The MED-7 transcriptional mediator encoded by *let-49* is required for gonad and germ cell development in *Caenorhabditis elegans*

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Abstract Transcription mediators are evolutionarily conserved from yeast to human. We previously reported the specific in vivo roles of mediators during development. Transcriptional mediators including *med-6*, *med-7*, and *med-10* were shown to be involved in the regulated transcription of specific genes, but not in the transcription of ubiquitous genes. In this report we have identified and characterized the *Caenorhabditis elegans med-7* gene. A genetic mutation in the *med-7* gene was identified by comparing genetic and physical maps and determining the molecular lesion. *let-49* was found to have a nonsense mutation in the coding region of the *med-7* gene. The identification of *let-49* as the *med-7* gene was confirmed by rescue experiments. The phenotype of the *let-49* mutation indicated that the *med-7* gene is required for normal postembryonic development. RNAi experiments showed that *med-7* is also involved in embryogenesis and the gonad and germ cell development. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Transcriptional mediator; *med-7*; *let-49*; Gonad development; *Caenorhabditis elegans*

1. Introduction

Transcriptional mediators form complexes associated with the basal transcription machinery, which are required to integrate diverse gene-specific regulatory signals and to recruit basal transcription machinery to specific promoters [1]. The mediator complexes were initially identified in yeast as distinct intermediary molecules that mediate signal transfer between gene-specific transcriptional activator proteins and the basal transcription machinery [2–4]. The yeast mediator complex is composed of the Med proteins (Med1, Med2, Med4, Med6, Med8, Med9, Med10, and Med11), Gal11, Rgr1, Sin4, Hrs1, Rox3, and the Srb family of proteins (Srb2, Srb4, Srb5, Srb6, and Srb7). These mediator components assemble into several functional modules that regulate distinct groups of genes [5,6].

To date, many mediator complexes have been identified in various multicellular species. These complexes include the human Srb/Med-containing cofactor complex (SMCC) [7], the negative regulator of activated transcription (NAT) complex [8], mouse and human mediator complexes [9,10], and the mediator complex in the nematode *Caenorhabditis elegans* [11].

Some experimental evidence of a physiological role for these mediator complexes in development has come from studies in *C. elegans*. The *med-6*, *med-7*, and *med-10* mediator genes were initially identified from a genome search for homologs of the yeast mediators [12], and we have previously reported that these mediators are required for regulated transcription of tissue- and stage-specific developmental genes, and loss of function in any of these genes causes embryonic lethality, confirming their essential roles in development [11]. We have recently reported that MED-6 may be the point of convergence at which diverse transcriptional signaling mediated by metazoan-specific transcription factors and mediator-related proteins converge, at least in metazoa. The *C. elegans med-6* gene was shown to play an important role in development by regulating the transcription of genes involved in conserved pathways such as the Ras and Wnt signaling pathways [13].

Most studies on the functions of the nematode mediators were performed using RNAi techniques. An advantage of the RNAi experiment is that it can reduce both maternal and zygotic gene functions. On the other hand, a limitation is that RNAi mimics the null phenotypes only in the F1 generation. Therefore, it is crucial to establish a genetic system in which mediators can be studied for their biological and biochemical functions. We previously reported that MED-6 is encoded by *let-425*. By characterizing and comparing the *let-425* mutant phenotypes and the *med-6* RNAi phenotypes, we were able to conclude that *med-6* has separable maternal and zygotic functions, and that *med-6* is required for embryogenesis, larval development, vulval development, and ray development. Mutations in other mediators have been identified in *C. elegans* by genetic screens. *sur-2* was first identified as a suppressor of a *ras* gain-of-function mutation [14], and *sop-1* was identified as a suppressor of a *pal-1* mutation [15]. While the SUR-2 and SOP-1 are conserved only in the metazoa, the mediators including MED-6 and MED-7 are conserved from yeast to humans, suggesting that the latter mediators may act as a convergence point of gene regulation [13]. In this study we have isolated and characterized the genetic mutation in the *med-7* gene. We have also examined the functions of *med-6* and *med-7* in the germ cell development of *C. elegans*. This report is the first to directly show that the MED-6 and MED-7 mediators are required for normal gonad growth and germ cell development.

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2. Materials and methods

2.1. Strains and culture

The Bristol N2 strain was used as wild-type. The alleles and strains used for cloning *med-7* were: MQ468 [*mad-1*(*qm39*) *I*]; RW3199 [*lev-11*(*x12*)*let-49*(*st44*)/*lev-11*(*x12*)*unc-54*(*e1152*) *I*]. The strains were obtained from the Caenorhabditis Genetics Center (CGC). The culture of *C. elegans* has been previously described [16].

2.2. Identification of let-49 as the med-7 gene

The physical location of *med-7* was determined to be on the YAC Y54E5 on chromosome I. Because *kin-22* and *unc-54* had been cloned to the left and right ends of the YAC Y54E5 respectively, we expected that the *med-7* gene would be located between *kin-22* and *unc-54*. Since the region is saturated with lethal mutations, we searched for lethal mutations mapped between the two genes. According to the genetic map available on the Internet (http://elegans.swmed.edu), *let-205*, *let-206*, *let-207*, *let-442*, *mad-1*, *let-49*, *let-50*, and *let-208* reside in this region. We decided that *mad-1* and *let-49* could be candidates for the *med-7* mutation based on the phenotypes described for animals containing each mutation. A *mad-1* mutation had been isolated as a larval lethal mutation with some embryonic lethality [17], and a *let-49* mutation isolated with mid-larval lethality [18]. We amplified the genomic region containing the entire coding sequence of the *med-7* gene from either homozygotes of *mad-1* or *let-49*. The primers used for amplifying the genomic region from single larvae were CM7F (5′-GGTCTAGACCCTTCCTTTTCAGCTCTG-3′) and CM7R (5′-GGCTCGAGCCCGGTAAAATAAATTAAAATG-3′). The amplified fragments were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA). We determined the sequences from two independent colonies with T7 Sequenase 2.0 DNA sequencing kits (Amersham, Amersham, UK). To confirm the identity of the mutation, we directly determined the sequence of the PCR-amplified fragments from eight *let-49* homozygous animals, and also digested the fragments with the restriction enzyme *Taq*I. To definitely show that *let-49* is the *med-7* gene, a rescue experiment was performed. The genomic DNA containing the full-length *med-7* gene was amplified by PCR using the primers CM7PF (5′-CGTCTGGTCGAGACACATGGCG-3′) and CM7PR (5′-CATTCCTGATGAGTGGAGGCG-3′). The amplified PCR product of 5.1 kb was co-injected with genomic DNA digested with *Eco*RI and the pRF4 (*rol-6*) marker DNA into the heterozygous animals of the genotype *lev-11*(*x12*)*let-49*(*st44*)/*lev-11*(*x12*)*unc-54*(*e1152*). The genomic DNA was co-injected to facilitate the transgene expression in the germline. Since the *unc-54*(*e1152*) mutation confers a dominant uncoordinated (Unc) phenotype, the heterozygous animal gives rise to fertile progeny with the Unc phenotype if there had not been any rescue of the *med-7* mutation because no homozygous *med-7* mutant animals reach adulthood. In the F2 generation after injection of the *med-7* genomic DNA, we were able to obtain the *med-7* homozygous animals which in turn gave rise to fertile progeny in the next generation. We obtained three independent stable lines.

2.3. Characterization of let-49 homozygous animals

The *let-49* mutation is maintained in the heterozygote form of the genotype *lev-11*(*x12*)*let-49*(*st44*)/*lev-11*(*x12*)*unc-54*(*e1152*) *I*. Heterozygotes are slow Uncs (uncoordinated movement) and segregate slow Uncs, mid-larval lethals and severe Uncs, because *unc-54*(*e1152*) is a dominant Unc. *lev-11* was isolated as having a levamisole resistant phenotype, which has no effects on the assays we performed in this study. In order to measure the lethality and sterility of the *let-49* homozygotes, we removed the *unc-54* balancer by mating with N2 males to produce heterozygote hermaphrodites of the genotype *lev-11*(*x12*)*let-49*(*st44*)/++, and *lev-11*(*x12*)*unc-54*(*e1152*)/++. Worms of the genotype *lev-11*(*x12*)*let-49*(*st44*)/++ were non-Uncs and viable in contrast to *lev-11*(*x12*)*unc-54*(*e1152*)/++ heterozygotes and self-progeny. The progeny of these viable heterozygotes were examined for phenotypes. The results indicate that the *let-49* homozygote mutation shows a high degree of mid-larval lethality.

2.4. RNAi, gonad staining, and microscopy

Standard microinjection procedure was carried out [19]. The *med-7* and *med-6* dsRNA used in this study was identical to the dsRNA used in the previous study [11]. For the RNAi, we injected 100 μg/ml of dsRNAs corresponding to *med-6* and *med-7* into wild-type N2. In order to observe the effect of *med-6* and *med-7* RNAi on germ cell development, we stained the gonads of F1 adults with Hoechst 33342. For gonad staining, the sterile adult F1 progeny laid at 6–24 h after microinjection were killed to extrude the gonads, which were immediately fixed in 3% formaldehyde with methanol. Gonads were washed in PTw (PBS+0.1% Tween 20) three times and then treated with Hoechst 33342. The numbers of the oocytes in the gonad were counted on a single focal plane of the microscopes for simple and accurate comparison of wild-type, *med-6* RNAi, and *med-7* RNAi animals. A Zeiss Axioplan2 microscope was used for observation of the stained gonads, and a Zeiss AxioCam digital camera (Carl Zeiss) was used for taking photographs.

3. Results and discussion

3.1. MED-7 is encoded by let-49

We have previously shown that mediators are essential for *C. elegans* development including embryogenesis and fertility [11]. We have also identified *let-425* as the *med-6* gene and have characterized its biological function [13]. Next, we attempted to search for mutant candidates of other mediators. In order to identify genetic mutations in the *med-7* gene, we compared the physical map to the genetic map (Fig. 1A). The *med-7* gene was physically mapped to the YAC Y54E5 by

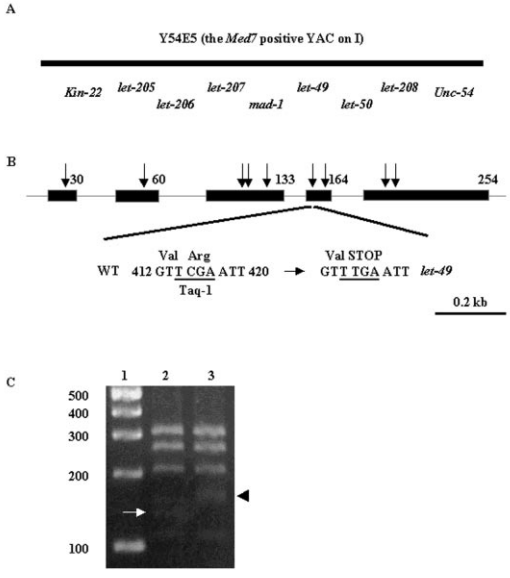

Fig. 1. Identification of the *med-7* mutation. A: The physical and genetic maps of the *med-7* region. The yeast artificial chromosome (YAC) Y54E5 contained the *med-7* gene. *kin-22* and *unc-54* were located on the most left and right parts on the YAC, respectively. Lethal mutations *let-205*, *let-206*, *let-207*, *let-442*, *mad-1*, *let-49*, *let-50* and *let-208*, which were mapped in that region, are shown. The map is not drawn to an exact scale. B: Genomic structure of *med-7* and the mutation identified in *let-49*. The *med-7* gene contains five exons separated by four introns. The *med-7* gene isolated from the homozygous *let-49* animals contained a point mutation that changes C to T, causing a change in the amino acid residue from Arg to a stop codon. The numbers above the genomic structure are the amino acid numbers encoded by the gene, and the arrows indicate *Taq*I digestion sites. C: Restriction fragment patterns digested with *Taq*I. Lane 1, size markers. Lane 2, the PCR product of wild-type *med-7* gene was treated with *Taq*I, and electrophoresed in a 2% agarose gel. The arrow indicates cleaved 130 bp DNA. Lane 3, those of *let-49* homozygote animals. The arrowhead indicates the uncleaved 153 bp DNA for mutation. The sizes of markers are indicated on the left.

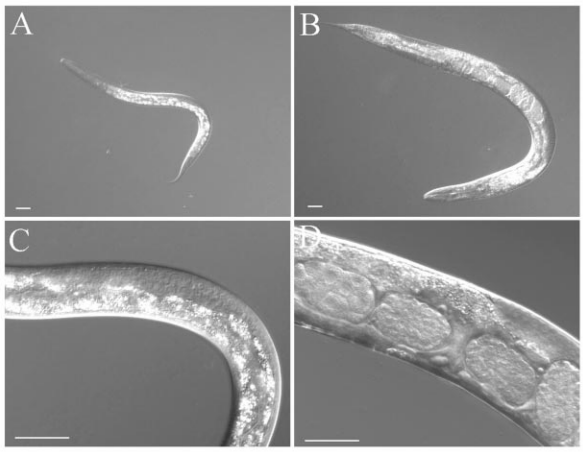

Fig. 2. The *let-49* mutation is rescued by the *med-7* gene. A, C: A typical *let-49* homozygous animal. The animal is arrested at a mid-larval stage, and eventually dies at this arrested stage without further development. B, D: A *let-49* homozygous animal rescued with the *med-7* gene. This animal has grown up to adulthood and bears several embryos in the gonad, which has never been observed in the *let-49* mutant animals. B and D are close-up views of A and C, respectively. The scale bars are 25 μm.

sequencing data from the Genome Project [20]. The *med-7* gene was located between *kin-22* and *unc-54*, and we reasoned that *med-7* should be encoded by a lethal mutation that mapped between these genes. Among the lethal mutations in this region that have not yet been cloned, we found that there was a single base substitution in the *med-7* gene from the *let-49* mutant animals (Fig. 1B, and Section 2). The mutation was a C to T transition at the location of nucleotide number 415, which we expect causes a truncated LET-49 protein with amino acids missing from the 139th residue. We confirmed the mutation by single worm PCR and sequencing of several homozygous animals containing two copies of the *let-49* mutation. The PCR product of the wild-type *med-7* gene is divided into 10 fragments – 146, 200, 246, 9, 51, 130, 23, 108, 11, and 303 bp – by digestion with the *Taq*I restriction enzyme. However, that of *let-49* animals was separated into nine fragments since the mutated region was not cleaved by *Taq*I (Fig. 1C). Rescue experiments also confirmed that *let-49* is the *med-7* gene (see Section 2; Fig. 2). The phenotypes of the *let-49* mutation showed similar phenotypes to those caused by *med-7* RNAi, also supporting our results (see below).

3.2. Phenotypes associated with the let-49 homozygote mutation

Originally *let-49* had been isolated as a mutation with larval lethality. It had been described that the *let-49* mutation is mid-larval lethal [18]. We examined whether *let-49* homozygote animals had other phenotypes such as embryonic lethality or adult sterility. As heterozygous animals containing a single copy of the *let-49* mutation displayed no visible defects comparable to those of the *let-49* homozygotes, we could know that the *let-49* mutation is fully recessive. In order to check if there was any embryonic lethality or other phenotype associated with the mutation, we examined the progeny from heterozygous mothers with the genotype *lev-11 let-49*/++. Among the 289 progeny laid from the heterozygous mothers, we found that 24% were mid-larval lethal animals. This percentage is comparable to the expected number of homozygous *let-49* progeny if there had been no other lethal phenotype associated with the *let-49* mutation. From the *let-49* phenotype, it can be concluded that *med-7* is required continuously during postembryonic stage. The mid-larval arrest or lethal phenotype caused by *let-49* is less severe than the phenotypes caused by high dose of *med-7* RNAi, which is embryonic lethal [11]. A possible explanation for this phenotypic discrepancy is that the homozygote animals containing the *let-49* mutation were from the heterozygous mothers containing a single copy of the wild-type *med-7* activity, which could complement the maternal functions of *med-7*. Consistent with this explanation, it has been reported that the mutation in *med-6* caused less severe phenotypes than those caused by *med-6* RNAi, and that it was due to the maternal rescue of the embryonic lethality by the maternal function of *med-6* [13].

3.3. med-7 is involved in the gonad and germ cell development

We have reported that mediators are essential for germ cell development since RNAi of any one of the mediators causes adult sterility [11]. We have also shown by RNAi experiments that *med-6* is required for both oogenesis and spermatogenesis. When RNAi-affected males and hermaphrodites were mated with wild-type partners, they could not produce any cross progeny [13]. In order to examine gonad development in the absence of mediators, we observed the gonads and the germ cells affected by either *med-6* or *med-7* RNAi. RNAi was used because the *let-425* and *let-49* mutant animals hardly reached adulthood and because sufficient numbers of adult animals could be obtained using RNAi. Most embryos born later than 24 h after microinjection of *med-6* or *med-7* dsRNA were embryonic lethal, but the embryos laid at 6–24 h arrested at the larval stage or became sterile at the adult stage. The gonads of almost all sterile hermaphrodite adults were significantly shorter than those of wild-type animals while the overall size of the animals did not show much difference from

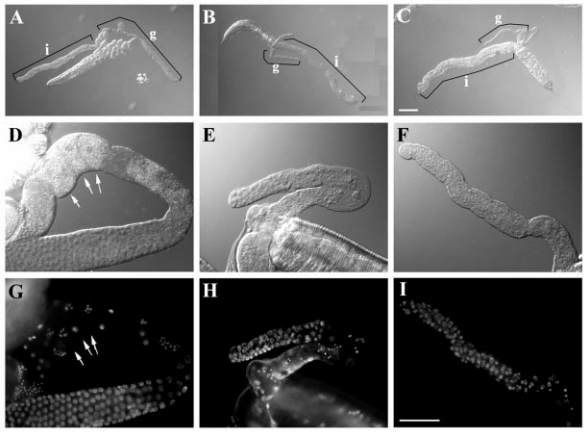

Fig. 3. Mediators are involved in germ cell development and gonad growth. A–C: The gonad arms of a wild-type animal (A), a *med-6* RNAi-affected animal (B), and a *med-7* RNAi-affected animal (C). 'i' indicates the intestine, and 'g' indicates a gonad arm. The size of the gonads in RNAi-affected animals is significantly smaller. D–I: Germ cell defects in *med-6* and *med-7* RNAi-affected animals. D–F: Nomarski image of wild-type (D), *med-6* RNAi- (E), and *med-7* RNAi- (F) affected animals. G–I: The Hoechst 33342 images of the same animals as D, E, and F, respectively. Mature oocytes are indicated by arrows. There is no mature oocyte in RNAi-affected animals. The scale bars are 100 μm (A–C) and 50 μm (D–I) respectively.

wild-type ($n = 50$, Fig. 3A–C). When the germ cells were observed under a fluorescence microscope after chromosomal staining with Hoechst 33342, the number of nuclei in the gonads of RNAi-affected animals was significantly smaller than that of wild-type adults. Specifically, while an average number of oocytes in one microscopic focal plane of the wild-type gonad was 480 ($n = 10$), the average numbers of oocytes in the *med-6* and *med-7* RNAi animals were 80 and 85, respectively ($n = 10$ each). Furthermore, we did not observe any mature oocyte in sterile adult animals affected by either *med-6* or *med-7* RNAi. Through these results we confirmed that mediators, at least MED-6 and MED-7, play an important role in the gonad and germ cell development. One possible reason for the small gonads in *med-6* or *med-7* RNAi-affected animals is impaired oocyte development. We have not detected any visible defects in the somatic gonad, thus it is possible that the gonad could not grow to a normal size due to reduced number of oocytes, which would normally fill the gonads. Since the oocyte development is a highly regulated process in terms of space and time, it is conceivable that the genes involved in this process are regulated through mediator complexes.

In conclusion, we have shown in this report that the *let-49* gene encodes the MED-7 transcriptional mediator, and that MED-7 is essential for many aspects of development including embryogenesis, postembryonic development, and gonad growth and development.

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