ANALYSIS OF THE *C. ELEGANS rpc-1* GENE

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

ANALYSIS OF THE *C. ELEGANS rpc-1* GENE

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TABLE OF CONTENTS

INTRODUCTION

RNA polymerase III and its activity

In eukaryotic organisms, three nuclear RNA polymerases carry out DNA-directed RNA transcription: RNA polymerase I (Pol I) transcribes the 5.8S, 18S, and 28S ribosomal RNAs (rRNAs); RNA polymerase II (Pol II) transcribes messenger RNA (mRNA) and most small nuclear RNA (snRNA); and RNA polymerase III (Pol III) transcribes transfer RNA (tRNA), 5S rRNA, and other small cellular and viral RNAs. While most Pol II mRNA products are translated into proteins, Pol I and Pol III transcribe functional RNA products. Mature Pol III transcripts are mostly shorter than 300 nucleotides (White, 2004), which play essential roles in cellular metabolism. tRNA and 5S rRNA are required for protein synthesis, 7SL RNA is involved in intracellular protein transport, and the U6, H1 and MRP RNAs are involved in RNA post-transcriptional processing (White, 1998a). Interestingly, Pol III can also transcribe some genes that are normally transcribed by Pol II. For example, in *Xenopus* oocytes, *c-myc* is transcribed by Pol II at low template concentrations and by Pol III at high template concentrations (Chung et al., 1987; Bentley et al., 1989).

RNA polymerases are enzymes, which are highly conserved among eukaryotic organisms from yeast to human (Sepehri and Hernandez, 1997). They are comprised of multiple subunits, some of which are shared among RNA polymerases (Table 1.) Pol III is the largest RNA polymerase, consisting of 17 subunits. In *S. cerevisiae*, 10 out of 17 subunits are found exclusively in Pol III, designated the C subunits; 2 subunits are shared with Pol I, designated AC subunits; and 5 subunits are shared with Pol I and Pol II, designated ABC subunits (Table 1). RPC-1 (C160) and

Table 1. Subunits of *S. cerevisiae* RNA polymerase III

RNA polymerase III subunits *	MW (kD)	Guide for nomenclature	Accession number
C160 (β'–like)	162.1	ScRPC1	P04051
C128 (β–like)	129.3	ScRPC2	AAB59324
C82	73.6	ScRPC3	CAA45072
C53	46.6	ScRPC4	P25441
C37	32.1	ScRPC5	NP_012950
C34	36.1	ScRPC6	P32910
C31	27.7	ScRPC7	P17890
C25	24.3	ScRPC8	P35718
C17	18.6	ScRPC9	P47076
C11	12.5	ScRPC10	AAD12060
AC40 (α–like)	37.6	ScRPAC1	P07703
AC19 (α–like)	16.1	ScRPAC2	P28000
ABC27	25.1	ScRPABC1	P20434
ABC23 (ω–like)	17.9	ScRPABC2	AAA34989
ABC14.5	16.5	ScRPABC3	CAA37383
ABC10α	7.7	ScRPABC4	AAA64417
ABC10β	8.2	ScRPABC5	P22139

*C subunits are Pol III exclusive subunits; AC subunits are shared by Pol I and Pol III; ABC subunits are shared by Pol I, Pol II and Pol III. Subunits evolutionarily related to the E. coli β′, β, α and ω subunits are indicated.

Modified from Schramm and Hernandez, 2002.

RPC-2 (C128), evolutionarily related to the β' and β subunits of prokaryotic RNA polymerase in *E. coli*, respectively, are the two largest subunits in Pol III. These two subunits form the core structure of the enzyme in collaboration with AC40, AC19 and ABC23. AC40 and AC19 are related to the α subunit of *E. coli* RNA polymerase, whereas ABC23 is related to the ω subunit of *E. coli* RNA polymerase (Reviewed by Schramm and Hernandez 2002). The high conservation of Pol III largest subunit between human and yeast has been reported (Sepehri and Hernandez, 1997). Mutations in RPC160 specifically suppress the synthesis of tRNA, H1RNA, and U6 snRNA (Gudenus et al., 1988; Lee et al., 1991; and Moenne et al., 1990).

Five conserved domains are found in the largest subunit of Pol III by BLAST (http://www.ncbi.nlm.nih.gov): 1) the discontinuous cleft domain (Rpb1 domain 5), required to form the central cleft or channel where the DNA template is bound; 2) the clamp domain (Rpb1 domain 1), which is a mobile domain involved in positioning the DNA strands, maintaining the transcription bubble and positioning the nascent RNA strand; 3) the pore domain (Rpb1 domain 3), to which the 3' end of RNA is positioned close, thought to form a channel through which nucleotides enter the active site and/or where the 3' end of the RNA may be extruded during back-tracking; 4) the funnel domain (Rpb1 domain 4), containing the binding site for some elongation factors; and 5) the RNA polymerase I subunit A N-terminus. These five domains are universally conserved in the largest subunits of Pols.

To initiate transcription, Pol III enzyme must first be recruited to Pol III promoters (promoters of Pol III target genes). This recruitment is executed by Pol III-specific transcription factors, TFIII C, TFIII B, TFIII A and/or SNAPc. Based on the

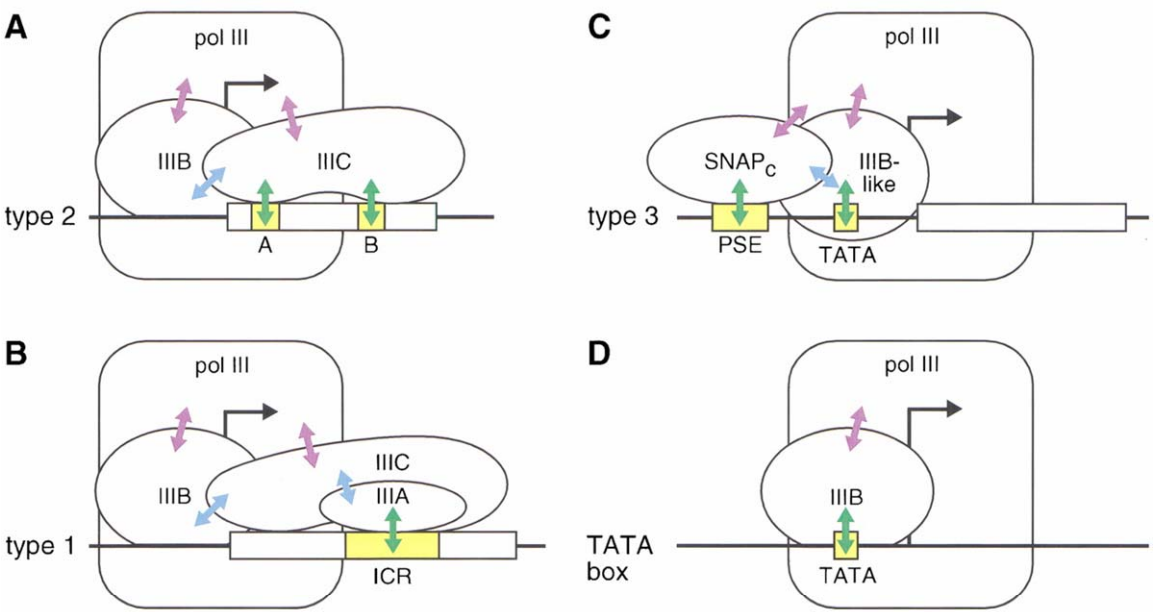
structure of Pol III promoters, they are divided into 3 types (Figure 1). Type I promoters drive transcription of the 5S rRNA genes; type II promoters drive tRNA genes; and type III promoters drive U6 snRNA genes, the 7SK gene, the H1 RNA gene and genes encoding RNAs with unknown function (reviewed by Schramm and Hernandez, 2002).

Different types of promoters recruit Pol III in different ways (Figure 1). Type II promoters are recognized by TFIII C (Lassar et al. 1983). TFIII B is then recruited to the promoter by DNA-TFIII C complex through Brf 1, a subunit of the TFIII B (Kassavetis et al. 1992), and the TATA box (Joazeiro et al., 1996). TFIII B binding to the promoter in turn allows the binding of Pol III enzyme mainly by protein-protein interaction. Type I promoter is first recognized by TFIII A (Engelke et al. 1980; Sakonju et al. 1981). TFIII A binding directs the placement of TFIII C onto the 5S rRNA promoter (Lassar et al., 1983). Then, similarly to type II promoters, recruitment of TFIII B and Pol III follows thereafter. In type III promoters, PSE–bound SNAPc and TATA–bound TBP (TFIIIB-like) function coordinately to recruit Pol III (Sepehri Chong et al., 2001). *in vitro* experiments showed that just a TATA box could direct some Pol III transcription with just recombinant TFIII B and highly purified Pol III (Kassavetis et al., 1995; Rüth et al., 1996). Thus, in general, TFIII B is the recruiter of Pol III to its target promoters, while TFIII A and TFIII C function to recruit TFIII B.

In *S. cerevisiae*, Pol III activity was found to correlate with growth conditions. When *S. cerevisae* undergoes a diauxic shift in response to nutritional exhaust, tRNA and 5S rRNA gene transcription is down-regulated (Clarke et al., 1996; Sethy et al., 1995). A similar phenomenon was found in mouse fibroblasts: growth-arrested cells by serum

Figure 1. Different pathways for recruitment of TFIIIB and RNA polymerase III. The transcription initiation complexes formed on type 2, 1, and 3 promoters, as well as on an artificial promoter consisting of just a TATA box, are shown. The green arrows show interactions of DNA-binding proteins with promoter elements, the blue arrows show protein-protein contacts among various transcription factors, and the purple arrows show protein-protein contacts between RNA polymerase III and transcription factors. In type II promoters, the A and B elements are first recognized by TFIIIC. The DNA-TFIIIC complex allows the binding of TFIII B and Pol III. In type I promoters, the first step is interaction between TFIIIA and the promoter element ICR. In type III promoters, it is the interaction between PSE-bound SNAPc and TATA-bound TFIII B that recruits Pol III. Panel D shows an *in vitro* recruitment of Pol III to a TATA box without TFIII C or SNAPc participation, which has not been found *in vivo* yet.

Adapted from Schramm and Hernandez, 2002.


A
pol III
IIIB
IIIC
type 2
A
B
C
pol III
SNAPc
IIIB-
like
type 3
PSE
TATA
B
pol III
IIIC
IIIB
IIIA
type 1
ICR
D
pol III
IIIB
TATA
box
TATA

starvation showed dramatic increase in Pol III transcripts, like tRNA and B2 RNA, when they were stimulated by serum (Mauck and Green, 1974; Edwards et al., 1985). It was also shown that Hela cells gained higher efficiency of Pol III transcription when they were grown in rich (5-10%) serum than in low (0.5%) serum (Sinn et al., 1995). These studies suggest that Pol III activity is associated with cell growth. Pol III activity is increased when cells grow rapidly, and Pol III activity is decreased when cells arrest growth. Moreover, this growth-related regulation was shown to be mediated by TFIII B and TFIII C (Sethy et al., 1995).

Some cell-transformation factors were found to be involved in regulating Pol III activity (Reviewed by Brown et al., 2000). Tumor suppressors, RB, p130 and p53 repress Pol III activity by binding TFIII B. The TFIII B binding of tumor suppressors, at least partially, contributes to their negative control of cell growth. Other studies suggested that some factors activate Pol III by targeting TFIII B, like c-Myc and several viruses, including SV40, HBV and HTLV-1. Since elevated Pol III activity is a feature of many cancers (reviewed by White, 2004), TFIII B may be a target of some oncogenic factors, which mediate the cell transformation signal to Pol III transcription. Besides the TFIII B activity regulations by these cell-transformation factors, over-expression of TFIII B and TFIII C has also been found in cancer cells (reviewed by White, 2004.). So far, no regulation of the *rpc-1* gene or protein has been observed.

Maternal effects during early embryo development

In many organisms, embryos inherit maternal transcripts and proteins from the mature oocytes for early development (Davidson, 1986). Maternal products are loaded

during oocyte maturation. After fertilization, the embryos undergo serial asymmetric cell cleavages. Maternal factor localization (e. g., transcription factors, transmembrane receptors) allows their lineage-specific activities (Bashirullah et al., 2001). Two classes of maternal transcripts have been described based on their spatial and temporal patterns during early embryogenesis in *C. elegans* and other organisms (Seydoux and Fire, 1994; Davidson, 1986): class I mRNA, encoding genes with ubiquitous "house-keeping" activities, are maintained in all blastomeres; class II transcripts, specifically associated with the early embryogenesis, are degraded in somatic blastomeres as early as the two-cell stage. Essentially, these maternal products function to control development until the developmental switch from maternal control to zygotic control, occurring at the mid–blastula transition. This transition is acccompanied by the destabilization of a subset of maternal transcripts. In *Drosophila*, this destabilization is triggered by egg-activation (Tadros et al., 2003). So far, the developmental switch from the maternal control to the zygotic control is poorly understood.

Involvement of the TGF-β signaling in the neurobiology

TGF-β signaling has been proven to be a relatively simple system (Figure 2), linking transmembrane TGF- β receptors to gene expression with Smad proteins as mediators (Massague and Wotton, 2000). TGF- β signaling has essential roles in diverse biological processes, such as embryogenesis, immune function, epithelial growth, bone morphogenesis, and the endocrine-reproduction axes (Massagué and Chen, 2000).

Increasing evidence shows that TGF- β signaling is involved in the neuronal development. Krieglstein et al. (2000) found that blocking TGF- β signaling prevents

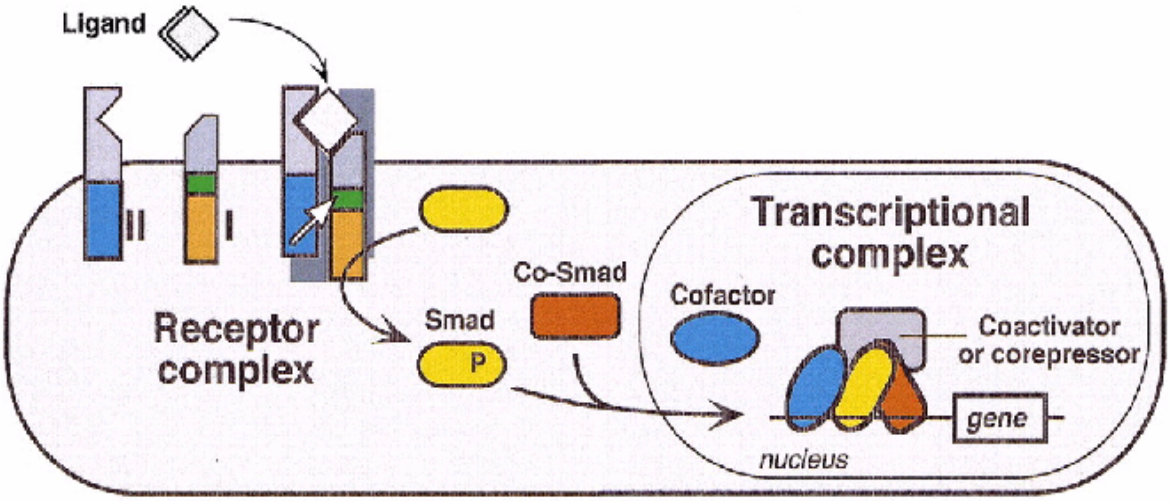
developmental cell death in the nervous system of the chicken embryo. TGF- β ligands inhibit neuronal proliferation and growth, and stimulate neuronal process outgrowth (Miller and Luo, 2002). The effect on neuronal process outgrowth was further confirmed in *Drosophila* by Sweeney and Ramaseami (2002). These studies indicate that TGF- β signaling may affect the nervous system by controlling neuronal development.

TGF- β may also directly regulate neuronal activity. Genetic evidence from *C. elegans* shows that TGF – β receptors on interneurons are targets of TGF – β released from chemosensory neurons. When active, these receptors inhibit dauer larval development or promote exit from the dauer stage (Ren et al., 1996; Schackwitz et al., 1996). In *Aplysia*, Wainwright et al. (2002) discovered that TGF-β induces synaptic facilitation without altering sensory neuron morphology. Brief treatment of TGF- β2 inhibits calcium influx into neurons. This inhibition is related to the control of the excitability of basal forebrain cholinergic neurons (Williams et al., 2002). Interestingly, some studies suggest that TGF-β signaling plays a protective role in response to neuronal injuries (Sakamoto et al., 2003; Docagne et al., 2002; Lesne et al., 2002). This protection is, at least partially, mediated by glial cell line-derived neurotrophic factor (GDNF) (Peterziel et al., 2002; Wang et al., 2002).

C. elegans as an animal model

C. elegans is a free-living soil nematode, isolated for laboratory research as a model organism. Under favorable conditions, *C. elegans* proceeds through four larval stages (L1~L4) to reach the reproductive adulthood; under unfavorable conditions, like

Figure 2. TGF–β signaling via Smads: Converging in and branching out of a simple signaling engine. "The basic signaling engine: The ligand assembles a receptor complex that phosphorylates Smads, and the Smads assemble a transcriptional complex that regulates target genes. The type II receptors are activators of the type I receptor. Smads are direct substrates of type I receptors. The assembly of receptor-phosphorylated Smads with co-Smads is essential for many transcriptional responses. Smads gain access to target genes by synergistically binding to DNA with cell-specific cofactors, many of which remain unknown. The Smad complex can recruit coactivators or corepressors" (Figure and Legend taken from Massague and Chen, 2000).


Ligand
II
I
Receptor complex
Smad
P
Co-Smad
Transcriptional complex
Cofactor
Coactivator or corepressor
gene
nucleus

food scarcity, high population density, they arrest development at the dauer stage (Figure 3). The dauer / non-dauer decision is mediated by amphid neurons, based on the environmental cues. The mean life span of *C. elegans* is about two weeks. This nematode was initially chosen to demonstrate how neurons function together to control behaviors, since it is anatomically simple, consisting of fewer than 1000 cells, 302 of which are neurons in the hermaphrodite (Riddle et al., 1997). With its ease of laboratory manipulation, convenient genetics of self-fertilizing and crossing, a wealth of molecular methods has been developed in *C. elegans* studies. Documentation of the cell lineage (Sulston, 1988) allows researchers to understand development at the cellular level, and associate the mutant phenotype reliably with individual cells. *C. elegans* has been intensively applied as an advantageous animal model in studies of different biological phenomena for nearly 40 years in areas including neurobiology, behavior, life span, embryogenesis, apoptosis, metabolism, molecular and cellular signaling.

Sequencing of the *C. elegans* genome was completed in 1998 (The *C. elegans* Sequencing Consortium, 1998). This landmark in *C. elegans* research provided a fundamental basis for the use of *C. elegans* as an experimental organism in the post-genomic era. Gene prediction with the known genome sequence makes it practical to perform genome-wide analysis of gene functions, while dramatically facilitating gene cloning. Much has been done in the genome-wide analyses, including RNAi analysis (Kamath and Ahringer, 2003; Simmer et al., 2003; Keating et al., 2003), and gene expression profiling (Hill et al., 2000; Jones et al., 2001; Holt and Riddle, 2003; Pleasance et al., 2003). These data broaden our insights into different biological processes and the corresponding genetic involvements. Since many aspects of cellular

function are conserved in evolution, knowledge of these processes in *C. elegans* are contributing to mechanistic studies on mammalian counterparts.

The *C. elegans rpc-1* gene was found in identifying the *C. elegans* homolog of RpII215, which encodes the largest subunit of Pol II in *Drosophila melanogaster*. (Bird and Riddle, 1989). The *C. elegans* genome sequence predicts one copy of *rpc-1* (The *C. elegans* Sequencing Consortium, 1998). *In situ* hybridization data showed that *rpc-1* mRNA is detectable during the embryo stage through adulthood, enriched in the gonad of the hermaphrodite (http://nematode.lab.nig.ac.jp/) (Figure 4). Other than these studies, this gene is poorly characterized.

The *rpc-1* mutant allele, *m654*, was identified in a collection of a dauer-like / lethal mutants, induced by EMS treatment (Caldicott, 1995). In a complementation test with several lethal mutations, which were believed to be *rpc-1* candidate mutations (Riddle, D., unpublished data), another *rpc-1* allele, *s1139*, was identified. In this study, I showed that *rpc-1 (s1139)* is a null mutation whereas *rpc-1 (m654)* is a severe, if not null, mutation. Maternally inherited *rpc-1* mRNA and/or RPC-1 allow animals to develop until the L3 stage. Starvation suppresses *rpc-1* promoter activity in most tissues. Re-feeding enhances *rpc-1* promoter acitivity and expression. Insulin signaling may not be involved in this regulation. This is the first time that *rpc-1* regulation has been observed. Interestingly, while starvation silences the *rpc-1* promoter activity in most tissues, this treatment does not silence the promoter activity in ASK neurons. The *rpc-1* expression in ASK neurons under starvation may be mediated by TGF-β signaling.

Figure 3. The life cycle of *C. elegans.* Under favorable conditions, *C. elegans* develops continuously larval stages (L1 ~ L4), followed by the productive adult stage. The larval stages are punctuated by molting. Under unfavorable conditions, like the high population density and food scarcity, *C. elegans* arrests development at the dauer stage. When environmental conditions are improved, dauer larvae will resume development by molting to the L4 stage.

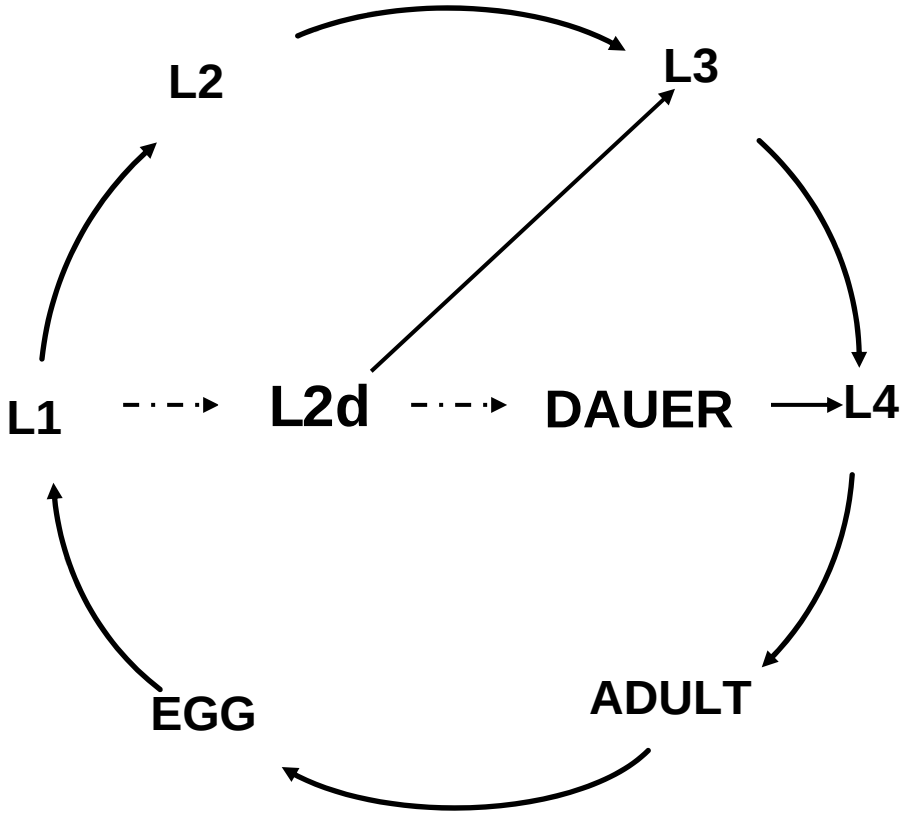

L2
L3
L1
L2d
DAUER
L4
EGG
ADULT

Figure 4. *rpc-1* expression during *C. elegans* development. **A.** *rpc-1* mRNA staining in embryos. Embryos were stained with the same conditions on the same slide. Different embryonic stages were identified visually. The decrease of *rpc-1* mRNA staining intensity with embryogenesis is shown. **B.** *rpc-1* mRNA staining in larvae and adults. Animals were staged with different culture times at 20^{o}C. P: pharynx; I: intestine; G: gonad. The enrichment of *rpc-1* mRNA in the gonad is shown.

(Data are extracted from Kohara, http://nematode.lab.nig.ac.jp/)

A

2 cell stage | 6~18 cell stage | Mid-gastrulation stage | 3 fold stage

B

L1~L2 stage | L2~L3 stage | L3~L4 stage | L4~adult stage

Pictures are adapted from http://nematode.lab.nig.ac.jp/

MATERIALS AND METHODS

Culture conditions

Animals were grown on NG Agar Petri dishes with *E. coli* strain OP50, as described by Brenner (1974). Genetic nomenclature follows the standard rules of Horvitz et al. (1979). The following abbreviations were applied: *ama* (amanitin resistance abnormal); *bli* (blistered cuticle); *daf-c* (dauer formation-constitutive); *daf-d* (dauer formation-defective); *dpy* (dumpy, short body length); *fem* (feminization); *hsp* (heat-shock promoter); *lin* (lineage abnormal); *rpc* (DNA-directed RNA polymerase III or C); *rrf* (RNA-dependent RNA polymerase family); *srg* (serpentine receptor family, class G); *unc* (uncoordinated movement); SNP (single nucleotide polymorphism). All mutants were derived from the wild-type Bristol N2 strain (Brenner, 1974). Wild type CB4856 strain was used for SNP mapping. All crosses were done at 20^{o}C and strains were maintained at 15^{o}C.

Strains

Wild type N2, *mEx165 [rol-6(su1006); rpc-1p::gfp], rpc-1(m654) unc-24(e138) / nT1(let-? m435), rpc-1(s1139) unc-24(e138) / nT1(let-? m435), mEx166 [rol-6(su1006); hsp16-41p::rpc-1::gfp], daf-2(e1370), fem(e1991) unc-24(e138) unc-22(s12) / stDf8, rrf-3(pk1426), daf-1(m40), daf-4(e1364), daf-7(e1372), daf-8(m85), daf-14(m77), daf-2(m41).*

DNA isolation and purification

DNA was isolated from bacteria using the ABI Prism™ Miniprep Kit (PE Applied Biosystems). DNA Clean & Concentrator™ -5 (Zymo Research) was used for DNA purification or concentration when needed.

***daf-30* SNP mapping**

daf-30 was genetically mapped in the interval between *lin-45* and *unc-24* (Caldicott, 1995). SNP markers between the wild-type Bristol N2 strain and Hawaii CB4856 strain in this interval was used for further mapping (Wick, et al., 2001). Since a *lin-45 daf-30* chromosome was not available, *bli-6 daf-30 unc-24 / nT1(let-? m435)* was used for SNP mapping. Briefly, CB4856 (HA) males were used to cross *bli-6 daf-30 unc-24 / nT1 (let-?)*. *bli-6 daf-30 unc-24 / HA* F1 progeny and allowed to segregate Unc-non-Daf and Bli-non-Daf recombinants. The presence of CB4856 SNP marker in the Unc-non-Daf recombinants would suggest that *daf-30* is to the left of this SNP site; while the presence of CB4856 SNP marker in the Bli-non-Daf recombinants would suggest that *daf-30* is to the right of this SNP site. All SNP markers, chosen in this mapping, can be recognized by individual restriction enzymes.

Germline transformation

Germline transformation was carried out as described by Mello, et al. (1991). The pRF4 [*rol-6(su1006)*] was used as the co-injection marker (100ng/μl). DNA of interest was injected at 10 ng/μl unless indicated. The roller phenotype was used to identify transgenic animals.

Transgene complementation for *daf-30* cloning

Germline transformation was performed to identify the cosmid carrying the *daf-30* activity from cosmids located in the region defined by SNP mapping. Candidate cosmids were injected into *daf-30(m654) unc-24(e138) / nT1(let-?) (IV, V).*

To test candidate genes carried by cosmid C42D4, each coding region in this cosmid (plus the 3.0 Kb 5' sequence to include the promoter) was amplified by PCR, using C42D4 as the template. PCR products were co-injected with pRF4 into the hermaphrodite gonad for germline transformation. Rescued animals were scored as Unc-Roller adults. Primers for each gene were:

*str-220/1*F: 5'- ACC ACT ACC GTA ATT CCT ACA A -3'

*str-220/1*B: 5'- TTC CTG GTC TAA ATT TCA ATC C -3'

*skr-16*F: 5'- GCA CAC CAA CTT GAT CGA TTC -3'

*skr-16*B: 5'- TAA TTT GAA CCT GCC GTG TTA C -3'

*str-44*F: 5'- TTC GTC GTC AAA TGC TAT CGA A -3'

*str-44*B: 5'- TGA GTA AGC AAA TAG AAC TAT GAA-3'

C42D4.2/3F: 5'- AAG TTT GGT GGA TAC GGA GTT A -3'

C42D4.2/3B: 5'- TCT CTC ATG AAA CGC TCA AAT C -3'

C34D4.1F: 5'- GCT GAT CTG ATC ATC ACT AGA AAG -3'

C34D4.1B: 5'- CTC TCA ACG TGA CGT GGT AAG TA -3'

C42D4.11F: 5'- TTT CCA AGC CAT TCC CTA CTT G -3'

C42D4.11B: 5'- GAT CTT GGT GTG AAC TCT CTC A -3'

C42D4.13F: 5'- TGT ACA TCG TTG AAT TAT TGT GTG -3'

C42D4.13B: 5’- GTG ATT CAA ATG TGT TTT AAC CCA -3’

5’-*rpc-1*: 5’- TAT CCA GGA ATG CAT TGG TTT CAG -3’

3’-*rpc-1*: 5’- GAT TCC AAG GCA ATT CGT GAG A -3’

Complementation test for *rpc-1* allele identification

The chromosome of *rpc-1(m654) unc-24(e138)* was used to identify more *rpc-1* alleles from a collection of *rpc-1* mutant candidates that were isolated by Dr. Donald L. Riddle. Males of the genotype *rpc-1(m654) unc-24(e138) / nT1(let-?) (IV; V)* were made by heat-shock of L4 hermaphrodites at 30^{o}C for 8 hours. *rpc-1 unc-24 / nT1* males were used to cross with *let-X unc-24(e138) / nT1 (let-?) (IV; V)* hermaphrodites. *let-X* here represents the *rpc-1* candidates: *let-289(s1133), let-290(s1140), let-291(s1139), and let-292(s1146)*. Parents were transferred to a new plate every day. Progeny were scored 48 ~ 72 hours at 20^{o}C after eggs were laid. Crossing was regarded as successful when ~40% progeny were males. If Unc L4 and/or adult animals were found among the cross progeny, the mutant was judged to complement *rpc-1(m654)*; if only Unc L1 or L2 animals were found among cross progeny, the mutant was identified as an *rpc-1* allele.

Worm PCR

Methods for single-worm PCR were modified from Williams et al. (1992). Worms (10 worms for *rpc-1 unc-24* homozygous animals) were transferred into 2.5μl of lysis buffer (10mM Tris pH 8.2, 50mM KCl, 2.5mM $MgCl_2$, 0.45% Tween-20, 60μg/ml proteinase K) in the cap of a 0.5ml PCR tube. Worms were spun down to the bottom and frozen at -70^{o}C for 15 minutes. The worm solution was exposed to 65^{o}C for one hour,

followed by 15 minutes at 95°C in a thermal cycler. The standard PCR procedure was then performed.

rpc-1 mutation sequencing

An Applied Biosystems 377 automated DNA sequencer was used to sequence *rpc-1* mutant genomic DNA, amplified by worm PCR. The coding region was split into 6 segments to be amplified by PCR. Each segment, ~1Kb long, overlaps the neighbor segments ~100 base pairs. The PCR product was cloned into pGEM ® -T Vector (Promega). Inserts were sequenced using primers, flanking the insert in the T Vector: T7: 5'- GTA ATA CGA CTC ACT ATA GGG -3'; SP6: 5'- TAC GAT TTA GGT GAC ACT ATA G -3'. When a mutation was found in one fragment, sequencing of this fragment was repeated using two independent worm PCR prep to confirm that mutation.

Primers used to amplify different segments of *rpc-1* gene were:

Fragment A: 5'- CTT CGC TAA ATC TCG ATG TTT GC -3' and 5'- GTA GTT CCA GCT TTG ACT TCT G -3'.

Fragment B: 5'- TAA TGG TTC GAT CCG GAG AGG -3' and 5'- TCA TTG AAT CTG AAC GTT CTT CC -3'.

Fragment C: 5'- CCT TGA TGA TAA CTA TGT GGT C -3' and 5'- CAG CAT GAT CAC GAA TCG TTG A -3'.

Fragment D: 5'- GCT CAA CCA GGT TGC ACT G -3' and 5'- TGC GGC AAT AGC TCC AAC TGC A -3'.

Fragment E: 5'- AAG ACC CAA CTC TGC AGT TTC A -3' and 5'- GAC TAC CAC ATT TGC GAG AGA A -3'.

Fragment F: 5’- CTC ATG GAA AGA CAA TGA TGG C -3’ and 5’- AGA CCA TTA CGA GAG ATT CCA -3’.

Semi-quantitative test for *rpc-1* expression

The response of *rpc-1* expression to food availability was tested by semi-quantitive RT-PCR. *C. elegans* eggs were purified by NaOCl treatment, as described by Emmons et al., (1979). Eggs were hatched in the M9 buffer for 48 hours to synchronize development at the L1 stage to prepare starved animals. Starved animals were fed with a 5% W/V suspension of *E. coli Chi 1666*. They were then washed in M9 buffer three times to remove bacteria and snap frozen in liquid nitrogen. Total RNA was extracted with an acid guanidinium thiocyanate-phenol-chloroform mixture (Chomczynski and Sacchi, 1987). Reverse transcription was performed using ImProm-II™ Reverse Transcription System (Promega). PCR was done with FailSafe™ PCR System (Epicentre Technologies). *rpl-21* was used as the loading control. The primers used were: *rpl-21*, 5' ATG ACT AAC TCC AAG GGT C 3' and 5' TCA CGC AAC AAT CTC GAA AC 3'; *rpc-1*, 5’ AAG ACC CAA CTC TGC AGT TTC A 3’ and 5’ GAC TAC CAC ATT TGC GAG AGA A 3’; *ama-1*, 5’ TGG AAA TAC GGA TGG AGG AGC A 3’ and 5’ TGT ATC CAC CTC CAC TTT CAT AA 3’; Y48E1A.1, 5’ CAA TCA ATC GGA GAA CCA TCA AC 3’ and 5’ CTT CAG CTG CAT CAG CTT CAC 3’.

***rpc-1p::gfp* construction**

To test the *rpc-1* promoter activity, *rpc-1p::gfp* was constructed. The 2.9Kb 5′ upstream sequence with the first two exons and introns of the *rpc-1* gene was used as the

rpc-1 gene promoter. The *rpc-1* promoter sequence was amplified by PCR, using cosmid C42D4 as the template. Primers used are: 5'-rpc-1: 5'- TAT CCA GGA ATG CAT TGG TTT CAG -3' and rpc-1AB: 5'- GTA GTT CCA GCT TTG ACT TCT G -3'. The PCR product was then digested by restriction enzymes Pst I and Hpa I to generate the *rpc-1* promoter. This promoter was cloned into pPD95.69, a *gfp* vector with the nuclear localization signal (Fire Lab Vector Kit, June 1995), at Pst I / Sma I clone sites. This construct was used for testing *rpc-1* promoter activity in transgenic animals.

***rpc-1* promoter activity test**

The *mEx165 [rol-6 (su1006);rpc-1p::gfp]* transgenic line was made with the *rpc-1p::gfp* construct (100 ng/μl) by germline transformation. The stable transgenic line (~17% transgenic animals could always been found in the progeny of the transgenic hermaphrodites) was used to test the *rpc-1* promoter activity by observing the GFP staining. L3 transgenic animals were used to observe the spatial pattern of *rpc-1* promoter activity.

To test the *rpc-1* promoter response to food availability, *mEx165 [rol-6 (su1006); rpc-1p::gfp]* animals at the L3 stage were transferred to an empty plate at 20^{o}C for 1 hour to deprive them off food. These animals were transferred again to a new empty plate in 6 hours for starvation treatment. 2 days later, animals in the plate without bacteria were scored as starved animals. Starved animals were transferred back to the normal bacteria food for re-feeding treatment. Re-fed animals were harvested at different times as indicated. *rpc-1* promoter activity was scored based on the posterior intestinal

GFP staining. After transgenic L3 animals were starved for two days at 20°C, no GFP staining was detected IN the posterior intestine.

RNA interference (RNAi)

A 1.7Kb fragment of the *rpc-1* genomic exon-rich sequence was amplified from cosmid C42D4 (5478 bp – 7184 bp) by PCR. Primers used were: Nco I-tagged *rpc-1*RF: 5' CAT GCC ATG GAT GTC AGG ACT TCC ACC AGA TA 3' and Xho I-tagged *rpc-1*RB: 5' CCG CTC GAG CAG TAT CTA CAA GAC CTT CAC G 3'. The PCR product was sub-cloned into the feeding RNAi plasmid vector L4440 (Timmons and Fire, 1998) at Nco I / Xho I cloning sites to generate the *rpc-1* RNAi construct. The RNAi construct was transformed into *E. coli* DH5α bacteria for replication.

The RNAi construct DNA harvested from *DH5α* was then transformed into *E. coli* HT115 (DE3), an RNase III-deficient strain with IPTG-inducible expression of T7 polymerase (Kamath et al, 2000). Transformed HT115 (DE3) bacteria were heavily inoculated into 2ml of LB containing ampicilin (100μg/ml). The overnight bacterial culture was then seeded onto NG agar plates containing ampicilin (100μg/ml) and isopropyl-thio-β-D-galactopyranoside (IPTG, 1mM), and incubated at room temperature for 16 hours. About 20 L4 animals were transferred to each RNAi plate. After 40-hour pre-treatment, animals were transferred to a fresh RNAi plate and allowed to lay eggs for 4 hours. The RNAi phenotype was scored 72-76 hours after eggs were laid. All tests were performed at 20°C.

Phenotypic test of *rpc-1* hemizygotes

A genetic test was used to evaluate whether *rpc-1(m654)* and *rpc-1(s1139)* are null alleles (complete loss of function). Since *stDf8* has a chromosomal deficiency, which covers the *rpc-1* locus and *unc-24* locus, *rpc-1 unc-24 / stDf8* is the hemizygous genotype. *fem-1(e1991) unc-24(e138) unc-22(s12) / stDf8* hermaphrodites were crossed with N2 males. Wild-type like F1 hermaphrodites (*fem-1) unc-24 unc-22 /* + or *stDf8 /* +) were isolated. *stDf8 /* + animals were identified from *fem-1 unc-24 unc-22 /* + mutants, based on their insensitivity to 1% Nicotine, since *fem-1 unc-24 unc-22 /* + animals twitch in 1% Nicotine. *stDf8 /* + L4 hermaphrodites were crossed with young adult *rpc-1 unc-24 / nT1 (let-?) (IV, V)* males. After 40 hours, the crossing parents were transferred to a new plate for 8 hours at 20^{o}C, then removed. Two days later at 20^{o}C, Unc larvae were transferred to a fresh plate for 3 more days until the phenotype was scored. In parallel the arrested homozygous Unc progeny of corresponding *rpc-1 unc-24 / nT1 (let-?)(IV, V)* mutants were used as controls.

***hsp16-41p::rpc-1::gfp* construction**

The *rpc-1* gene with 2.0 Kb promoter was amplified from the cosmid C42D4 by PCR, using primers: 5'- AAG GAA AAA AGC GGC CGC TAG ATG GAG TTC TTC GAT TTG GC -3' and 5'- CGG GGT ACC GAT TCC AAG GCA ATT CGT GAG ATC -3'. The PCR product was digested with Not I and Kpn I. The digestion product was then cloned into pBlueScript SK+ (Stratagene) to generate the *rpc-1* construct. No missense mutation was found by DNA sequencing. A *gfp* cDNA fragment (A gift from John Walker's Lab) was amplified by PCR (primers: 5'- GGA GGC CGA CAA GGC

CAG TAA AGG AGA AGA ACT TTT C -3' and 5'- CAA GGC CTT GTC GGC CGA TTT GTA TAG TTC ATC CAT GCC -3'). The PCR product was digested by Sfi I and cloned into the above *rpc-1* construct to generate *rpc-1::gfp* construct. This construct was proven to be functional by rescue of the *rpc-1* mutant phenotype.

The *rpc-1::gfp* coding region was amplified by PCR from the above *rpc-1::gfp* construct. Primers were: 5'- ATT GCA GCT AGC ATG GGT AAA GAG CAG TTT CGG -3' and 5'- CGG GGT ACC GAT TCC AAG GCA ATT CGT GAG ATC -3'. This product was then cloned into pPD49.83, a vector that carries the heat-shock promoter *hsp16-41p* (Fire Lab Vector Kit, June 1995), at the clone sites, Nhe I and Kpn I. The product (100ng/μl) was used to generate the *rpc-1(m654) unc-24(e138) / nT1(let-?) (IV; V); mEx166 [rol-6 (su1006); hsp16-41p::rpc-1::gfp]* transgenic line by germline transformation. The stable transgenic line was used to test effects of the induced *rpc-1::gfp* gene products by heat-shock. Heat shock was performed at 30^{o}C.

construction of *daf-c; mEx165 [rol-6(su1006); rpc-1p::gfp]*

To construct strains carrying *mEx165 [rol-6(su1006); rpc-1p::gfp]* and one of the *daf-c* mutations, *daf-7(e1372), daf-4(1364), daf-8(m85), daf-14(m77),* or *daf-2(m41)* strains, the phenotype of constitutive dauer formation at 25^{o}C and the roller phenotype were used to identify the desired strains. First, *mEx165 [rol-6(su1006); rpc-1p::gfp]* hermaphrodites were crossed with N2 males to make a *mEx165 [rol-6(su1006); rpc-1p::gfp]* male strain. These males were used to cross with *daf-c* hermaphrodites at 25^{o}C. Since the mating ability of *mEx165 [rol-6(su1006); rpc-1p::gfp]* males is poor, 15 males were crossed with 5 hermaphrodites. Roller F1 hermaphrodites (*daf-c/+; mEx165 [rol-*

6(su1006); rpc-1p::gfp]) were transferred to a new plate 3-5 days later, and incubated at 25°C for another 3 days. F2 roller dauers (*daf-c; mEx165 [rol-6(su1006); rpc-1p::gfp]*) were allowed to recover at 15°C for 2 days and one animal was cloned to generate the stock.

The *daf-1(m40)* mutant phenotype is maternally rescued (homozygous F2 *daf-1(m40)* mutants segregated from F1 *daf-1(m40)/+* parents do not show the Daf-c phenotype at 25°C). Here, L4 roller animals (F2 generation) were transferred to fresh plates to produce F3 roller dauers at 25°C, which were allowed to recover at 15°C. One recovered animal was used to generate the stock (*daf-1(m40); mEx165 [rol-6(su1006); rpc-1p::gfp]*).

Construction of *daf-7(e1372); mEx56 [rol-6(su1006); srg-8p::gfp]*

N2 males were crossed with *mEx56 [rol-6(su1006); srg-8p::gfp]* hermaphrodites to generate *mEx56 [rol-6(su1006); srg-8p::gfp]* roller males, which were then crossed with *daf-7(e1372)* hermaphrodites at 25°C. Roller L4 hermaphrodites (F1, *daf-7(e1372) / +; mEx56 [rol-6(su1006); srg-8p::gfp]*) were transferred to a new plate for one generation at 25°C. Roller F2 dauers (*daf-7(e1372); mEx56 [rol-6(su1006); srg-8p::gfp]*) were recovered at 15°C, and one animal was used to generate the stock.

Construction of *daf-5(e1386); mEx165 [rol-6(su1006); rpc-1p::gfp]*

Fifteen *mEx165 [rol-6(su1006); rpc-1p::gfp]* males were crossed with five *daf-5(e1386)* hermaphrodites. F1 roller L4 progeny were transferred to a new plate and F2 roller animals were isolated and cultured at 25°C until starvation, and dauer formation

was scored 5 days later. Progeny failed to form dauers under dauer-inducing conditions (on the starved plates), were identified as *daf-5; mEx165 [rol-6(su1006); rpc-1p::gfp]*. One transgenic animal was isolated to generate the stock.

Construction of *daf-6(e1377); mEx165 [rol-6(su1006); rpc-1p::gfp]*

daf-6(e1377) mutants have the *dyf* (dye filling of neurons defective) phenotype. This phenotype was used to score *daf-6(e1377)* homozygous mutants. The *dyf* phenotype was scored using FITC (fluorescein isothiocyanate isomer I) staining as described by Hedgecock et al. (1985). *daf-6(e1377)*; *mEx165 [rol-6(su1006); rpc-1p::gfp]* was identified by the Rol-Dyf phenotype. One animal was isolated to generate the stock strain.

RESULTS

Physical mapping of the *daf-30*

daf-30(m654) was previously positioned in between *lin-45* and *unc-24* (Caldicott, 1995) by genetic mapping (Figure 5). DNA polymorphisms in this region between the *C. elegans* Bristol strain N2 and the Hawaiian strain CB4856 were used as physical markers to refine the mapping. Totally, 5 SNP sites were used: ZK381:5047, Y43B11AR:1108, D2024:25027, T12B3:3235, R05G6:2917 (letters and digits before colons represent names of cosmids or YACs in which the corresponding SNPs are located, and numbers after colons represent the base pair positions of the SNPs in that sequence). All used SNP sites could be identified individually by specific restriction enzymes, as exemplified in Figure 6. SNP mapping placed *daf-30* between D2024:25027 and ZK381:5047, which are ~260Kb apart (Figure 7).

Transgene rescue by germline transformation

It is assumed that germline transformation of the wild type *daf-30* gene would rescue the *daf-30* mutant phenotype, which is the case for most genes except for germline genes. Based on this assumption, all candidate cosmids (cosmids in the above defined interval, Figure 8A), ZK381, C25A8, C06G3, ZC477, C34D4, C42D4, R13A1 and D2024, were introduced into *daf-30(m654) unc-24(e138) / nT1(let-?)* animals by germline transformation to test which one carried *daf-30*. The stable transgenic line was used for each cosmid to test if the mutant phenotype of transgenic progeny were rescued. After testing all candidate cosmids, it was found that only C42D4 partially rescued the

daf-30(m654) phenotype, i.e., transgenic *daf-30(m654) unc-24(e138)* animals grew up to adulthood, but were sterile. This result indicates that C42D4 may carry the *daf-30* activity.

There are ten predicted genes in the cosmid C42D2: C34C4.1, *rpc-1*, *skr-16*, *str-220*, *str-1*, *str-44*, C42D4.3, C42D4.2, C42D4.11, C42D4.13, and C42D4.1; and one cloned gene, *rpc-1* (Figure 8B). All these genes were isolated using PCR. Germline transformation of these isolated genes was applied individually to identify *daf-30* gene candidate by transgene complementation as above. It was found that *rpc-1* was the only gene that could complement *daf-30* mutation partially, i.e., rescued animals were sterile. Partial rescue may be caused by insufficient expression of the transgene in germline cells, which is supported by the post RNAi phenotype (See below). Based on this result, the *daf-30* gene was identified as the *rpc-1* gene. Since the *rpc-1* gene was cloned by Bird and Riddle in 1989, *daf-30* is assigned as an *rpc-1* allele, *rpc-1(m654)*. This is the first *rpc-1* mutant allele ever identified in *C. elegans*.

rpc-1(s1139) identification

A collection of *rpc-1* candidate mutants was identified by Dr. Donald L. Riddle (unpublished data). They are*: let-289(s1133), let-290(s1140), let-291(s1139)* and *let-292(s1146)*. To determine if there were any *rpc-1* alleles in this collection, the *rpc-1(m654) unc-24(e138) / nT1(let-?)* strain was used to perform genetic complementation tests. Only *let-291 (s1139)* failed to complement *rpc-1(m654)* (See Materials and Methods), and this mutant was renamed *rpc-1(s1139)*.

Figure 5 Physical map of the *daf-30* region on the right arm of the chromosome IV. The position of *daf-30* is shown in relation to other genetic markers and SNP markers. *lin-45* and *unc-24* have been cloned and their positions on the chromosome IV are shown. *bli-6* is not cloned yet, but genetic mapping indicates that *bli-6* is 0.04 map units to the left of (up from) *lin-45*. All SNP (shown as "Cosmid/Yac name: SNP site relative to the Cosmid/Yac") markers can be recognized by individual restriction enzymes. Physical position is indicated by Mb (Mega base) on chromosome IV. Interval size between markers is indicated to the right in Mb.

LG IV Right

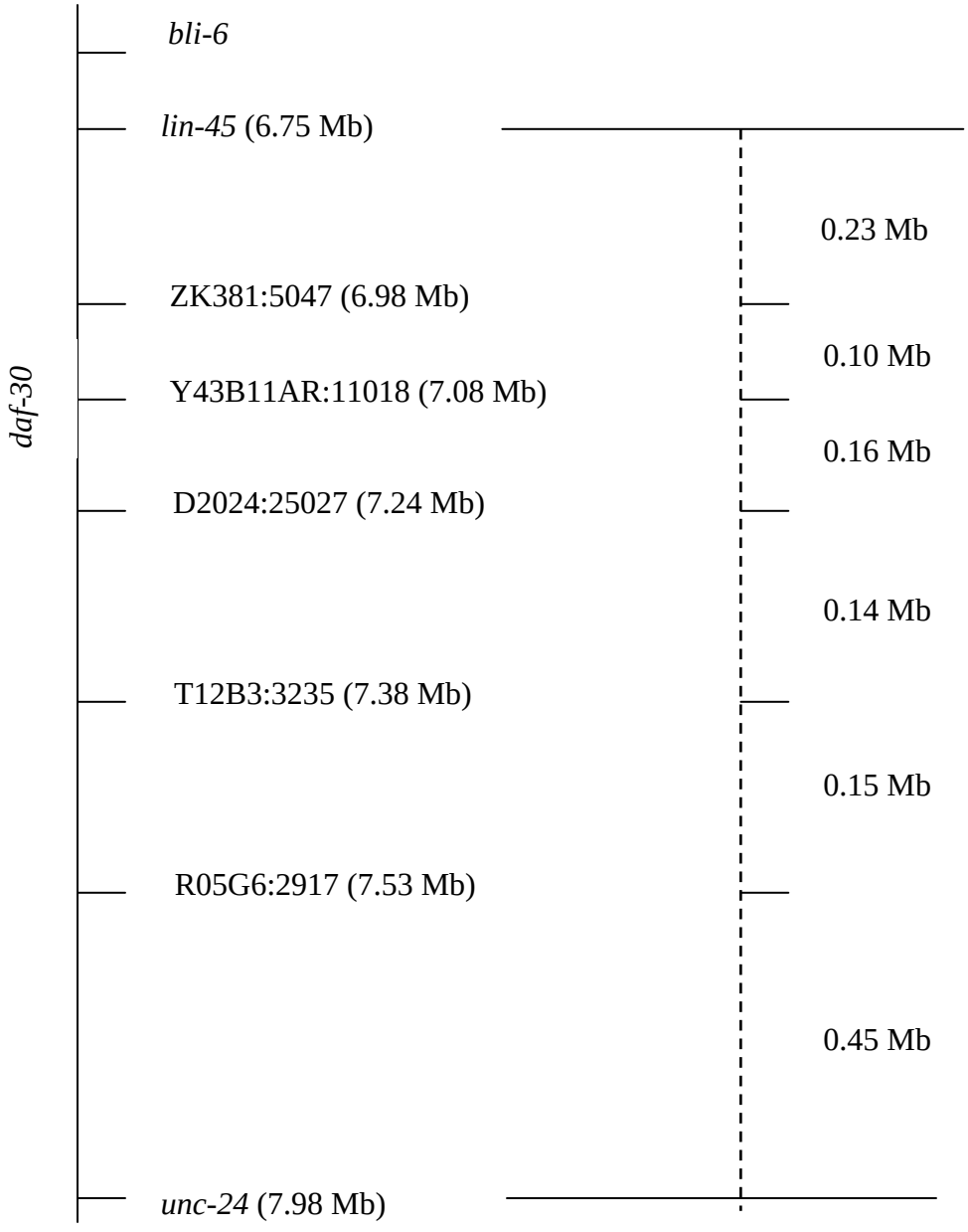
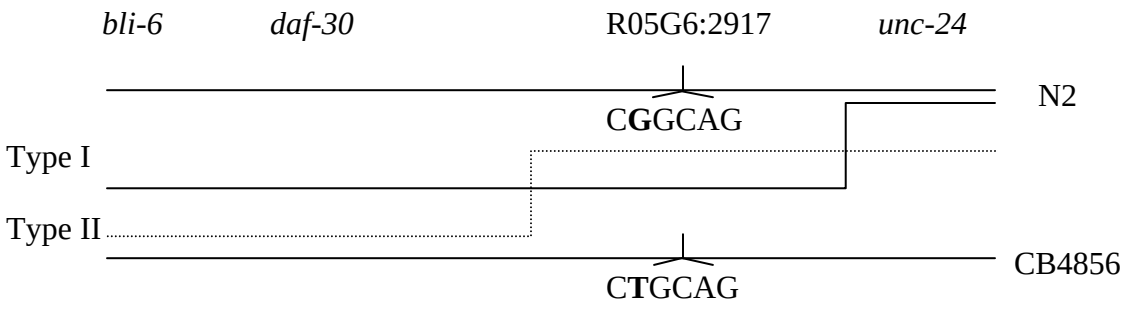
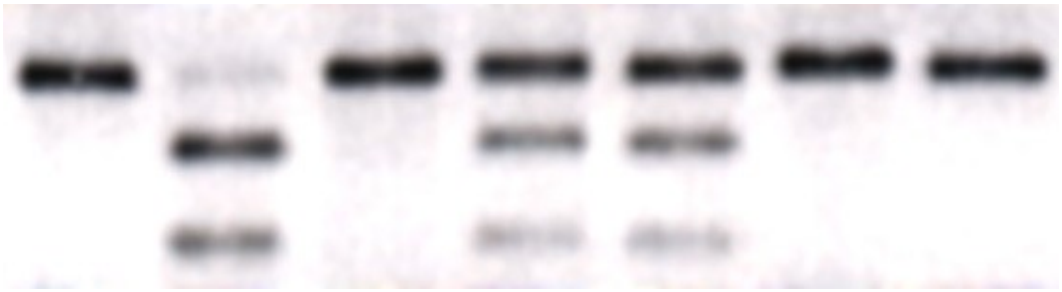

bli-6
lin-45 (6.75 Mb)
0.23 Mb
ZK381:5047 (6.98 Mb)
0.10 Mb
daf-30
Y43B11AR:11018 (7.08 Mb)
0.16 Mb
D2024:25027 (7.24 Mb)
0.14 Mb
T12B3:3235 (7.38 Mb)
0.15 Mb
R05G6:2917 (7.53 Mb)
0.45 Mb
unc-24 (7.98 Mb)

Figure 6. Example of SNP used in *daf-30* mapping. SNP mapping example at R05G6:2917 is shown here. (A) Diagram of the SNP mapping principle at this site. SNP between Bristol N2 strain and Hawaii CB4856 strain results from a G→T substitution at the 2919 base pair position of cosmid R05G6. This substitution introduces the Pst1 restriction site C**T**GCAG in CB4856 relative to the N2 sequence C**G**GCAG. Unc-non-Daf recombination, which occurs to the right of the R05G6:2917 (shown by solid line, recombination type I), will be recognized by Pst1at this site. However, Unc-non-Daf recombination, which occurs to the left of the R05G6:2917 (shown by dotted line, recombination type II), will be recognized by Pst1 cleavage at this site. (B) The result of the SNP mapping at this site. Worm PCR products of all Unc-non-Daf recombinants were tested by Pst1. Primers used were: GGT GTT CAA ACA TGC GAC G and TTT GGA CGG ATA GCT ACA TAC G.

A



B



N2 CB485 Type II Type I* Type I* Type I Type II

*The band on the top results from the incomplete digestion.

Figure 7 *daf-30* SNP mapping results. Since *daf-30* was isolated from N2, CB4856 males were used to cross *bli-6(sc16) daf-30(m654) unc-24(e138) / nT1(let-?)* hermaphrodites. Unc-non-Daf and Bli-non-Daf recombinants were selected. Individual restriction enzymes were used to detect SNP inheritance, as indicated in Figure 6. Numbers by the curve represent recombinations identified by the restriction enzyme. 58 recombinants were tested totally. Each SNP site is indicated below.

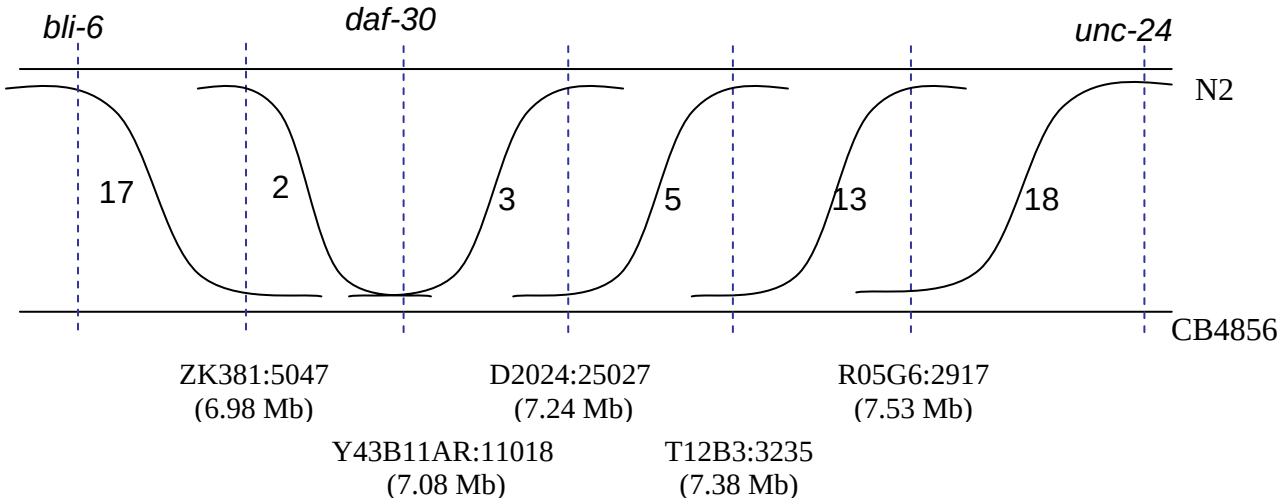
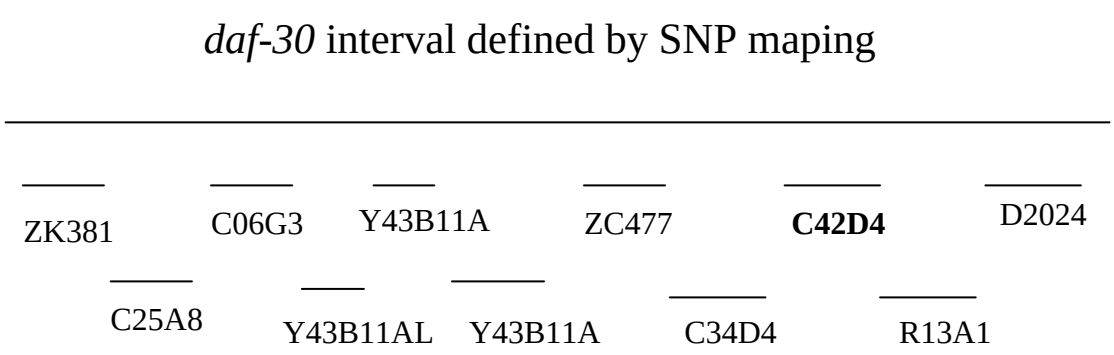
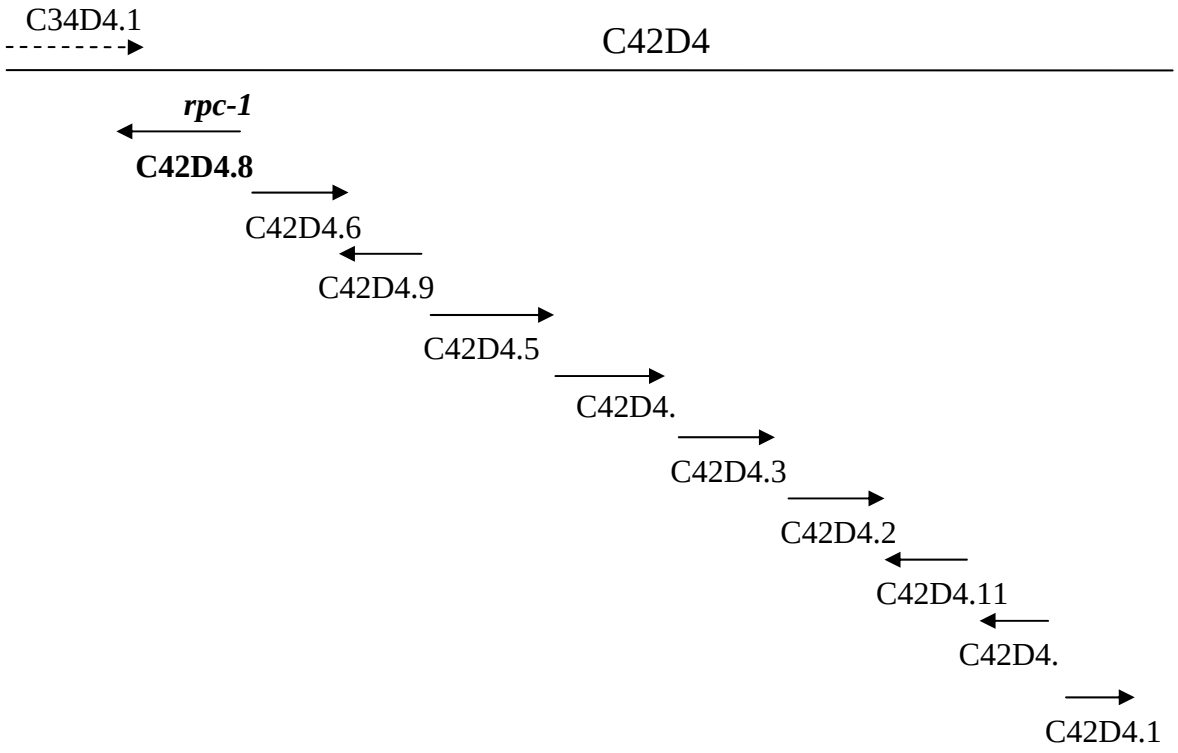

bli-6
daf-30
unc-24
N2
17
2
3
5
13
18
CB4856
ZK381:5047
(6.98 Mb)
D2024:25027
(7.24 Mb)
R05G6:2917
(7.53 Mb)
Y43B11AR:11018
(7.08 Mb)
T12B3:3235
(7.38 Mb)

Figure 8. Identification of the *daf-30* gene. (A) Cosmids and YACs covered by the defined *daf-30* region with SNP mapping. These sequences were tested for *daf-30* activity by germline transformation. C42D2 was shown to be the candidate cosmid. (B) Predicted genes in Cosmid C42D4, shown with direction of transcription. The predicted C43D4.1 gene sequence is incomplete in C42D4. These genes were PCR amplified with ~3 Kb upstream sequences as individual promoters. All PCR products were tested for *daf-30* activity by germline transformation. *rpc-1* was identified as the *daf-30* gene.

A


daf-30 interval defined by SNP maping
ZK381
C06G3
Y43B11A
ZC477
C42D4
D2024
C25A8
Y43B11AL
Y43B11A
C34D4
R13A1

B


C34D4.1
C42D4
rpc-1
C42D4.8
C42D4.6
C42D4.9
C42D4.5
C42D4.
C42D4.3
C42D4.2
C42D4.11
C42D4.
C42D4.1

rpc-1 encodes the largest subunit of Pol III

The *rpc-1* gene encodes the largest subunit of Pol III, a protein of 1400 amino acids. BLAST search results suggest that it is highly conserved in different species from yeast to humans (Table 2). A conserved domain search defined five conserved domains in the RPC-1 protein (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Figure 9): Rpb1 domain 1 (the clamp domain, residues 14-352), RNA polymerase I subunit A N-terminus (residues 244-549), Rpb1 domain 3 (the pore domain, residues 524-699), Rpb1 domain 4 (the funnel domain, residues 720-830), and Rpb1 domain 5 (the discontinuous cleft domain, residues 837-1332). These five domains play essential roles in gene transcription.

rpc-1p::gfp expression pattern

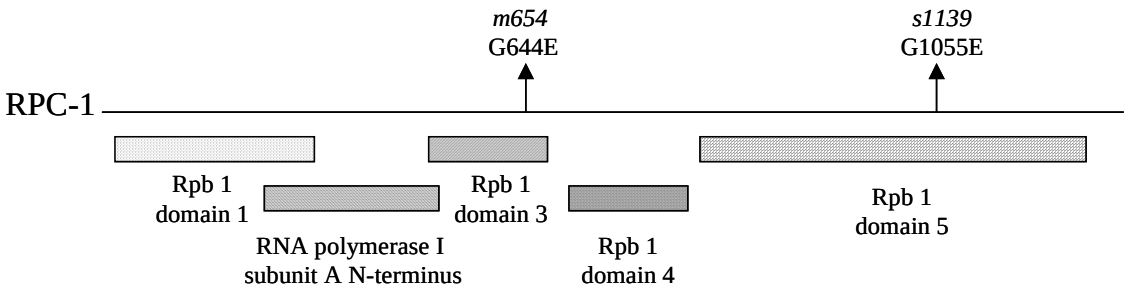
GFP was used as a reporter to test the *rpc-1* expression pattern. First, *gfp* cDNA was fused in frame to the *rpc-1* genomic sequence to make the *rpc-1::gfp* construct, trying to generate the functional RPC-1::GFP fusion. When this construct was introduced into *rpc-1 unc-24 / nT1(let-?)* by germline transformation, it rescued the *rpc-1* mutant phenotype as efficiently as the wild-type gene. However, no GFP staining was observed (data not shown). The fusion coding region was then fused to a heat-shock promoter, *hsp16-41pp*, to generate the *hsp16-41pp::rpc-1::gfp* construct. When this construct was introduced into *rpc-1 unc-24 / nT1(let-?)* animals, *rpc-1* phenotype was rescued and GFP staining was observed, mainly in the intestine, after heat-shock treatment (Table 4).

Table 2. Blast result of the *C. elegans* RPC-1 with Pol III largest subunits from other species*

Species	Identity to RPC-1	Similarity to RPC-1	Accession number
Homo sapiens	56%	69%	AAH41089.1
S. cerevisiae	46%	62%	CAA99314.1
Arabidopsis thaliana	40%	56%	NP_200812.1
Rattus norvegicus	55%	68%	XP_341389.1

(*http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi)

Figure 9. The schematic structure of RPC-1. The conserved domains are indicated below, relative to their corresponding positions in the diagram of the protein first order structure. Rpb1 domain 1 covers residues 14-352; RNA polymerase I subunit A N-terminus domain covers residues 244-549; Rpb1 domain 3 covers the residues 524-699; Rpb1 domain 4 covers residues 720-830; and Rpb1 domain 5 covers residues 837-1332. Amino acid substitutions in *m654* and *s1139* are shown above the line. The Gene Bank accession number of RPC-1 is NP_501127.


m654
G644E
s1139
G1055E
RPC-1
Rpb 1
domain 1
RNA polymerase I
subunit A N-terminus
Rpb 1
domain 3
Rpb 1
domain 4
Rpb 1
domain 5

The results suggest that RPC-1::GFP itself is fluorescent, but the fluorescence is silenced when RPC-1::GFP is integrated into the Pol III enzyme with other subunits. Heat-shock is assumed to induce *hsp16-41pp::rpc-1::gfp* transgene over expression to generate more RPC-1::GFP products than necessary for Pol III enzyme formation. So, it is the over-produced free RPC-1::GFP in *hsp16-41pp::rpc-1::gfp* transgenic animals that emits fluorescence. These results suggest that *rpc-1::gfp* construct cannot be used to test *rpc-1* expression.

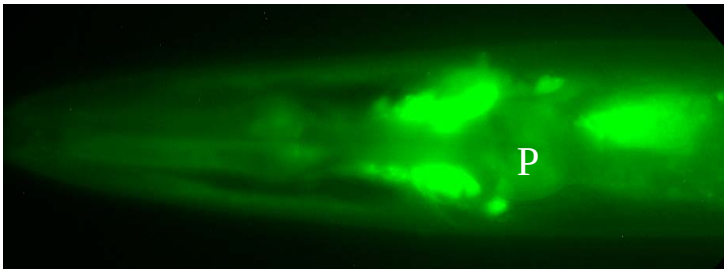
To avoid this problem, the *rpc-1* promoter driven *gfp* expression was used as a reporter to characterize *rpc-1* gene expression. A *rpc-1p::gfp* construct (See Materials and Methods) was introduced into N2 animals by germline transformation to generate the *mEx165 [rol-1(su1006); rpc-1p::gfp]* transgenic animals. GFP staining in transgenic animals was observed (Figure 10). Interestingly, it was shown that GFP was mainly detectable in some head neurons, the intestine (especially in the posterior end), and some tail neurons. These results suggest that the *rpc-1* gene may not be expressed equally in all cells in *C. elegans*.

Mutation analysis

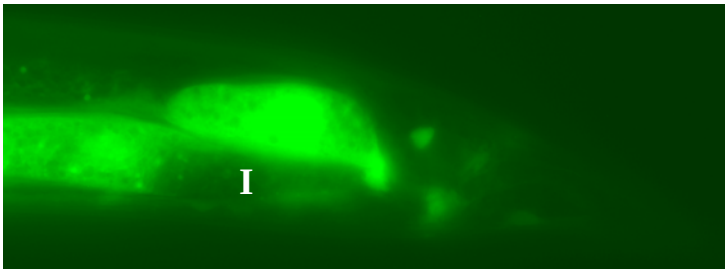
A PCR method was applied to sequence the two *rpc-1* alleles, *(m654)* and *(s1139)*. In the allele *m654*, a GC-AT mutation was found in the Exon 8 of *m654*, which results in the amino acid change G644E in the pore domain of the RPC-1 protein. In the allele *s1139*, a GC-AT mutation was found in the Exon 11, which results in an amino acid change G1055E in the cleft domain. Both amino acids, mutated in two alleles respectively, are highly conserved not only in the large subunit of Pol III from different

Figure 10. Expression of *gfp* driven by the *rpc-1* promoter. The 3Kb upstream sequence, including the first two exons, of the *rpc-1* gene is used as the *rpc-1* promoter. The *rpc-1p::gfp* construct includes a nuclear localization signal (See Materials and Methods). Labels indicate the pharynx (P) and the intestine (I). L3 larva was used to observe the expression pattern. (A) *gfp* expression in the anterior part of the body. (B) *gfp* expression in the posterior part of the body. Images were taken by krypton/argon gas laser excitation at 488 nm on a Biorad MRC-600 confocal microscope.

A



B



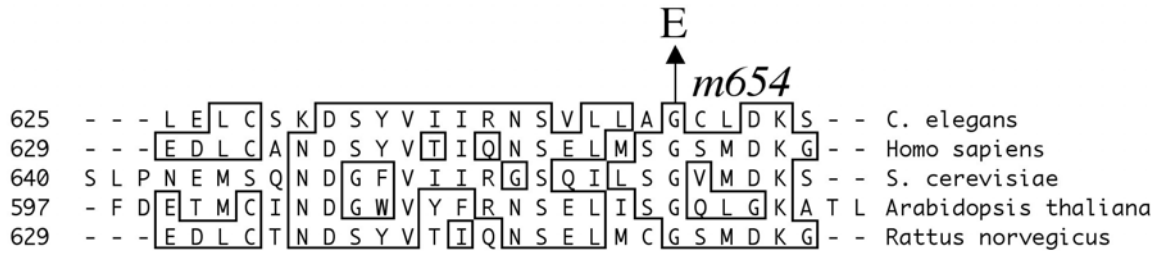
species (Figure 11), but also in all three large subunits of three RNA polymerases in *C. elegans* (Figure 12). Also, these two amino acid residues are conserved in yeast RNA Pol II largest subunit. Using a Swiss-PDB viewer (Guex and Peitsch, 1997), the three-dimensional structure of yeast RNA Pol II (Protein Data Bank accession number 1R9R) was visualized (Figure 13). It is predicted that, in the structure of an yeast RNA polymerase II-transcribing complex in the post-translocation state with a vacancy at the growing end of the RNA-DNA hybrid helix (Westover, et al., 2004), the Rpb1 Gly615 (the corresponding amino acid of RPC-1 Gly644) is 32.72Å away from the Mg^{++} at the catalytic center; whereas the Rpb1 Gly1073 (the corresponding amino acid of RPC-1 Gly1055) is 19.35Å from the Mg^{++}.

***rpc-1* mutations in hemizygotes**

To evaluate the severity of *rpc-1* mutations, the hemizygous phenotypes of *rpc-1* mutants were scored. First, a chromosome deficiency strain was used to generate *rpc-1* hemizygotes. Since the *stDf8* deficiency covers the *rpc-1* locus and *unc-24* locus, *rpc-1 unc-24* / *stDf8* was assumed to be the *rpc-1 unc-24* hemizygous genotype. The *unc-24* phenotype was used as a marker of the linked *rpc-1* mutations (Figure 14). It was shown that both homozygotes and hemizygotes arrested development at the L3 stage. The *rpc-1(m654) unc-24* homozygous mutant was slightly bigger and darker than the hemizygote, *rpc-1(m654) unc-24* / *stDf8*, whereas *rpc-1(s1139) unc-24* homozygous mutant was essentially the same as the corresponding hemizygote. These results suggest that *rpc-1(s1139)* mutation is null (no residual activity), whereas *rpc-1(m654)* is not completely null.

Figure 11 Aligment results of the RPC-1 sequence with its orthologs. The RPC-1 sequence is aligned with Pol III largest subunits of *Homo sapiens, S. cerevisiae, Arabidopsis thaliana* and *Rattus norvegicus*, using Clustal method with PAM250 residue weight table (MegAlign program from DNASTAR, Madison, WI). Part I shows the alignment of the RPC-1 sequence around Gly644, which is mutated in the allele *m654*; and Part II shows the alignment of the RPC-1 sequence around Gly1055, which is mutated in the allele *s1139.* Invariant residues are boxed. Mutations in both alleles are indicated. Numbers to the left of sequences indicate positions of the beginning amino acid residues in corresponding consequences.

Part 1


E
m654
625 - - - L E L C S K D S Y V I I R N S V L L A G C L D K S - - C. elegans
629 - - - E D L C A N D S Y V T I Q N S E L M S G S M D K G - - Homo sapiens
640 S L P N E M S Q N D G F V I I R G S Q I L S G V M D K S - - S. cerevisiae
597 - F D E T M C I N D G W V Y F R N S E L I S G Q L G K A T L Arabidopsis thaliana
629 - - - E D L C T N D S Y V T I Q N S E L M C G S M D K G - - Rattus norvegicus

Part 2

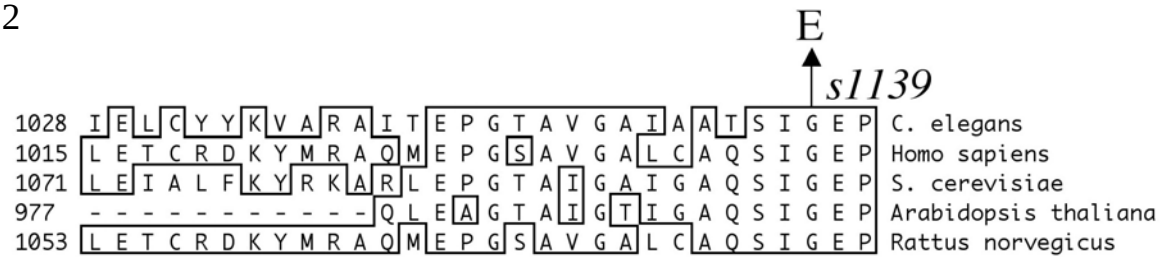
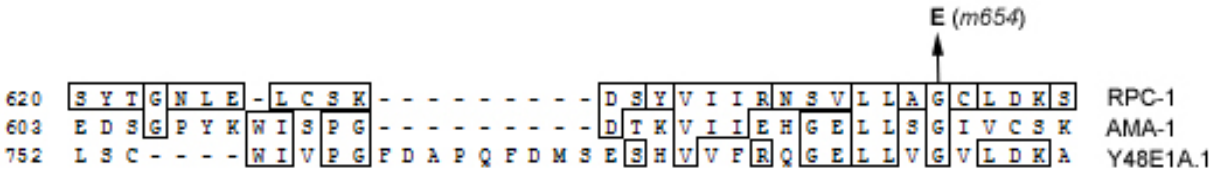

E
s1139
1028 I E L C Y Y K V A R A I T E P G T A V G A I A A T S I G E P C. elegans
1015 L E T C R D K Y M R A Q M E P G S A V G A L C A Q S I G E P Homo sapiens
1071 L E I A L F K Y R K A R L E P G T A I G A I G A Q S I G E P S. cerevisiae
977 - - - - - - - - - - - Q L E A G T A I G T I G A Q S I G E P Arabidopsis thaliana
1053 L E T C R D K Y M R A Q M E P G S A V G A L C A Q S I G E P Rattus norvegicus

Figure 12. Aligment results of the RPC-1 sequence with the largest subunits of *C. elegans* Pol I and Pol II. *ama-1* encodes the largest subunit of *C. elegans* Pol II; and Y48E1A.1 encodes the largest subunit of *C. elegans* Pol I. Invariant residues are boxed. Alignment was performed as in Figure 11. Part I shows the alignment around Gly644; and Part II shows the alignment around Gly1055. Mutations at both positions are indicated.

Part 1


E (m654)
620 S Y T G N L E - L C S K - - - - - - - - - D S Y V I I R N S V L L A G C L D K S RPC-1
603 E D S G P Y K W I S P G - - - - - - - - - D T K V I I E H G E L L S G I V C S K AMA-1
752 L S C - - - - W I V P G F D A P Q F D M S E S H V V F R Q G E L L V G V L D K A Y48E1A.1

Part 2

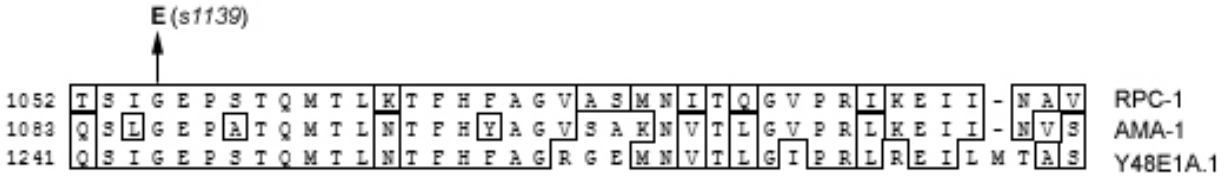

E (s1139)
1052 T S I G E P S T Q M T L K T F H F A G V A S M N I T Q G V P R I K E I I - N A V RPC-1
1083 Q S L G E P A T Q M T L N T F H Y A G V S A K N V T L G V P R L K E I I - N V S AMA-1
1241 Q S I G E P S T Q M T L N T F H F A G R G E M N V T L G I P R L R E I L M T A S Y48E1A.1

Figure 13. The yeast Rpb1 and Rpb2 domains in the Pol II crystal structure. Rpb1 is indicated in yellow, and Rpb2 is indicated in blue. The magenta sphere indicates the catalytic center. The brown sphere indicates the residue Gly1073. The violet sphere indicates the residue Gly615 (Protein Data Bank accession number 1R9R).

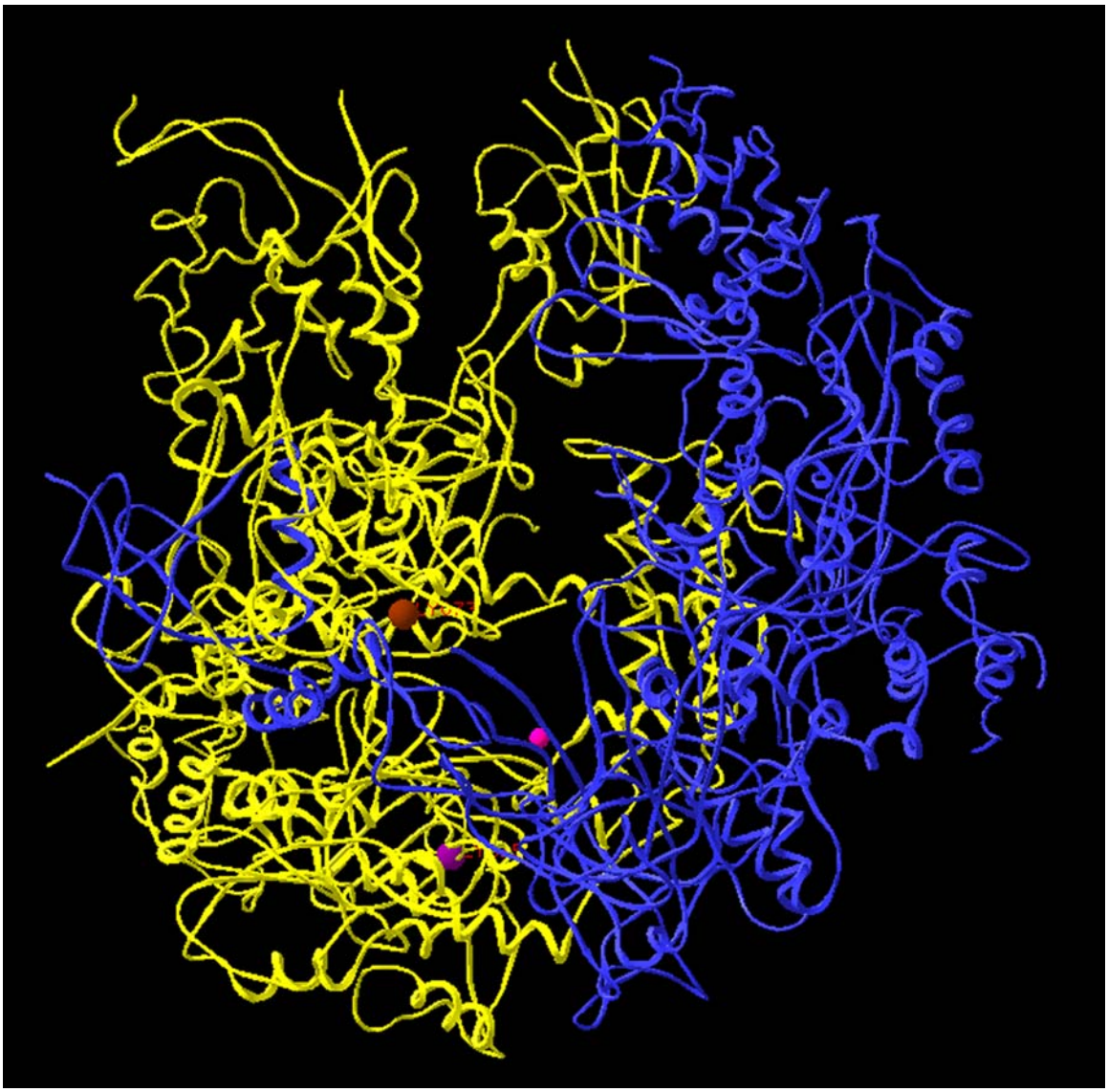
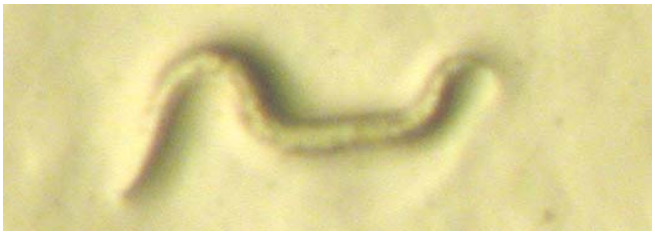


Figure 14. Hemizygote arrest of the *rpc-1 unc-24* mutants. *rpc-1 unc-24* is maintained in the heterozygote *rpc-1 unc-24/nT1(let-?)*. *unc-24* is used as the marker of *rpc-1*. Unc progeny of the *rpc-1 unc-24 / nT1(let-?)* hermaphrodite were tested as the *rpc-1 unc-24* homozygote. *rpc-1 unc-24 / stDf8* hemizygotes were obtained by crossing *fem-1 unc-24 unc-22 / stDf8* hermaphrodites with *rpc-1 unc-24 / nT1(let-?)* males (see Materials and Methods). Animals were scored 5 days after eggs were laid at 20^{o}C. 10-15 animals were scored for each genotype. Images of representative animals are taken at the same settings.

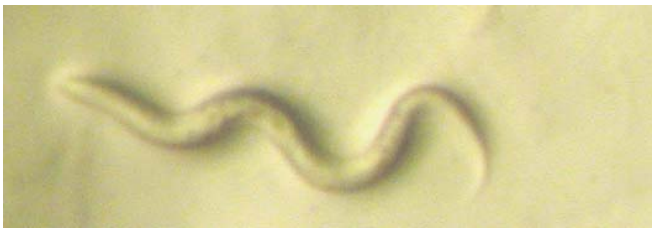
rpc-1 (m654) unc-24

rpc-1 (m654) unc-24/stDf8

rpc-1 (s1139) unc-24



rpc-1 (s1139) unc-24 / stDf8



rpc-1 RNAi treatment arrests development earlier than *rpc-1* mutations

To characterize *rpc-1* gene activities, RNAi treatment was performed using wild type N2 animals and *rrf-3(pk1426)* mutants. The *rrf-3(pk1426)* mutant strain shows enhanced sensitivity to RNAi (Simmer, et al., 2003). With *rpc-1* RNAi treatment, N2 animals were developmentally arrested at the embryo stage, the L1 stage, or the L2 stage, while *rrf-3* mutants were arrested at the embryo stage or the L1 stage (Table 3). These arrested stages are all earlier than *rpc-1(m654)* and *rpc-1(s1139)* mutation arrested stage, L3. Since the *rpc-1(s1139)* mutation was genetically shown to be null, it is possible that *rpc-1* RNAi arrested development by reducing maternally derived *rpc-1* products.

The *rpc-1* RNAi arrested animals were transferred to the normal OP50 food for 7 days at 20^{o}C. All N2 arrested animals (n=10) grew up to adulthood, but they were sterile. None of arrested *rrf-3* animals recovered, consistent with their sensitivity to RNAi treatment. In general, RNAi treatment is believed to silence gene expression in somatic cell equally to germline cells. The infertility of recovered anmals indicates that germline cell development is more sensitive to *rpc-1* expression than somatic cell development.

This is the supportive evidence that germline transformation of wild type *rpc-1* gene could only partially rescue the *rpc-1* mutant phenotype, since germline transformation can not drive transgene expression efficiently in germline cells.

Table 3. *rpc-1* feeding RNAi test (20°C).

Tested strain	Un-hatched eggs	L1 larvae	L2 larvae	Total animals tested*
N2	8.5%	68.3%	23.2%	142
rrf-3(pk1426)	11.2%	88.7%	0%	98

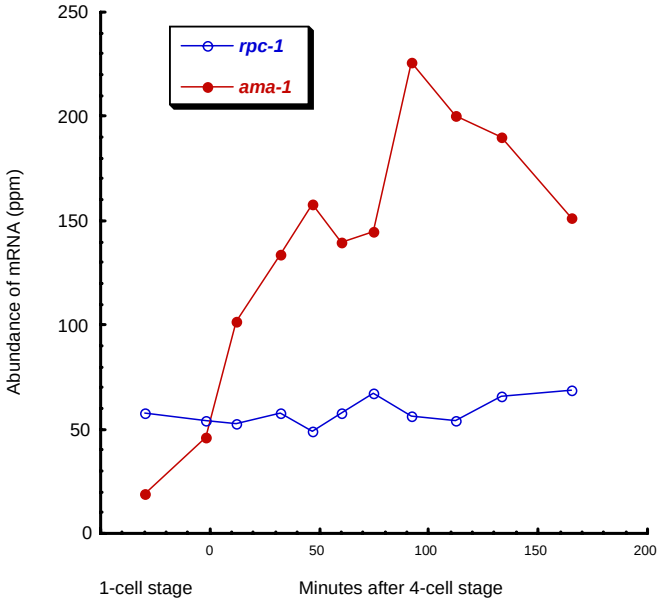
rpc-1 gene transcription may be inactive during early embryonic development

The *rpc-1* mutant phenotype and RNAi tests suggest that maternally inherited *rpc-1* gene products are essential for early development. The next question addresses the issue of maternal expression versus embryonic expression of the *rpc-1*. It is known that *rpc-1* mRNA abundance is stable through the early embryo stage at least until 165 minutes after the 4-cell stage, while *ama-1* mRNA abundance is increased with early embryo development (Figure 15). This result suggests that *rpc-1* gene transcription may be inactive during the early embryo development(Baugh, et al., 2003).

Late artificial expression of the *rpc-1* gene rescued the mutant phenotype

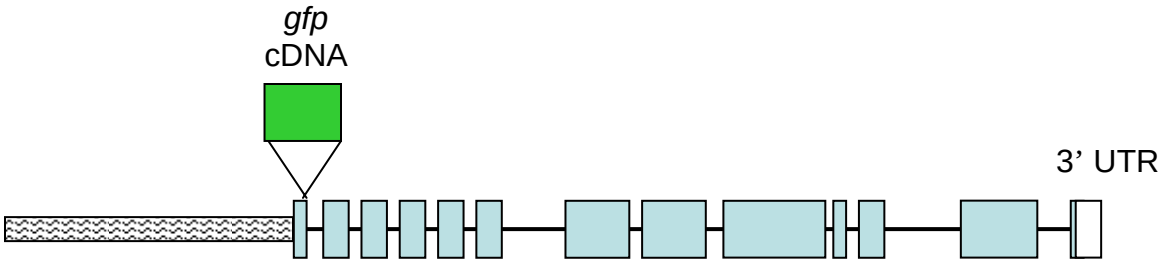
If maternally derived *rpc-1* gene products are sufficient for early development, late artificial expression of *rpc-1* should rescue the *rpc-1* phenotype. To test this hypothesis, *hsp16-41p*::*rpc-1::gfp* was constructed, and *rpc-1(m654) unc-24 / nT1(let-?); mEx166 [rol-6(su1006); hsp16-41p::rpc-1::gfp]* transgenic animals were tested. Heat-shock was used to induce gene expression (see Materials and Methods). GFP staining was detected 6-8 hours after different heat-shock treatments. No difference in rescued animals with different heat-shock treatments was observed (Table 4). To test when *rpc-1* gene expression was required to rescue the phenotype, heat-shock (30^{o}C, 1 hour) was chosen to induce temporal gene expression (Table 5, Figure 17 and Figure 18). Heat-shock, performed in a range of 12-50 hours after eggs were laid, could rescue the phenotype. However, heat-shock, performed 4-5 hours after eggs were laid or earlier, failed to rescue the phenotype (Table 5). This result suggests that late *rpc-1* gene

Figure 15. Dynamic mRNA abundance of the *rpc-1* gene and the *ama-1* gene during the early embryo stage. The *ama-1* gene was characterized as a class I (stable everywhere) maternal gene (Seydoux and Fire, 1994). Transcript abundance estimates are defined in units of parts per million (ppm) by reference to a standard curve of eleven spiked *in vitro* transcripts (Baugh et al., 2003). Data were extracted from the data base of www.mcb.harvard.edu/hunter.



Data were extracted from the data base of www.mcb.harvard.edu/hunter

Figure 16. The schematic structure of the *hsp16-41p::rpc-1::gfp* construct. *gfp* cDNA is inserted in frame at the Sfi restriction site in the first exon of the *rpc-1* gene. All exons are shown by shaded boxes, whose sizes are proportional to their individual sequence length. The 3'-UTR is shown by an open box. This construct was introduced into the *rpc-1(m654) unc-24(e138) / nT1(let-?)* strain by germline transformation to generate the *mEx166 [rol-6(su1006); hsp16-41p::rpc-1::gfp]* transgenic line (see Materials and Methods).



hsp16-41 promoter

rpc-1 genomic sequence

Table 4. Heat-shock induced expression of *hsp16-41p::rpc-1::gfp* in transgenic *rpc-1(m654) unc-24(e138) / nT1(let-?); mExIx166 [rol-6(su1006); hsp16-41p::rpc-1::gfp].*

Heat-shock treatment (30°C)	Rescue*	GFP staining		
		Intestine	Pharynx	Tail cells
1 hr	+	+	-	-
2 hr	+	++	+/-	-
4 hr	+	++	+	+
6 hr	+	++	++	++

*No difference of rescue was observed with different heat-shock treatment.

Table 5. Temporal rescue test of *rpc-1(m654) unc-24(e138) / nT1(let-?); mEx166 [rol-6(su1006); hsp16-41p::rpc-1::gfp]* transgenic animals by heat-shock treatments.

Development stage of heat-shock treated animals*	Rescue (%)	Tested transgenic animals
0 ~ 1 hr	0	10
4 ~ 5 hr	0	10
12 ~ 14 hr	100	15
20 ~ 24 hr	100	10
46 ~50 hr	100	10

*Developmental stage was defined as hours after eggs were laid.

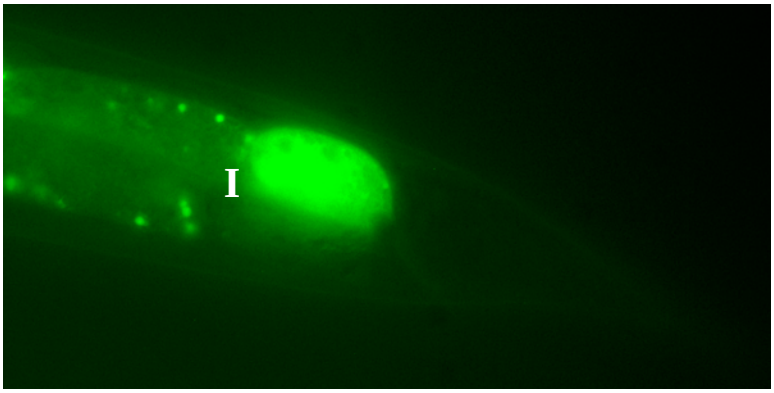
Figure 17. Heat-shock induced rescue of *rpc-1(m654) unc-24(e138)* by *hsp16-41p::rpc-1::gfp* transgene. Progeny of *rpc-1(m654) unc-24(e138) / nT1(let-?); mEx166 [rol-6(su1006); hsp16-41p::rpc-1::gfp]* transgenic animals were used for heat-shock treatment. Roller-Unc were picked up 24-36 hours after eggs were laid. *rpc-1(m654) unc-24(e138)* progeny of *rpc-1(m654) unc-24(e138) / nT1(let-?)* hermaphrodites were used as controls. Heat-shock (1 hour, 30^{o}C) was performed 12-14 hours after eggs were laid. Animals were cultured at 20^{o}C for 4 days before images were taken.

rpc-1(m654) unc-24(e138)

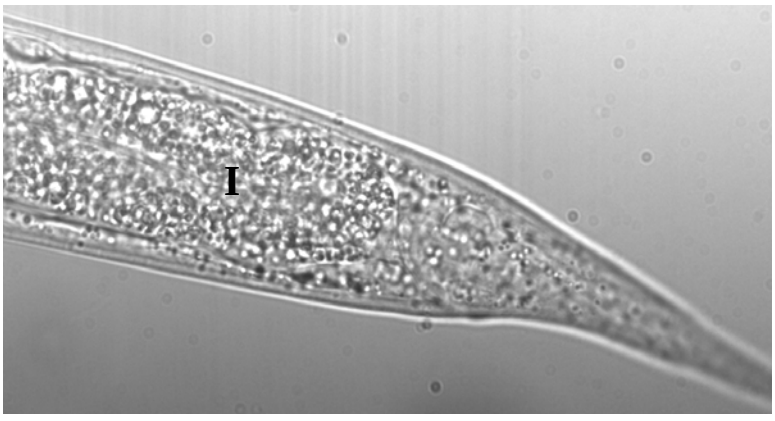
rpc-1(m654) unc-24(e138); mEx166 [rol-6(su1006); hsp16-41p::rpc-1::gfp]

Figure 18. Heat-shock induced *gfp* expression in *rpc-1(m654) unc-24(e138) / nT-1(let-?); mEx166 [rol-6(su1006); hsp16-41p::rpc-1::gfp]*. Heat-shock was performed at 30^{o}C for 1 hour. Images were taken 12 hours after heat-shock. Shown was an L3 transgenic animal. GFP staining is only observed at the posterior end of the intestine (I). (A) GFP staining in the intestine. The images was taken by krypton/argon gas laser excitation at 488 nm on a Biorad MRC-600 confocal microscope. (B) Nomarski image of the same scope in (A).

A



B



expression is necessary and sufficient to complete larval development (rescued animals reached adulthood, but were sterile).

rpc-1 promoter activity in ASK neurons under starvation conditions

GFP staining in *mEx165 [rol-6(su1006); rpc-1p::gfp]* transgenic animals was used to test *rpc-1* promoter activity. After transgenic animals were starved at 20^{o}C for 2 days, GFP staining in most tissues was damped. However, two cells were stained continuously by GFP (Figue 19), indicating that the *rpc-1* promoter in these two cells stays active under starvation conditions. These two cells are between the two pharynx bulbs, closer to the posterior one. And in starved animals, GFP staining in the cell processes toward the amphid was occasionally observed. Based on the morphology and spatial pattern, these two cells were identified as a pair of amphid neurons.

There are eight pairs of amphid neurons in this area: ASE, ADF, ASG, ASH, ASI, ASJ, ASK, and ADL. To further identify these two neurons, the GFP fluorescence image was aligned with the Nomaski microscopy of the same field (Figure 20). The GFP stained neurons were positioned at the anterior end of a trio cell pattern, which consists of ASK, ADL and ASI (Troemel, et al., 1995). The ASK cell body is the biggest of the three. By combining the cell spatial pattern with the GFP expression pattern, these two neurons were identified as the bilaterally symmetrical pair of ASK neurons.

TGF-β signaling may mediate *rpc-1* promoter activity in ASK neurons

It was observed that specific ASK staining is not present in *rpc-1p::gfp* transgenic dauers, but ASK staining appeared earlier than other tissues in dauer recovery on the

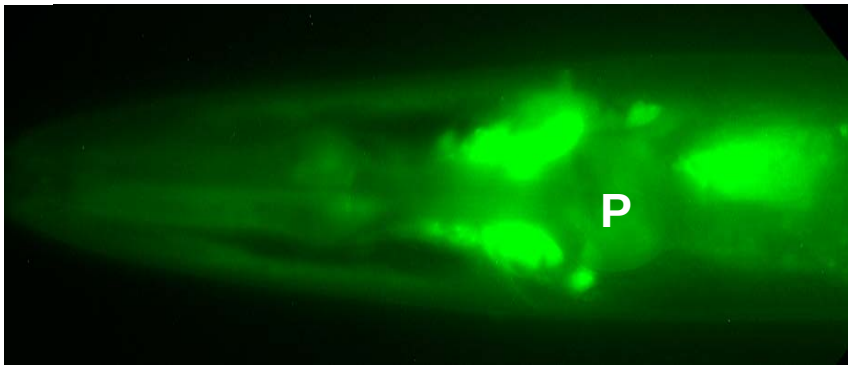
fresh OP50 food at 20^0C (data not shown). Since starvation is a factor inducing dauer formation, it is possible that the *rpc-1* promoter activity in ASK neurons is associated with dauer development. To test this assumption, I observed the *rpc-1* promoter activity in ASK neurons exposed to starvation in two *daf-c* backgrounds, *daf-7(e1372)* and *daf-2(m41)*. *daf-7* and *daf-2* are involved in two different signaling pathways in dauer development: the TGF-β signaling pathway and the Insulin-like signaling pathway, respectively. At 15^oC, transgenic *daf-2* animals showed the same ASK staining as transgenic wild-type animals, whereas ASK staining in the *daf-7* transgenic animals was damped (Table 6). This result indicates that the TGFβ signaling may be involved in mediating *rpc-1* promoter activity in ASK neurons.

To further characterize the involvement of the TGF-β signaling in this phenomenon, other related mutants in this pathway were tested (Table 6). Among these mutations, *daf-1(m40)* and *daf-4(e1364)* mutations suppress the ASK staining (*daf-1* mutation reduces the fluorescence intensity); whereas *daf-8(m85)* or *daf-14(m77)* failed to suppress, consistent with the redundancy between *daf-8* and *daf-14*. *daf-5(e1386)*, as a *daf-d* mutation, had no effect on ASK staining. These results suggest that TGF-β signaling may be involved in *rpc-1* expression in ASK neurons.

With reduced *rpc-1* promoter activity in ASK neurons of *daf-7* mutants under starvation conditions, shown by GFP staining, there is a possibility that ASK neurons are abnormal in the *daf-7(e1372)* background. To test this possibility, a *srg-8p::gfp* transgene was used as a reporter, since *srg-8* is only expressed in ASK neurons (Troemel et al., 1995). The *daf-7(e1372); mEx56 [rol-6 (su1006); srg-8p::gfp]* transgenic animals

Figure 19. Expression of *gfp* driven by the *rpc-1* promoter in response to food availability. Pharynx (P) is indicated. *mEx165 [rol-6(su1006); rpc-p::gfp]* transgenic animals were used. (A) Expression of *gfp* in a representative well-fed transgenic L3 larva. (B) Expression of *gfp* in a representative starved animal. The transgenic L3 larva was starved on an plate with no food for 2 days. Images were taken by krypton/argon gas laser excitation at 488 nm on a Biorad MRC-600 confocal microscope.

A



B

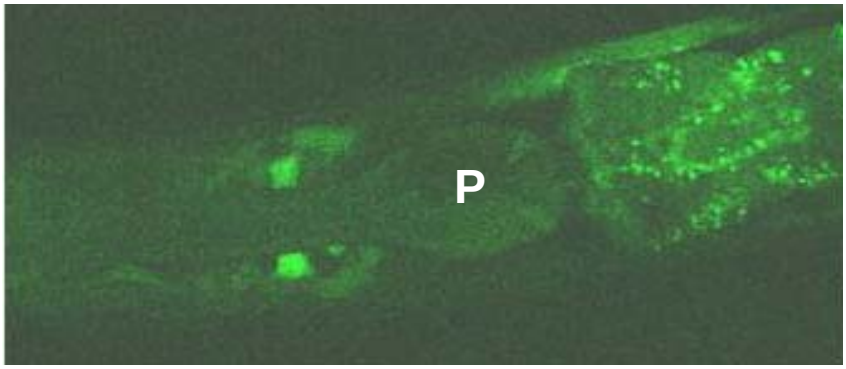


Figure 20. Identification of ASK neurons in starved animals. As shown is Figure 19 (B), a pair of cells in the head show *gfp* expression in *mEx165 [rol-6(su1006); rpc-1p::gfp]* transgenic animals under starvation conditions. The trio cell pattern of ASK, ADF and ASI is diagramed in the middle. A Nomarski interference contrast image of these three neurons is shown to the left and a combined Nomarski and fluorescence image is shown to the right.

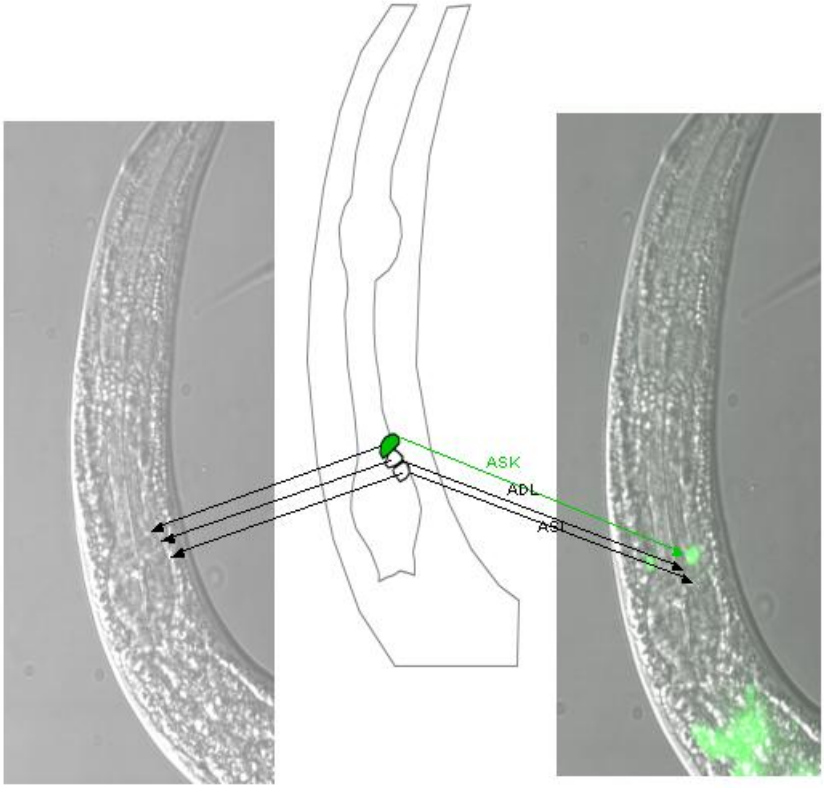

ASK
ADL
ASI

Table 6. GFP expression in ASK neurons in different mutation backgrounds under starvation conditions (15°C*).

Mutation backgrounds of *mEx165 [rol-6(su1006); rpc-1p::gfp]*	GFP expression*		Animals tested
	Detectable	Not detectable	
N2	85%	15%	20
daf-2 (m41)	75%	25%	20
daf-7 (e1372)#	25%**	75%	20
*daf-1 (m40)****	80%**	20%	20
daf-4 (e1364)#	20%**	80%	15
daf-8 (m85)	90%	10%	20
daf-14 (m77)	85%	15%	20
daf-5 (e1386)	83%	17%	18

*GFP was scored as detectable in a animal when the ASK neuron showed visually detectable GFP fluorescent signal. Percentages indicate the percent of animals.
**No difference of ASK staining between N2 and *daf-7(e1372)* was observed at 25°C.
***Fluorescence intensity is reduced in this mutant background.
#P<0.001, chi-squared test.

were generated by crossing. No abnormalities were observed in ASK neurons in this *daf-7* background (data not shown).

rpc-1 promoter activity is regulated by food availability

Using *rpc-1p::gfp*, the response to food availability was tested by scoring the GFP staining in the intestine (Figure 21). After L3 transgenic animals of genotype *mEx165 [rol-6(su1006); rpc-1p::gfp]* were starved on empty plates for two days at 20^{o}C, GFP staining in the intestine was gone. When starved animals were transferred to fresh food (OP50), GFP staining was recovered within 4 hours (Figure 21). Since formation of the GFP fluorophore takes ~4 hours at 22^{0}C in *E. coli* (Heim et al. 1994), it was inferred that food exposure to starved animals activates *rpc-1* promoter rapidly.

Amphid sensory neurons are not involved in *rpc-1* rapid response to food availability

Since the *rpc-1* promoter in starved animals responds to food availability rapidly, it is possible that this response is mediated by sensory neurons. To test this possibility, *rpc-1* promoter activity in the *daf-6(e1377)* background was observed as above. *daf-6* mutation results in closing of the amphid channel (Albert et al., 1981), i.e., mutants cannot sense environmental cues. Closure of the amphid channel in *daf-6* mutants had little effect on the *rpc-1* promoter response, indicating that this response is not medicated by amphid sensory neurons(Figure 21).

rpc-1 expression is regulated by food availability

To further characterize the *rpc-1* gene response to food availability, *rpc-1* mRNA levels of wild-type N2 animals exposed to starvation-feeding treatment were tested (see Materials and Methods). mRNA level was determined by semi-quantitative RT-PCR. It was shown that (Figure 22) feeding treatment increased *rpc-1* expression in a time-dependent pattern, while no obvious effect was found in expression of *ama-1* and Y48E1A.1. (The *ama-1* gene encodes the largest subunit of Pol II. Y48E1A.1 encodes the largest subunit of Pol I.) This result suggests a special regulation pattern of *rpc-1* gene expression by food availability.

Insulin signaling may not be involved in regulating *rpc-1* gene response to food availability

It is known that insulin/PI3-Kinase signaling plays important roles in cell proliferation, cell growth and metabolism (Taha and Klip, 1999; and Bateman and McNeil, 2004). In *Drosophila*, PI3-Kinase inhibition arrests larval development, mimicking starvation responses (Britton et al., 2002). With specific expressions of the *dilp* genes (*Drosophila* insulin-like peptides) in the brain and gut of *Drosophila* and genetic studies of other components, involvement of insulin signaling in nutritionally regulated cellular mechanisms has been proposed (Brogiolo et al., 2001). The bias of *rpc-1* promoter activity in the *C. elegans* intestine and some head/tail neurons and its response to food availability encouraged me to ask if insulin signaling is involved in regulating *rpc-1* gene expression responses. To achieve this goal, *rpc-1* gene expression

of *daf-2* mutant animals to food availability was determined by semi-quantitative RT-PCR as above.

In *C. elegans*, *daf-2* encodes the insulin/IGF receptor, mutations in which affect metabolism, development and lifespan (McElwee et al., 2004; and Gems et al., 1998). *daf-2(e1370)* was chosen as the test strain since this allele is the best characterized in which *daf-2* activity is lost at 25^{o}C. I found that *rpc-1* gene expression in *daf-2(e1370)* mutants shows the same response to food exposure as in wild-type N2 animals (Figure 23), indicating that insulin/PI3-Kinase pathway may not be involved in regulating *rpc-1* gene response to food availability in *C. elegans*. I speculate that this response may be mediated by other molecule(s) or other pathway(s).

Figure 21. Response of *rpc-1* promoter activity to food availability (20^{o}C).

mEx165 [rol-6(su1006); rpc-1p::gfp] transgenic animals and *daf-6(e1377); mEx165 [rol-6(su1006); rpc-1p::gfp]* transgenic animals were tested for food response of the *rpc-1* promoter activity by scoring GFP staining in the intestine. 20 animals were tested for each time point. L3 transgenic animals were starved on empty plates without food for 2 days. Starved animals were then exposed to OP50 bacterial food for re-feeding treatment.

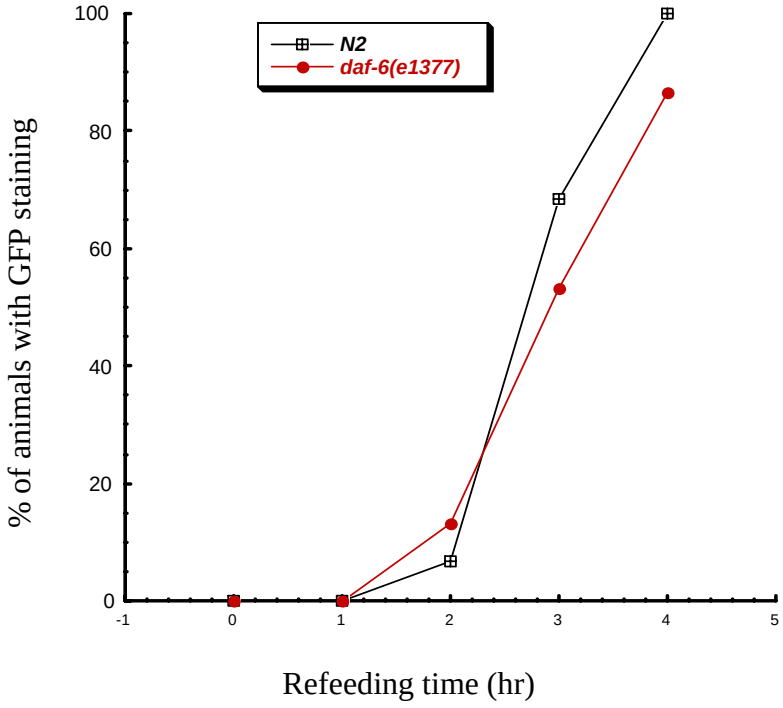

100
80
60
40
20
0
-1
0
1
2
3
4
5
N2
daf-6(e1377)
% of animals with GFP staining
Refeeding time (hr)

Figure 22. Regulation of *rpc-1* expression by food availability (20^{o}C). Wild-type N2 animals were used for testing. Re-feeding time indicates the exposure of starved L1 animals to liquid bacterial food (see Materials and Methods). Expression levels were determined by semi-quantitative RT-PCR. *rpl-21* is used as an equal loading control.

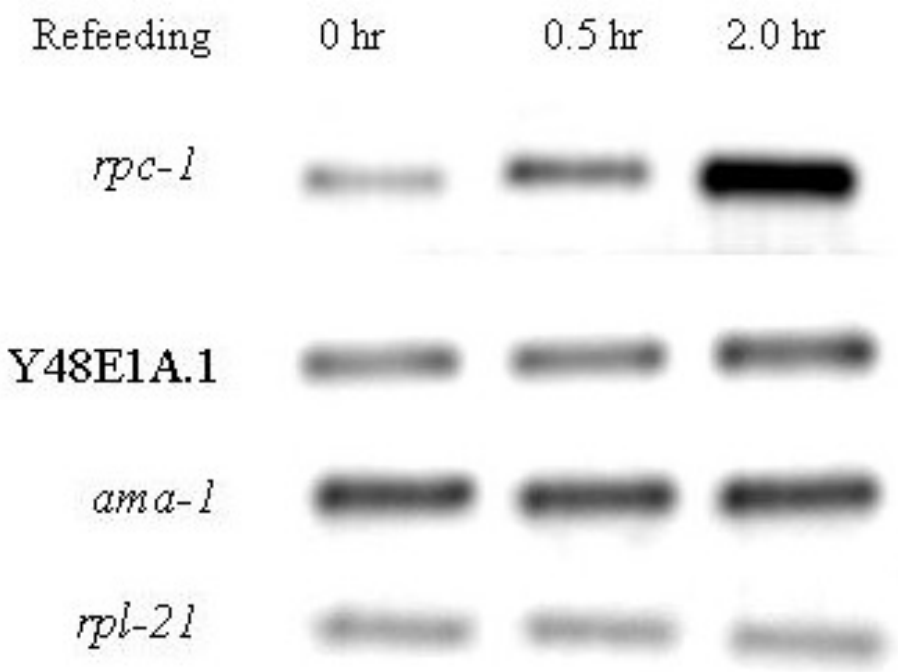
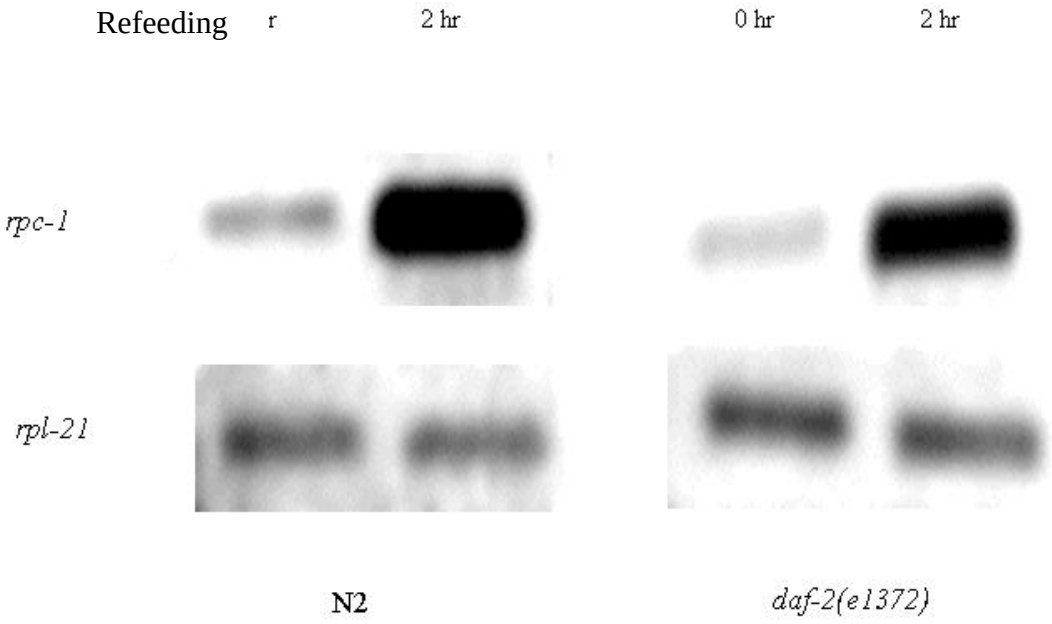

Refeeding
0 hr
0.5 hr
2.0 hr
rpc-1
Y48E1A.1
ama-1
rpl-21

Figure 23. *rpc-1* response to food availability in *daf-2(e1370)* mutants(25^{o}C). Gene expression levels were determined as in Figure 22. Animals were starved and exposed to liquid food at 25^{o}C. Animals were harvested 2 hours after re-feeding treatment.


Refeeding
r
2 hr
0 hr
2 hr
rpc-1
rpl-21
N2
daf-2(e1372)

DISCUSSION

Two critical structural components in *C. elegans* RPC-1

I have identified *daf-30(m654)* as an *rpc-1* allele, the gene encoding the largest subunit of *C. elegans* Pol III. In the *C. elegans* chromosome, there is one copy of the *rpc-1* gene. Although it was cloned before and designated as *rpc-1* (Bird and Riddle, 1989), no mutation had yet been identified in *C. elegans* prior to this work. In this study, I identified two *rpc-1* alleles with single amino acid substitutions, *m654*(G644E) and *s1139*(G1055E).

Since *s1139* appears to be a null mutation, whereas *m654* is phenotypically less severe, it is proposed that Gly1055 contributes more to Pol III activity than Gly644. This is consistent with the alignment result that RPC-1 Gly1055 is in the highly conserved region, whereas there is no highly conserved amino acid(s) around Gly644 along the peptide chain. The conserved area where Gly1055 lies is not only present in the largest subunits of Pol IIIs from different species, but also in the largest subunits of *C. elegans* Pol I and Pol II. Alignment results (data not shown) also show that Gly644 and Gly1055 are conserved in Rpb1, the largest subunit of yeast RNA polymerase II. In the yeast Rpb1, Gly615 (the RPC-1 Gly644 counterpart) lies in the channel domain of RNA polymerase; and Gly1073 (the RPC-1 Gly1055 counterpart) lies in the cleft domain, close to the Mg^{++} at the catalytic active site (Cramer, et al., 2001). Furthermore, the Rpb1 Gly1073 of the yeast Pol II is closer to the Mg^{++} than Rpb1 Gly615 in the three-dimensional structure of an RNA polymerase II-transcribing complex (Westover, et al. 2004). It is interesting that although the Gly644 lies in a less conserved region, Gly644 *per se* is universally invariant among the largest subunits of Pol IIIs from different

species and the largest subunits of all three *C. elegans* RNA polymerases. Since Gly is a small amino acid residue whereas the substitute Glu is a relatively big amino acid residue, the Gly→Glu substitution may act as a structural component to block the RNA polymerase activity by changing the conformation of the enzyme.

The lethal phenotype of *m654* and *s1139* suggests that Gly644 and Gly1055 are two critical structural components in maintaining the RPC-1/Pol III functional conformation. Further elucidation of involvement of these two residues in the RNA polymerase functional conformation should be instructive for us to understand the structural basis of gene transcription mechanism(s).

Maternal effect of the *rpc-1* gene

Since Pol III activities are required for development, especially for cell growth (reviewed by Mauger and Scott, 2004), it is interesting to note that the *rpc-1* null mutation arrests development at the L3 stage, instead of the embryo stage. The simple explanation is maternal effects of the *rpc-1* gene. It is supported by the low and flat dynamic mRNA abundance pattern of the *rpc-1* gene during early embryo development, a pattern characteristic of maternal genes (Baugh et al., 2003). An RNAi test, aimed to deprive the maternal source of *rpc-1* gene expression, showed that loss of maternal *rpc-1* products arrests animals earlier than null mutation, indicating that maternally deposited wild-type *rpc-1* gene products contribute to Pol III activity during early development. A similar phenomenon was observed with the *ama-1* gene: an *ama-1* null mutation arrests development at the L1 stage (Rogalski et al., 1988) whereas *ama-1* RNAi treatment of the mother arrested development of its progeny at the embryo stage (<100-cell stage)

(Powell-Coffman et al., 1995). Compared to the phenotype of the *ama-1* null mutation, the *rpc-1* null mutation arrests development later. Considering their general requirement in development and the dynamic expression patterns of these two genes during early development, maternal *rpc-1* products may stay functional longer than maternal *ama-1* products. Given the fact that Pol III transcripts, like tRNAs and 5sRNA, are stable, I cannot exclude the possibility that maternally inherited Pol III transcripts contribute to the *rpc-1* mutant phenotype.

Observation of the maternal phenotype of the *rpc-1* gene is supported by previous studies, showing that strongest *rpc-1* gene expression is restricted in the gonad of adult hermaphrodites (Figure 4). This spatial expression pattern allows oocytes to be fully loaded with *rpc-1* gene products during oogenesis. Actually, the assay of the *rpc-1* mRNA abundance during early embryo development failed to detect any *rpc-1* gene transcription increase (Figure 15). Interestingly, our results of heat-shock induced expression of the *rpc-1* gene suggest that early artificially-induced embryonic expression (=<4-5 hours after eggs were laid) failed to rescue the mutant phenotype, indicating that *rpc-1* expression at this stage results in no functional Pol III complex formation. It is possible that additional endogenous Pol III subunits other than RPC-1 may not be available during the early embryo stage. Without other subunits, a functional Pol III complex cannot be formed even in the presence of RPC-1 at this stage. These results are consistent with the idea that maternally derived *rpc-1* gene products contribute to Pol III activity for early development.

Regulation of *rpc-1* expression by food availability

If *rpc-1* gene expression is quiescent during early development, the next question is how it is controlled or regulated. It is known that Pol III plays an important role in cell growth-related processes by producing protein synthesis components like tRNA and 5s rRNA (Reviewed by Mauger and Scott, 2004). Overall, its activity is "turned off" during cell mitosis, and "turned on" during cell growth (White et al., 1995; and Scott et al., 2001). It has also been shown that nutrient availability is obviously one of the environmental cues of Pol III transcriptional regulation in *S. cerevisiae*. So far, mechanistic studies suggest that these responses are mediated by TFIII B and TFIII C (see introduction). However, in my study, I found that food availability regulates *rpc-1* gene expression. *rpc-1* promoter activity, silenced by starvation, rapidly responds to food exposure (Figure 21). This response is consistent with the semi-quantitative RT-PCR result: feeding of starved animals efficiently activates the *rpc-1* mRNA transcription within 2 hours.

It is known that Pol I and Pol III coordinate to promote protein synthesis by producing rRNAs and tRNAs. I expected an co-regulated expression of *rpc-1* and Y48E1A.1, which encodes the largest subunit of Pol I. However, no similar regulation of transcript abundance was observed on expression of Y48E1A.1 (Even though there is a increase of this gene transcription, it is quite marginal and not repeatable in my independent tests). Indeed, the lack of coordinated regulation of *rpc-1* and Y48E1A.1 is not surprising, given the previous observation of uncoupling of rRNA and tRNA syntheses in response to nutrient exposure. rRNA synthesis (mainly by Pol I) of starved yeast increases faster than tRNA synthesis (by Pol III), i.e., Pol I activity increases faster

than Pol III activity (Waldron, 1977; Ludwig et al., 1977; and Kief and Warner, 1981). It may be at least partially explained by our observation: compared to the mRNA abundance in two-hour fed animals, Y48E1A.1 mRNA may be sufficiently available while the *rpc-1* mRNA is much less available than necessary for growth in animals exposed to starvation. It may take time for sufficient transcription of *rpc-1* to form functional Pol III complex in response to food exposure. The mRNA availability of the Pol I largest subunit gene may account for the rapid response of Pol I activity to nutrients, relative to Pol III activity.

Insulin/PI3-Kinase signaling has been found to be involved in cell growth regulation by nutrient availability in *Drosophila* (Britton et al., 2002). However, *daf-2(e1370)* executes no effect on *rpc-1* gene expression response (Figure 23), indicating that insulin/PI3-Kinase signaling may not be involved in this response.

The differential promoter activity of the *rpc-1* gene

Given the requirement of Pol III activities in protein synthesis, the *rpc-1* gene was assumed to express ubiquitously in all tissues. Curiously, the expression pattern of the *rpc-1p::gfp* transgene, constructed to characterize *rpc-1* expression, showed that it is restricted. GFP staining is only observed in a subset of neurons in the head and tail, the intestine and occasionally in some other cells. In addition, the *in situ* hybridization data (http://nematode.lab.nig.ac.jp) suggest that *rpc-1* is strongly expressed in germline cells. Insufficiency of transgene expression from the repetitive gene extra-chromosome arrays in germline cells (Seydoux and Schedl, 2001) is thought to result in the absence of GFP staining in the gonad of the transgenic animals. So, the gonad is likely an *rpc-1*

expressing organ, even though no GFP staining is observed in transgenic animals. Failure to observe the GFP staining in other tissues of *rpc-1p::gfp* transgenic animals may result from low *rpc-1* promoter activity, whereas detectable GFP staining in the neurons and the intestine suggests that RPC-1 synthesis in these tissues is more active than other tissues. Moreover, this observation implies that these tissues are more active in metabolism, given that Pol III activity is closely involved in cell metabolism.

After *rpc-1p::gfp* transgenic animals are starved, the frequency of detectable GFP staining in most tissues and cells is reduced, indicating that this expression may be regulated by food availability. Data of the *rpc-1* gene response to food availability are consistent with this observation: starved animals show a rapid increase in *rpc-1* expression in response to food exposure (see above). The rapid response indicates that it may be mediated by sensory neurons. This assumption is supported by the *rpc-1p::gfp* expression pattern, the biased expression pattern in some head neurons, some tail neurons and the intestine. These tissues or cells are important chemosensory systems, used to acquire environmental information. It is likely that they are involved in the *rpc-1* rapid response to food availability. However, when amphid neurons are closed to the environment by the *daf-6* mutation (Albert et al., 1981), the *rpc-1* promoter of *daf-6* mutants show essentially the same response pattern as wild-type N2 animals, indicating that amphid neurons may not be involved in this response, or other tissues are involved redundantly with amphid neurons.

It is interesting to note that, while GFP staining in most tissue of the starved *rpc-1p::gfp* transgenic animals is absent, a pair of neurons in the head is still stained by GFP. These two neurons were identified as ASK neurons, based on the cell body position.

ASK neurons have been found to promote dauer formation (Schackwitz et al., 1996) and influence life span (Alcedo and Kenyon, 2004). It is possible that they regulate dauer formation and life span by sensing environmental cues. My observation of the GFP staining in ASK neurons under starvation conditions may suggest that ASK neurons are still active while other head neurons are silenced in starved animals. ASK neurons have been shown to function as gustatory neurons that sense food or its metabolic products, such as amino acids, in the environment (Bargmann and Horvitz, 1991). The ASK activity may keep starved animals responsive to environmental feeding cues: once the food is available, animals start feeding quickly. The involvement of ASK neurons in sensing food availability is supported by the observation that, during dauer recovery of transgenic animals, GFP staining in ASK comes out first over other tissues (data not shown). This temporal expression pattern may indicate that ASK neurons are involved in the early phase of dauer recovery, possibly by controlling feeding behavior.

Since ASK neuronal activity promotes dauer formation and influences life span, I decided to observe effects of the TGF-β signaling and Insulin signaling on *rpc-1* expression in ASK neurons. Previous studies showed that ASK ablation shortens the lifespan of *daf-2* mutants. In this study, I did not observe the effect of Insulin signaling on *rpc-1* promoter activity in ASK neurons of starved *daf-2(m41); mEx165 [rol-6(su1006); rpc-1p::gfp]* animals, indicating that the Insulin-like pathway may not be involved in regulating *rpc-1* expression in these cells. Interestingly, ASK expression of the *rpc-1p::gfp* transgene in *daf-7(e1372)* mutants is silenced when animals are starved at 15^{o}C. Observations of *rpc-1p::gfp* transgene expression in other TGF-β signaling mutants suggest that this signaling may be involved in mediating *rpc-1* expression in

ASK neurons. Further investigation of the functional significance of this regulation will be intriguing.

SUMMARY

Above all, in this study, I identified two amino acid residues critical for function in RPC-1, the largest subunit of *C. elegans* Pol III. Since these two residues are universally conserved in largest subunits of RNA polymerases, characterization of their involvement in the transcription complex will contribute to our understanding of the structural basis of gene transcription mechanisms by all three RNA polymerases in eukaryotes. It is also suggested that maternally derived products of the *rpc-1* gene are responsible for early embryonic development of *C. elegans*. Expression of *rpc-1* is regulated by food availability whereas genes of largest subunits of *C. elegans* Pol I and Pol II were not found to be regulated. Insulin/PI3-Kinase signaling may not be involved in this response.

In addition, the *rpc-1* gene promoter is not equally active in all tissues. While starvation silences the promoter activity in most tissues, the *rpc-1* promoter constitutes to be active in amphid ASK neurons. TGF-β sinaling may be involved in mediating the *rpc-1* promoter activity in these cells.

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