresponding dsRNA fragments when transformed into *E. coli* HT115 (see Material and Method). After feeding the worms for 72 hours gpb-1 produced 100% dead embryos and unc-22 produced 98% worms with uncoordinated movements (Kamath *et al*., 2001). The down-regulation of unc-22 and gpb-1 was further verified by Northern blots (Fig. 1), showing the efficacy of the RNAi method. Actin was used as a control for Northern blot performed for gpb-1 and unc-22. Actin mRNA level remained same for both control and sample subjected to RNAi. To further testRNAi system, worms expressing green fluorescent protein (GFP) were fed with bacteria expressing double-stranded RNA (dsRNA) homologous to the gfp gene. Visual inspection under microscope clearly indicated the reduction in GFP fluorescence in the worms.

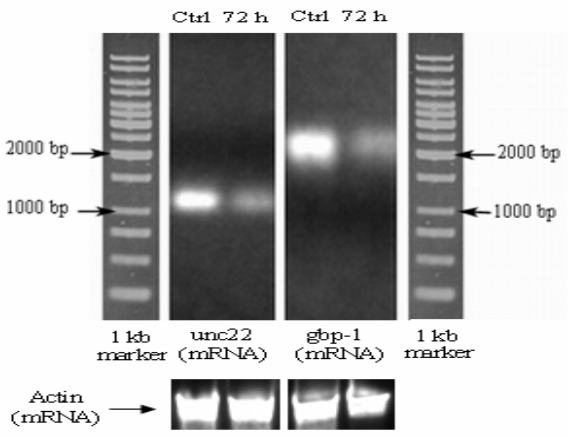


Fig. 1. Northern blot of the unc-22 (1151 bp) and gpb-1 (2098 bp) mRNAs. Northern blots after 72 hours of feeding of *C. elegans* with *E. coli* expressing double-stranded RNA homologous to the unc-22 and gpb-1 gene.

**Depletion of Y associated proteins produce distinct phenotypes:** The effect of RNAi against Y RNP mRNA on the Y RNA expression levels was investigated. RNAi against each of the two proteins produced distinct phenotypes (Table 1). Depletion of Ro60 resulted in majority of worms (~85%) showing the sterile worms (Fig. 2A). Most of these sterile worms (~80%) also showed protruding bulges of varying size located at the vulva. When sterile worms with and without protruding bulges were examined under high magnification, it was observed that both types of worms lacked eggs. Previous Ro60 depletion studies in *C. elegans* also resulted in sterile phenotypes (Bryne *et al*., 2006). Depletion of Ro60 in *Saccharomyces cerevisiae*led to slower and defective growth (Cheng *et al*., 1996; Bouffard *et al*., 1999). Similarly, depletion studies of La protein resulted in ~95% dumpy worms that was in accordance with the previous studies (Fig. 2B) (Ceron *et al*., 2007).

**Table 1. Phenotypes observed after application of RNAi against two Y-associated proteins mRNAs.**

| S. No. | Y-associated proteins | Phenotype | % Age of affected worms |
|---|---|---|---|
| 1. | Rh-60 (RPO-1) | Sterile worms | ~85% |
| 2. | La | Dumpy worms | ~95% |

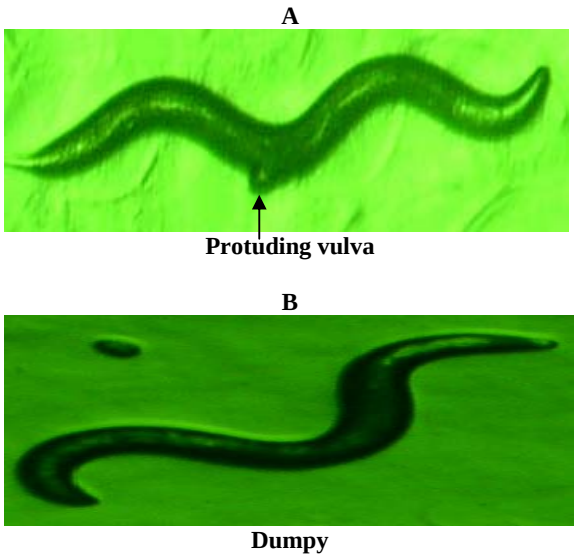


Fig. 2. Pictures of *Caenorhabditis elegans* showing (A) protruding vulva after depletion of Ro60 and (B) dumpy phenotype after depletion of La protein.

For down-regulated Y gene, total RNA was extracted after 72 hours of feeding. Northern blots were performed to confirm the RNAi depletion of the Y associated proteins mRNA genes compared with control worms (Fig. 3). For each target protein gene (Y-associated protein mRNA) Northern analysis confirmed a strong reduction in mRNA levels (Fig. 3), and it is reasonable to think that the obtained phenotypes may be a consequence of a loss of function of the targeted protein (or the corresponding RNP complex) rather than an effect of the reduction of Y ncRNA (see below).

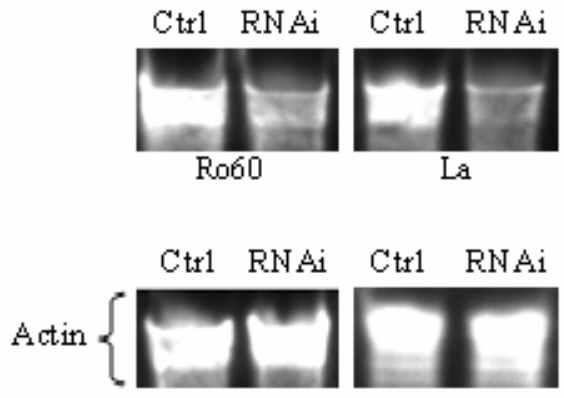


Fig. 3. Northern blots of mRNAs encoding protein components of Y-RNPs. Ro60 and La mRNAs after depletion by RNAi.

**Y proteins depletion specifically reduces Y RNA expression levels:** The effect of RNAi against Y mRNAs on the Y RNA was then investigated. For this purpose, total RNA was extracted at equal time intervals (24 h, 48 h and 72 h) after depletion of each of the Y RNP component proteins. Northern analyses of Y RNA (CeN9) showed that the levels of Y RNA reduced considerably after depletion of both of its respective protein components of Y RNPs (Fig. 4). The control snRNA also showed consistent levels of expression after 24, 48 and 72 hours.

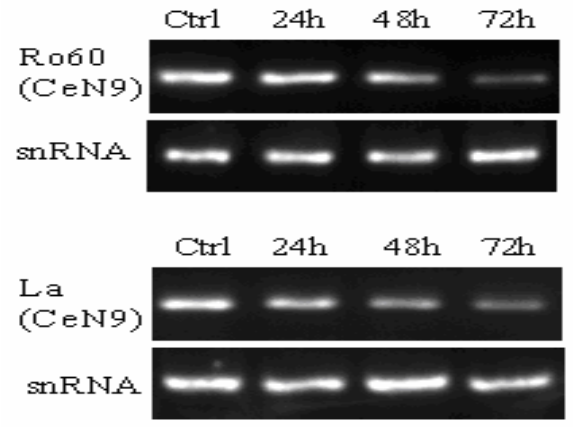


Fig. 4.Effects of RNAi against protein components of Y RNPs on Y RNA expression levels. Northern blots of Y RNA (CeN9) after RNAi against both proteins (Ro60 and La) of Y RNA. snRNA was used as a control for each of the Northern blot.

**RNAi employed directly against Y RNA:** In a further attempt, RNAi technique was employed to target the Y RNA directly. For this purpose, gene for Y RNA was amplified by using primer pairs. The PCR product was cloned in plasmid L4440 for producing dsRNAs fragments when transformed in *E. coli* HT115. After feeding the worms for 72 hours, no abnormality was observed in the worms. Total RNA was isolated after 24h, 48h and 72 h of feeding. Northern blot was performed and the results verified that no change in the expression level of Y RNA was observed in the control and treated samples (Fig. 5). The RNAi was also employed in F1 and F2 generations but no abnormality was observed in the worms. The Northern blot results also verified these findings (Fig. 5 B and C). Results indicating down regulation of non-coding RNAs by RNAi are not reported till now.

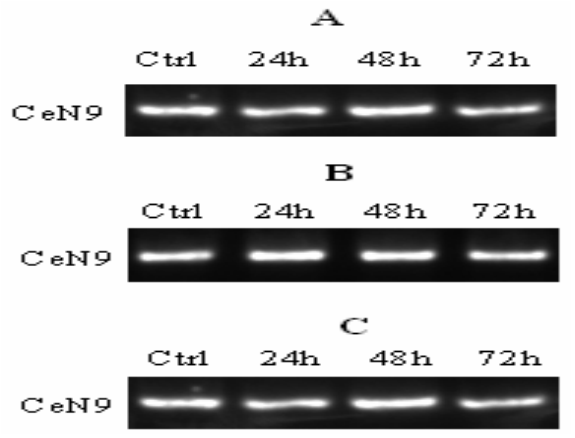


Fig. 5. Effect of RNAi against Y RNA. Northern blot of Y RNA (CeN9) after 24h, 48h and 72 h in (A) Parental worms (B) F1 worms and (C) F2 worms.

## Conclusion

Ro60 and La proteins are essential for the stability of the Y RNA in *C. elegans* and RNAi is not effective against non-coding RNAs like Y RNA in *C. elegans*.

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