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Use of RNAi in *C. elegans*

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Summary

RNA-mediated interference (RNAi) has been a valuable tool for the analysis of gene function in *Caenorhabditis elegans* (*C. elegans*). In *C. elegans*, the injection of double-stranded RNA (dsRNA) or plasmid DNA expressing dsRNA under the control of a *C. elegans* promoter results in gene inactivation through the specific degradation of the targeted endogeneous mRNA. It is also possible to initiate RNAi either by soaking worms in a solution of dsRNA or by feeding worms with *E. coli* expressing the dsRNA. Using studies of the DNA repair in *C. elegans* as an example, we describe the use of RNAi against the *C. elegans POLH* gene (*Ce-POLH*), which encodes DNA polymerase η (pol η).

Pol η has the ability to catalyze translesion synthesis (TLS) past UV-induced cyclobutane pyrimidine dimers (CPDs) and some other lesions as well. Loss of pol η in humans results in increased photosensitivity and the cancer-prone genetic disorder xeroderma pigmentosum variant (XPV). We provide an example of the feeding RNAi technique, in which downregulation of pol η in *C. elegans* results in increased sensitivity of several development and differentiation processes, including meiosis and embryogenesis to UV radiation.

Key Words: *C. elegans*; RNAi; DNA polymerase η; UV-C; embryogenesis.

1. Introduction

DNA can be damaged by a wide range of physical and chemical agents, both endogenous and environmental. To maintain the integrity of the genetic material, cells possess multiple pathways for the repair of various types of DNA damage ***(1)***. Nucleotide excision repair (NER) is the main pathway for the removal of CPDs and ***(4–6)*** photoproducts produced by UV radiation,

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although CPDs are repaired fairly slowly by this pathway. Unrepaired damage can interfere with basic processes such as DNA replication and transcription, resulting in increased mutation rates and even cell death ***(2)***. In eukaryotes, another major pathway that circumvents such threats involves DNA polymerases that can synthesize past sites of DNA damage, a process known as translesion synthesis (TLS).

C. elegans possesses TLS polymerases ***(3,4)*** and is a convenient organism for the study of different aspects of the DNA damage response, such as checkpoint activation, apoptosis, and DNA repair ***(5)***. For the functional analysis of DNA damage response genes in *C. elegans*, the RNAi technique is ideal as it can easily and rapidly produce gene-specific loss of function. It is possible to initiate RNAi by injecting dsRNA prepared by *in vitro* transcription ***(6,7)*** or plasmid DNA designed to express dsRNA from a *C. elegans* promoter ***(8)***. Alternatively, the worms may be soaked in a solution of dsRNA ***(9)*** or fed with *E. coli* expressing the target gene dsRNA ***(7,10)***. Following RNAi treatment, RNAi-induced phenotypes can be observed in progeny and be maintained over the course of several generations with continuous feeding ***(10)***.

Additionally, *C. elegans* can be used to study the effects of DNA-damaging agents on development and differentiation, including meiosis and embryogenesis, which have been greatly facilitated by the conventional classification of gametogenesis and embryogenesis in *C. elegans* into several distinct periods ***(11)***.

Downregulation of pol η in *C. elegans* has been shown to increase sensitivity to UV radiation during germline development ***(12)***. Human pol η bypasses CPDs by incorporating the correct nucleotide opposite the lesion ***(13–18)***, indicating that pol η is involved in the relatively accurate TLS pathway for the bypass of UV-induced lesions *in vivo* and *in vitro*. To further understand the roles of pol η in multicellular organisms, we examined the sensitivity of *C. elegans* to UV radiation at various stages of embryogenesis using the RNAi feeding method. Transcripts of the *Ce-POLH* (*Polh - 1*) gene were suppressed by feeding worms *E. coli* strains containing plasmid vectors that were designed to produce dsRNA by the bidirectional transcription through the inserts. *Ce-POLH* transcript loss following RNAi treatment was confirmed by performing RT-PCR on total RNA isolated from the adult worms. The UV sensitivity of *C. elegans* embryos was determined by measuring the hatching rate of eggs laid by RNAi-treated adult hermaphrodites following UV irradiation. This approach is suitable for determining the requirement for DNA repair at various embryonic stages in *C. elegans*. Additionally, *C. elegans* treated by RNAi provides a good model system to investigate DNA repair in germ and embryonic cells.

2. Materials

Worms were grown on nematode growth medium (NGM) plates seeded with either *E. coli* OP50 or *E. coli* HT115 (DE3) for RNAi treatment. Plates were incubated at 20 °C.

2.1. C. elegans and Bacterial Strains

1. The *C. elegans* strain Bristol N2 (kindly provided by Dr. I. Katsura, National Institute of Genetics, Mishima, Japan) ***(19)***.
2. *E. coli* OP50 (kindly provided by Dr. I. Katsura) ***(19)***.
3. *E. coli* HT115 (DE3) (W3110, *rnc14* ::Δ Tn*10*) (kindly provided by Dr. A. Fire, Stanford University School of Medicine, Stanford, CA) ***(7,10)***.

2.2. C. elegans and E. coli Culture

1. Nematode growth medium (NGM) plates. To 600 mL of double distilled water (ddw) in a flask containing a stirring bar, add 1.8 g NaCl, 1.5 g Bacto peptone (Becton, Dickinson, Spark, MD), and 18 g Bacto ager (Becton, Dickinson). After autoclaving, mix the solution on a stirring plate and cool down to about 60 °C. Add the following sterile reagent: 0.3 mL of 2% cholesterol (prepared in ethanol), 0.6 mL of 1 M $MgSO_4$, 0.6 mL of 1 M $CaCl_2$, and 15 mL of 1 M potassium phosphate (pH 6.0). In addition, add 0.6 mL of 50 mg/mL Carbenicillin and 1.2 mL of 0.5 M isopropyl-1-thio-β-D-galactoside (IPTG) to the NGM plates to be used for the RNAi experiments. Distribute 30 mL of the solution per 100 mm in a Petri dish, and dry at room temperature overnight.
2. 20 °C incubator (for *C. elegans* culture).
3. Sterile LB media.
4. 37 °C shaker (for *E. coli* culture).
5. Platinum picker.

2.3. Vector for Bacteria-Mediated RNAi

1. L4440 (pPD129.36) plasmid (kindly provided by Dr. A. Fire) ***(20)***.
2. The DNA plasmid for RNAi containing the sequence of the target gene located between the two T7 RNA polymerase promoters of L4440 (pPD129.36).

2.4. RT-PCR Analysis of Target Gene Expression Following RNAi

1. Disruption buffer: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, pH 8.0, and 0.02% lysozyme (Seikagaku Corp., Tokyo, Japan).
2. Total RNA Isolation kit (Macherey-Nagel, Inc., Duren, Germany).
3. SuperScript One-Step RT-PCR System (Invitrogen, Carlsbad, CA).
4. PCR machine.

2.5. UV Light Irradiation

1. Germicidal lamp (254 nm) (GL15, Toshiba, Tokyo, Japan)
2. UV dose calibrator (UVR-2, TOPCON, Tokyo, Japan)

3. Methods

The methods described below outline (1) the construction of the plasmid for bacteria-mediated RNAi, (2) the preparation of feeding RNAi plates, (3) RT-PCR analysis of gene expression following RNAi, and (4) the UV sensitivity of RNAi-treated worms.

3.1. Plasmid for Bacteria-Mediated RNAi

Amplify a DNA fragment by PCR from a *C. elegans* cDNA library (*see* **Note 1**), and insert it between the two T7 RNA polymerase promoters of L4440 (pPD129.36). The plasmid vector is designed to produce dsRNA by bidirectional transcription of the insert.

3.2. Preparation of NGM Plates Seeded with E. coli for RNAi

1. Transform the resulting plasmid containing the sequence from the target gene into HT115 (DE3) cells, which have an IPTG-inducible T7 RNA polymerase gene and are deficient in RNase III ***(7,10)***.
2. Add IPTG (final concentration: 1 mM) to the transformed cells in log phase.
3. Spread the mixture (100 µL/100 mm plate) over the NGM agar plates containing 1 m*M* of IPTG and 50 µg/mL of Carbenicillin, and incubate the plates for 24 h at 20 °C. HT115 (DE3) cells transformed by the L4440 vector alone are used as a control.

3.3. Targeting Gene Expression by RNAi

1. Place four hermaphrodites at the young adult stage on fresh RNAi plates (*see* **Note 2**) and transfer them to new RNAi plates after 12 h of feeding at 20 °C (*see* **Fig. 1** and **Note 3**).
2. Incubate the RNAi-treated worms over the next 5 h and then remove the adult worms. F1 embryos from the RNAi-treated worms are also fed on RNAi plates.
3. To confirm the suppression of transcripts from the targeted gene, collect the RNAi-treated F1 adult worms, suspend them in 100 µL of disruption buffer, quickly freeze them at −80 °C for 10 min, and then incubate them at 37°C for 3 h. Extract RNA from the worms using the Total RNA Isolation kit (Macherey-Nagel, Inc.), and carry out RT-PCR using the SuperScript One-Step RT-PCR System (Invitrogen) with appropriate primers. Separate the RT-PCR products on agarose gels (*see* **Fig. 2** and **Note 4**).

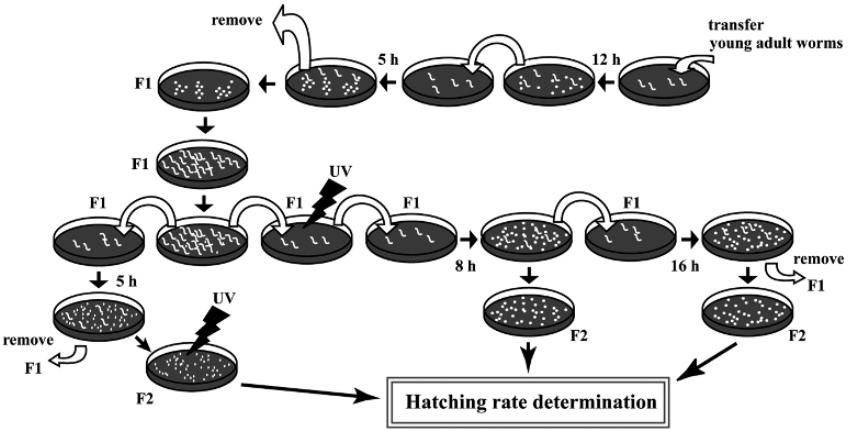

Fig. 1. Schematic representation of the RNAi experiments. Young adults were placed on RNAi plates that contained bacteria producing *Ce-POLH*-dsRNA. After feeding for 12 h, the worms were transferred to fresh RNAi plates. F1 eggs laid over the next 5 h were used in the experiments. Young F1 adults were transferred to fresh RNAi plates, and the hatching rates of F2 eggs from F1 adults irradiated with UV 0–8 h and 8–24 h before laying and the hatching rates of F2 eggs irradiated with UV 0–5 h after laying were determined.

3.4. UV Sensitivity of RNAi-Treated Worms

The experimental scheme is shown in **Fig. 1**. The UV sensitivity of RNAi-treated worms was determined by monitoring the hatching rates of F2 embryos.

1. Prewarm a UV 254-nm lamp for at least 15 min before irradiation and monitor the energy of the lamp in the UV-C region by using a UV dose meter (*see* **Note 5**).
2. Irradiate young gravid F1 worms and F2 eggs laid by F1 worms with several doses of UV.
3. Transfer these F1 worms to new RNAi plates, and incubate the F1 worms at 20°C for 5 h, 8 h and 16 h, as shown in **Fig. 1**.
4. Following removal of the F1 worms from the RNAi plates, count the number of eggs.
5. After incubation for 36 h at 20 °C, count the number of unhatched eggs and score hatching rates.
6. Examples of the hatching rates of RNAi-treated eggs and a schematic representation of gametogenesis and early embryogenesis in a young gravid hermaphrodite are shown in **Fig. 3**. We examined the hatching rates of F2 eggs from F1 adults irradiated with UV 0–8 h and 8–24 h before laying and the hatching rates of F2 eggs irradiated with UV 0–5 h after laying, which correspond to the early embryogenesis stage, the meiotic pachytene nuclei stage, and the stage from gastrulation to early morphogenesis, respectively ***(11)***.

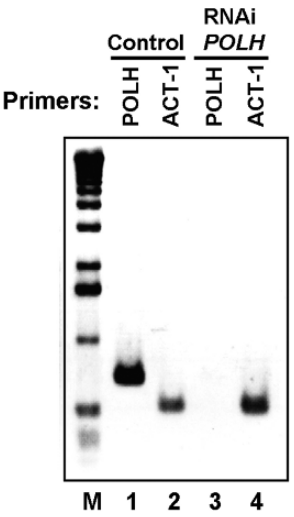

Fig. 2. RT-PCR of total RNA isolated from F1 adult worms treated by RNAi. RT-PCR with specific primers for *Ce-POLH* and *ACT-1* (encoding the body wall actin gene) as a control was carried out on total RNA prepared from RNAi-treated F1 adult worms. The figure is a negative image of an ethidium bromide-stained agarose gel. *POLH* mRNA is depleted in pol η-targeted adults (lane 3) as compared with control worms (lane 1), while *ACT-1* mRNA is expressed in both the control and treated animals (lanes 2 and 4). M refers to 1-kb DNA size marker.

4. Notes

1. The dsRNA region should be derived from the coding region and not from introns. Generally, dsRNA molecules for RNAi experiments in *C. elegans* can be longer than siRNAs, because worms do not display the interferon response to longer molecules ***(21)***. For example, we used the full-length coding region of *Ce-POLH* ***(12)***.
2. It is better to use freshly seeded plates rather than plates that have been stored.
3. *C. elegans* is predominantly a self-fertilizing hermaphrodite and should be used as such for RNAi experiments. Males also exist, but they may be less affected by dsRNA delivered through feeding ***(10)***. The transfer of worms should be handled gently with the platinum picker to avoid damaging them. RNAi plates should be sealed with vinyl tape to prevent them from drying out over long incubation times.
4. The RT-PCR is performed as described ***(22)***. The reaction mixture (100 μL) is split into two tubes into which either the *Ce-POLH* gene or *ACT-1* (*act-1*)-specific primers are mixed. After 30–40 cycles of amplification,the RT-PCR products are separated on a 1% agarose gel.
5. To avoid accidental exposure to UV-C light, UV-opaque glasses with side shields and appropriate gloves should be worn.

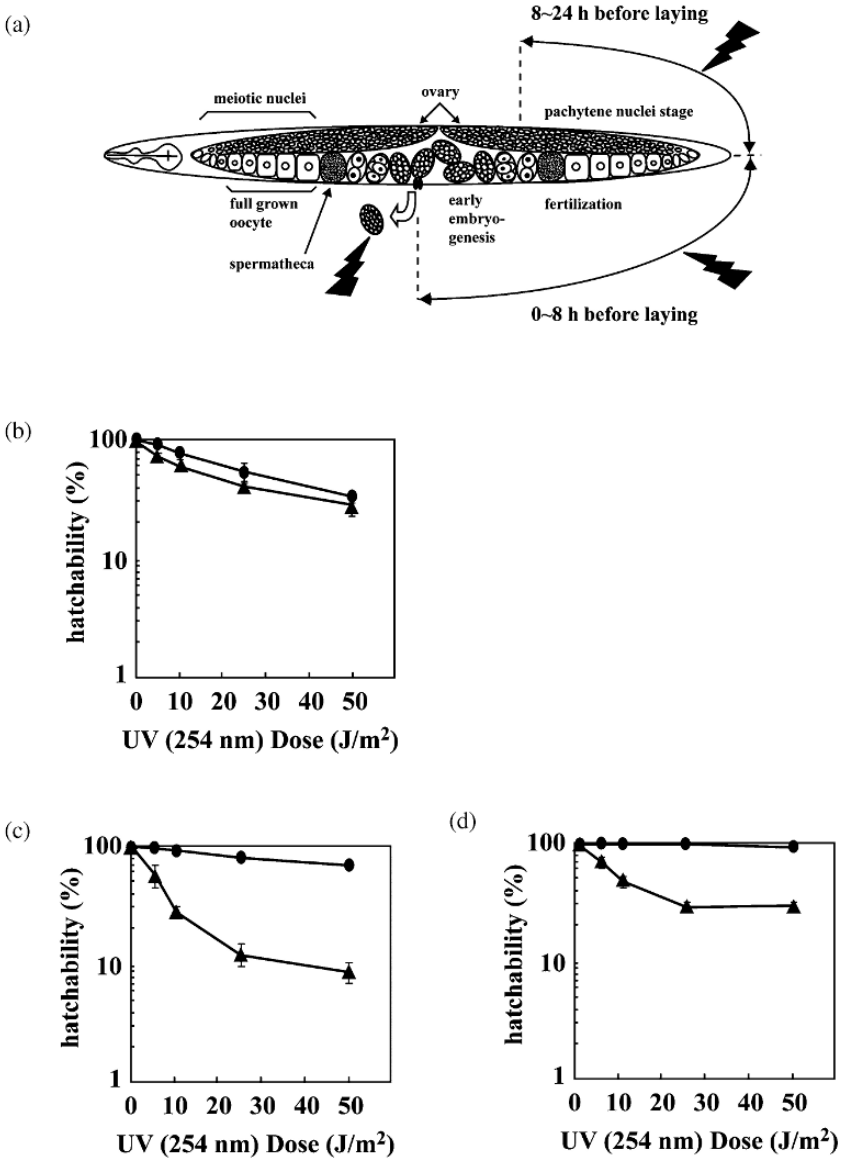

Fig. 3. (a) Schematic representation of gametogenesis and early embryogenesis in a young gravid hermaphrodite. (b) F2 eggs laid over the course of 5 h by F1 worms were UV-irradiated and hatching rates were scored. (c) Young F1 adults were UV-irradiated, and the hatching rates of F2 eggs laid up to 8 h after irradiation were scored. (d) The hatching rates of F2 eggs laid 8–24 h after irradiation were scored. Error bars indicate standard deviations. Closed circles: control worms; closed triangles: *Ce-POLH* RNAi-treated worms.

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