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Phenotypic Suppression of Caenorrhabitis Elegans NAS 31 Gene by RNA Interference

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Abstract:
The genetic interference phenomenon in the nematode Caenorhabditis elegans has been described in which expression of an individual gene can be specifically reduced by soaking or microinjecting a corresponding fragment of double-stranded (ds) RNA. One striking feature of this process is a spreading effect whereby interference in a broad region of the parasite is observed following the soaking of dsRNA into the extracellular body cavity. In this paper showed that C. elegans can respond in a gene-specific manner to dsRNA encountered in the environment. C. elegans normally feed on E. coli OP50 bacteria strain, ingesting and grinding them in the pharynx and subsequently absorbing bacterial contents in the gut. Here we report that soaking C. elegans with dsRNAs confers specific interference effects in the larvae transcripts as judged by RT-PCR.

Keywords: *Phenotypic suppression, Caenorrhabitis elegans, NAS 31 gene, RNA interference*

1. Introduction

When double-stranded RNA (dsRNA) corresponding to a sense and antisense sequence of an endogenous mRNA is introduced into a cell, in organisms ranging from trypanosomes to mice, the cognate mRNA is de-graded and the gene is silenced (Fire, 1999). This type of posttranscriptional gene silencing (PTGS) was first discovered in *C. elegans* (Fire et al., 1998) and is called RNA interference, or RNAi. RNA interference (RNAi) is increasingly being used to identify gene function and lethal targets for vaccine antigens. In this paper we report phenotypic suppression of *Caenorrhabitis elegans* NAS 31 gene associated with reproduction and blood-feeding of the parasite and predominantly expressed in late larval and adult stages by RNAi interference.

2. Materials and Methods

2.1. Bioinformatics

The *Caenorhabditis elegans* genome was accessed through the Wormbase interface (Stein et al., 2001) (www.wormbase.org). Analyses of selected sequence and the deduced amino acid sequences were performed using the DNAstar software program (DNAstar Inc.). Database searches using BLAST algoritms (Altschul et al., 1997) were performed on the NCBI server (www.ncbi.nlm.nih.gov/blast). Genes corresponding the NAS genes from other nematode species were identified by BLAST search of the Nembase nematode EST datasets. Multiple alignments of ESTs and predicted proteins were generated using CLUSTALX (Thompson et al., 1997) and adjusted by eye where necessary.

2.2. Reverse Transcriptase Coupled PCR

2.2.1. Primers

Four primers with cloning sites specific for expression of *C. elegans* NAS31gene designated CEF1-Nco-Ⅰ(5'-**CCATGG**TATAGTTTATTCACGCAT-3'); CER1-Xho-Ⅰ (5'-**CTCGAG**TAGTTTCTGGCTGGCATTGC-3'); CEF2-Nco-Ⅰ (5'-**CCATGG**AGTTGGACATGCT
TTGA -3'); CER2 - Xho-Ⅰ (5'-**CTCGAG**ATTTGAAGAAGCACTCGTTGC -3'), were used as forward and reverse primers respectively, to amplify specific 3' or 5'end regions by PCR, were synthesized (EurofinS MWG Operon, Germany). Restriction enzyme (RE) nucleotide recognition sequences (underlined) were included in the primers to facilitate subsequence in-frame cloning of the coding sequence for expression.

2.2.2. RNA Extraction

Total RNA was extracted from exsheathed L3, L4 and adult male and female parasites according to Hashmi et al. (2002). In brief, parasites were re-suspended in 200µl of lysis buffer (0.5% SDS, 5% β-mercaptoethanol, 10 mM EDTA, 10

mM Tris–HCl, pH 7.5, and 0.5 mg/ml proteinase K), quick frozen at -80 ^{0}C for 10 min, followed by incubation at 55 ^{0}C for 1 h. The RNA was extracted using Total RNA Isolation Reagent (Advanced Biotechnologies Ltd.) and stored at -80^{0}C until use.

2.2.3. Single step RT-PCR

Reverse transcriptase coupled PCR (RT-PCR) was used to determine the specificity of ***C. elegans*** **NAS 31** mRNA transcription using SuperScript One-Step RT-PCR System (Invitrogen) according to the manufacturer's instructions. The thermo cycle conditions were cDNA synthesis and pre-denaturation phase of 55^{o}C (30min) (1-cycle) and 94^{o}C (5min) (1-cycle), followed by PCR amplification of 30cycles at 94^{o}C (30s), 56^{o}C (1min), 72^{o}C (2min), and a final extension for 7min at 72^{o}C in the last cycle. The C. elegans NAS31 amplified products were analyzed by gel electrophoresis on 1% (w/v) agarose gels (Sigma*), stained with red gel (BIOTIUM*) and submerged in 1xTAE electrophoresis buffer. The gels were visualized under ultraviolet illumination and recorded on a Polaroid 667-film.

RT-PCRs for two housekeeping genes, extracellular (SODe) and cytoplasmic superoxide disumutases (SODc) (Liddell and Knox, 1998), were used to control the uniformity of the RNA purifications. Primers used for the RT-PCR were: F54D5.3_F ATGAACCGTTTCAGCTGCG; F54D5.3_RCTCCATTTTGAACTGCGTCGTAG; F54D5.4_F AGGATGCTTGAATACTGGA ACTG; F54D5.4_R ATCAGTGTCAGAAACAGAGTCG, respectively and products also analysed on 1.0% agarose gels.

2.4. RNA Interference in C. Elegans

2.4.1. Synthesis of dsRNA

The double-stranded RNA interference (RNAi) procedure was essentially carried out as described by Fire *et al* (1998) and Tabara *et al* (1998). A 200 bp fragment from the *C. elegans* NAS 31 gene (accession no. AM159505) was PCR amplified from *C. elegans* t-RNA by One-Step RT-PCR System (Invitrogen) as per manufacturer's instructions. Amplified 3'and 5'end DNA fragments of *C. elegans* NAS 31 were purified utilizing the QIAqiuck Gel extraction Kit(QIAGEN*), and cloned into pGEM*-T cloning vector (Promega*) and verified by sequencing (EurofinS MWG Operon, Germany).

The 3'and 5'end DNA fragments were subsequently excised from pGEM*-T using *Nco* Ⅰ and *Xho* Ⅰ restriction enzymes and ligated in the L4440 RNAi vector (Promega*). The L4440 constructs were linearised with *Nco* Ⅰ and *Xho* Ⅰ restriction enzymes separately and dsRNA were prepared from these templates using the T7 Ribomax Express RNAi System (Promega*) according to manufacturer instructions.

2.4.2. Soaking of *C. Elegans* Worms with dsRNA

RNAi using the soaking protocol was done on ~15-20 L_3 and L_4 worms. The worms were soaked in 30µl of PBS containing 1 mg/ml dsRNA pre-mixed with 3 µl of Lipofectin reagent (Invitrogen). After 24 h incubation at room temperature, the larvae were transferred to NGM plates seeded with the *E. coli* OP_{50} bacteria strain and their development monitored for 5 days. Larvae soaked in either PBS alone or lipofectin reagent without dsRNA served as the controls.

2.4.3. Detection of dsRNA Treated Worms

The loss of Ce-NAS 31.3 and Ce-NAS31.4 transcripts following RNAi was detected by RT-PCR with pairs of Ce-NAS 31.3 and Ce-NAS31.4 specific primers CEF1-Nco-Ⅰ(5'-TATAGTTTATTCACGCAT-3'); CER1-Xho-Ⅰ(5'-TAGTTTCTGGCTGGCATTGC-3'); CEF2-Nco-Ⅰ(5'-AGTTGGACATGCTCTTGA -3'); CER2 - Xho-Ⅰ(5'-ATTTGAAGAAGCACTCGTTGC -3') respectively. Approximately 100 adult hermaphrodites were harvested from NGM/OP_{50} plates and washed twice in PBS. Wild-type adults were used as controls for normal gene expression levels. CPR-4 (acc. nm_072281) was used as a 'housekeeping' gene to control the uniformity of the RNA purifications (Larminie and Johnstone, 1996). The specific primers used for detection of the CPR-4 transcript were F 5´ ATC GTT TCT GCA TCG CCT CC and R 5´ CTG AGC CTC GTA AGA TCCTCC GGT. Purification of the tRNA and the RT-PCRs were performed as described above (Section 2.2.2 and 2.2.3). After 35 cycles of amplification, the RT-PCR products were separated on 1% agarose gels.

3. Results

The alignment of the nucleic acid sequences of Ce-NAS-31 is shown in Fig. 1. The Ce-NAS-31.3 and Ce-NAS-31.4 fragments of the Ce-NAS-31 sequence were cloned in the L4440 RNAi vector to produce dsRNA.

Figure 1: Alignment of the Nucleic Acid Sequence of Ce-NAS-31 Gene Highlighting Ce-NAS-31.3 and Ce-NAS-31.4 Fragment, the Overligned Parts of the Ce-NAS-31 Sequence Were Cloned in the L4440 RNA Interference Vector Separately To Produce Dsrna

3.1.Ce-NAS-31.3 and Ce-NAS-31.4 PCR Products.

The PCR products of Ce-NAS-31.3 and Ce-NAS-31.4 fragments migrated to expected size of ~200bp (Fig. 2.0), following gel electrophoresis on 1% (w/v) agarose (Sigma) gels, stained with 0.5mg/ml red gel.

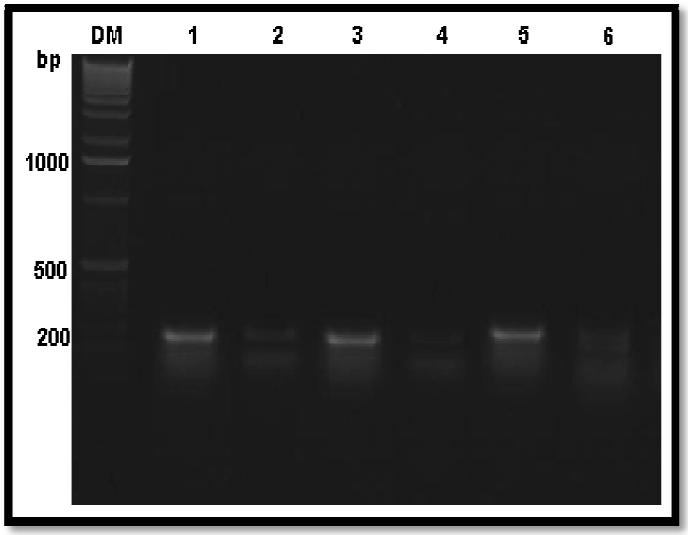

Figure 2: C. elegans NAS 31 PCR products. Lane DM: DNA molecular weight marker (0.075 – 12.21 kbp). The C. elegans Nas 31 DNA amplified in Lane 1:CP4F =CPFR; Lane 2: CP4F =CPFR (water as control); Lane 3: Ce-NAS-31.3 (CEF1 =CER1); Lane 6: CEF1=CER1 (water as control); Lane 7: and Ce-NAS-31.4(CEF2 =CER2); Lane 8: CEF2 =CER2 (water as control). Lanes 1, 3 and 7 are positive C. elegans NAS 31 gene PCR products of ~200bp.

3.2. Recombinant pGEM–T/ C. elegans NAS 31 DNA*

The purified plasmid DNA of *C. elegans* NAS 31 gene 3' and 5'ends that were amplified with primers CEF1Nco-Ⅰand CER1Xho-Ⅰ; CEF2Nco-Ⅰand CER1Xho-Ⅰrespectively, migrated to expected size of ~200bp on 1% agarose gels, Fig. 3.

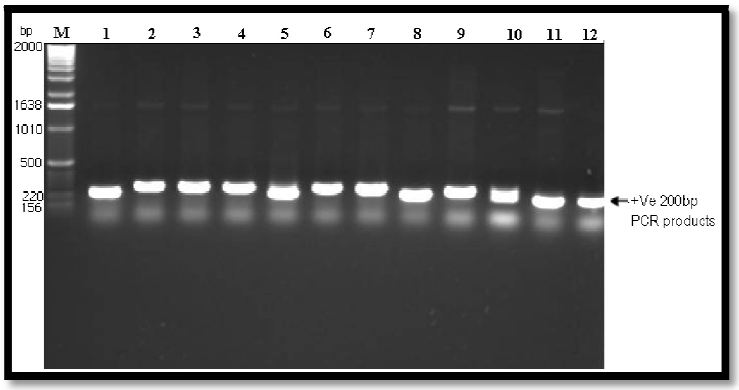

Figure 3: Gel Electrophoresis of PCR Products for C. Elegans NAS 31 DNA Amplified Utilizing Primers with Restriction Enzyme Sites for Cloning Into Rnai Vector L4440. M- DNA Molecular Weight Marker (0.075 – 12.21 Kbp). Lanes 1- 8: C. Elegans Nas 31 DNA 3'end Amplified Using CEF1- Nco-Ⅰand CER1-Xho-Ⅰ Primers. Lanes 9-12: C. Elegans Nas 31 DNA 5'end PCR Products Amplified Using CEF2- Nco-Ⅰand CER1-Xho-Ⅰ Primers. The C. Elegans NAS 31 DNA PCR Products of ~200bp

3.3. Recombinant pGEM-T/C. Elegans NAS 31*

The recombinant pGEM*-T/*C.elegans* NAS 31 DNA fragments digested with Nco-Ⅰand *Xho*Ⅰ restriction enzymes followed by gel electrophoresis migrated to the expected size of ~200bp (Fig. 4).

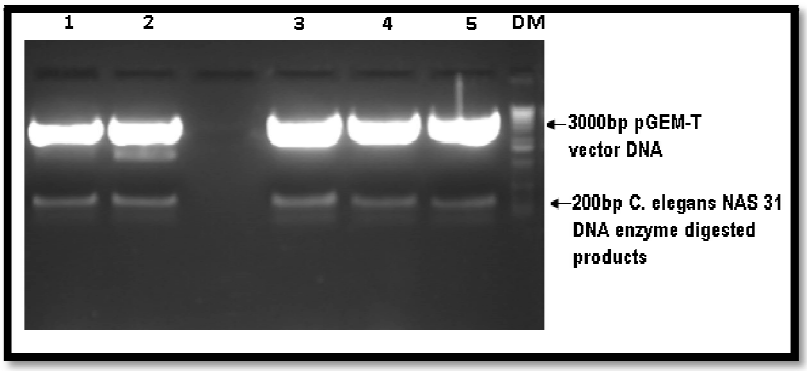

Figure 4: Identification of Recombinant Plasmids Containing Inserts of Recombinant Pgem-T/C. Elegans NAS 31 DNA with Nco-Ⅰand Xho Ⅰ Restriction Enzymes Key: DM: DNA Molecular Weight Marker X (0075 – 12 21kbp); Lanes 1~5: The Positive C. Elegans NAS 31 Enzyme Digested DNA Products of ~200bp.*

3.4. RNA_i vector L4440 DNA

Digested RNA_i vector L4440 DNA of ~2790 (Fig. 5), was cut from agarose gels, purified and ligated to purified *C. elegans* NAS 31 ~200bp for construction of RNA interference recombinant expression vector of *C. elegans* NAS 31.

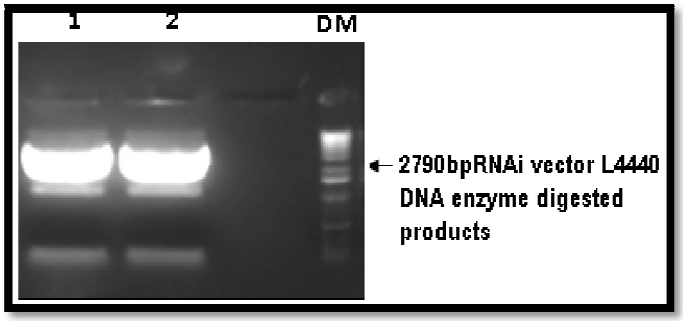

Figure 5: The Plasmid of Rnai Vector L4440 Digested with Nco-Ⅰand Xho Ⅰ Restriction Enzymes Key: DM: DNA Molecular Weight Marker X (0075 – 12 21kbp); Lanes 1 and 2: Digested Rna_i Vector L4440 DNA Of ~2790bp.

3.5. Recombinant C. elegans NAS 31/ L4440 DNA

The recombinant *C. elegans* NAS 31/ L4440 plasmid DNA digested with Nco Ⅰ and Xho Ⅰ restriction enzymes followed by electrophoresis on 1% agarose gels migrated to the expected size of ~200bp (Fig. 6.0).

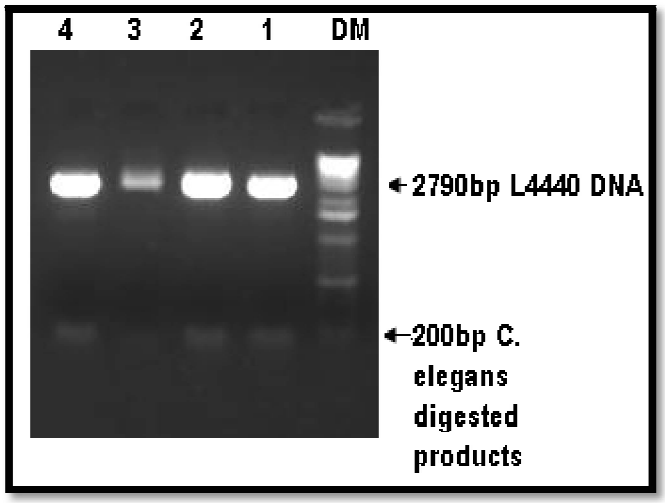

Figure 6: The Recombinant C. Elegans NAS 31/ L4440 DNA Digested with Nco- Ⅰ and Xho Ⅰ Restriction Enzymes for Identification Key: DM: DNA Molecular Weight Marker X (0075 – 12 21kbp); Lanes 1-4: The Positives C. Elegans NAS 31 DNA Fragments

3.6. Rnai in C. Elegans

No visible phenotype was detected after the RNAi experiment. To confirm the absence of the two transcripts following RNAi treatment, RT-PCRs using gene specific primers were carried out on treated as well as wild-type worms. Fig. 7 shows the absence of Ce-NAS 31.3 and Ce-NAS 31.4 cDNA from RNAi-treated worms, while the control gene CPR-4 is present in both control and treated worms.

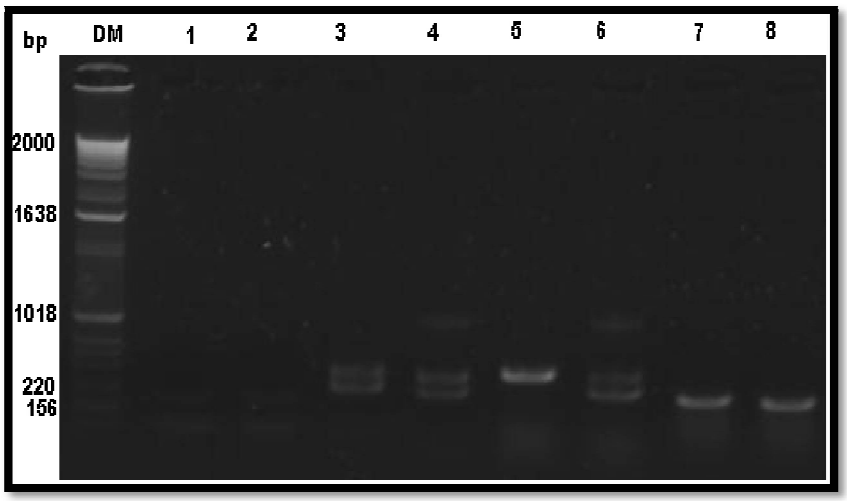

Figure 7: Loss of Ce-NAS31.3 and Ce-NAS 31.4 Transcripts Following Rnai. RT-Pcrs Were Carried Out Using Total RNA Isolated From 15 Wild-Type and 15 Rnai-Treated Worms. CPR-4 Was Used as a Control to Check The Uniformity of the RNA Purifications. DM: DNA Molecular Weight Marker X (0075 – 12 21kbp); Lanes 1 and 2: Rnai-Treated Worms, Lanes 3 ~ 6: Negative Control Worms, Lanes 6 ~ 8: CPR- 4 Control Worms. The Major Band at 200 Bp Is Absent from Dsrna Treated Worms in Lanes 1 and 2 For Both Ce-NAS 31.3 and Ce-NAS 31.4 Transcripts

4. Discussion

In this study, interference in expression of *C. elegans*-NAS-31 gene has been demonstrated *in vitro*. The RNAi experiments were set up to knock down expression of each ce-NAS-31 gene fragment individually. The effects on transcripts levels and specificity of the silencing following RNAi treatment was shown by RT-PCR's using gene-specific primers. The expression levels for both genes' fragments were significantly lower or non-existent compared to control samples. The RT-PCR results further revealed that the new dsRNA samples were gene-specific. However, it is worthy of note that no abnormal phenotype was associated with the F1 and F2 generations, feeding and growth appearing normal in both cases after RNAi.

Lack of phenotype in ce-NAS-31RNAi *C. elegans* worms observed here is in agreement with findings elsewhere (Geldhof *et al* 2006), who reported that worms treated with individual dsRNA or mixed dsRNA samples did not show any visible phenotypes changes as judged by egg production and viability. This is in contrast to findings by (Kamath *et al* 2003), who reported defective phenotypes evidenced by larval death, stunted growth, sterility and in-coordinated

movements in offspring of the parasite. Targeting *C. elegans* genes for which there are conserved counterparts in other eukaryotes (such as yeast, fruit flies and the plant *Arabidopsis thaliana*) is more likely to produce an abnormal phenotype than targeting less conserved genes (Kamath *et al* 2003), The *C. elegans*-NAS-31 gene is a less evolutionary conserved and unlikely to be essential.

Although most genes of this organism have already been knocked out either individually in large scale RNAi screenings (Kamath *et al* 2003), or combinatorially (Knox *et al* 2006), care is needed in the interpretation. Majority of genes analysed belong to multicopy gene families which are highly prevalent in *C. elegans*. Genes within such large families probably show high levels of functional redundancy and may compensate for loss of function. It is therefore important to search and study single gene targets directly in the parasites during RNAi experiments.

RNAi is a powerful tool for gene discovery and functional validation out of the vast amount of information available in the EST datasets. Traditional molecular approaches are a time-consuming and expensive. Gene identification and functional genomics for novel control candidate antigens in this big pool of genes is complex. Consider a search for possible vaccine candidates, one of the important questions in the screening process is whether an antigen is accessible by an immune response. This can only be addressed by performing a localization study. Starting from an EST sequence it takes months to make a recombinant antigen, raise antiserum and localize the native protein on worm sections, perform *in-vitro* functional tests and run animal vaccination trials.

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