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Control of DAF-7 TGF-β expression and neuronal process development by a receptor tyrosine kinase KIN-8 in *Caenorhabditis elegans*

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SUMMARY

KIN-8 in *C. elegans* is highly homologous to human ROR-1 and 2 receptor tyrosine kinases of unknown functions. These kinases belong to a new subfamily related to the Trk subfamily. A *kin-8* promoter::*gfp* fusion gene was expressed in ASI and many other neurons as well as in pharyngeal and head muscles. A *kin-8* deletion mutant was isolated and showed constitutive dauer larva formation (Daf-c) phenotype: about half of the F_1 progeny became dauer larvae when they were cultivated on an old lawn of *E. coli* as food. Among the cells expressing *kin-8::gfp*, only ASI sensory neurons are known to express DAF-7 TGF-β, a key molecule preventing dauer larva formation. In the *kin-8* deletion mutant, expression of *daf-7::gfp* in ASI was greatly reduced, dye-filling in ASI was specifically lost and ASI sensory processes did not completely extend into the amphid pore. The Daf-c phenotype was suppressed by *daf-7* cDNA expression or a *daf-3* null mutation. ASI-directed expression of *kin-8* cDNA under the *daf-7* promoter or expression by a heat shock promoter rescued the dye-filling defect, but not the Daf-c phenotype, of the *kin-8* mutant. These results show that the *kin-8* mutation causes the Daf-c phenotype through reduction of the *daf-7* gene expression and that KIN-8 function is cell-autonomous for the dye-filling in ASI. KIN-8 is required for the process development of ASI, and also involved in promotion of *daf-7* expression through a physiological or developmental function.

Key words: Receptor tyrosine kinase, Dauer larva formation, TGF-β, ROR family, Neuroendocrine signaling, *Ceanorhabditis elegans*

INTRODUCTION

Many kinds of receptor-type tyrosine kinases (RTKs) have been found in animals (Hanks et al., 1988) and have thus been placed into a large family. Some of them are well defined as cell surface receptors for specific protein ligands, which control cell proliferation and differentiation. For a large part of them, their functions and ligands in vivo are still unknown. Elucidating the functions of those orphan RTKs may help to reveal novel intercellular signaling pathways important in development or physiology. For this purpose, the soil nematode *C. elegans* may be one of the best model animals. In this organism, a reverse genetic method was developed for any gene in general (Zwaal et al., 1993).

The nematode *C. elegans* develops to adulthood through four larval stages (L1-L4) under favorable conditions (Brenner, 1974). Under less favorable conditions, *C. elegans* forms a special third stage larva, called a dauer larva, which is nonfeeding, developmentally arrested and resistant to harsh conditions (Cassada and Russell, 1975). The dauer-inducing pheromone, food supply and temperature are important environmental signals known to influence dauer formation (Golden and Riddle, 1982; Golden and Riddle, 1984a and, 1984b). A high ratio of the pheromone to food favors the induction of dauer formation. A high temperature also promotes dauer formation. The pheromone is a stable, non-volatile, and fatty acid-like compound or a family of compounds, which is produced at all stages in the life cycle of the animal (Golden and Riddle, 1982). The concentration of dauer pheromone thus reflects the population density.

These environmental signals are perceived by sensory neurons (Bargmann and Horvitz, 1991). Those sensations are thought to couple with a transforming growth factor β (TGF-β) signaling pathway (Ren et al., 1996) and probably with an insulin-related signaling pathway (Kimura et al., 1997). DAF-7 (a TGF-β homolog; Ren et al., 1996), DAF-4 (a type II TGF-β receptor; Estevez et al., 1993), DAF-1 (a type I TGF-β receptor; Georgi et al., 1990) and DAF-3 (a Smad protein; Patterson et al., 1997) are known components in the TGF-β pathway. DAF-2 (an insulin receptor homolog, Kimura et al., 1997), AGE-1 (a phosphatidylinositol-3-OH kinase; Morris et al., 1996) and DAF-16 (a fork head transcription factor; Ogg et al., 1997) are identified members in the insulin-related pathway. Mutations in *daf-7*, *daf-4*, *daf-1*, *daf-2* and *age-1* genes leading to loss or reduction of their functions cause dauer formation even under favorable conditions (constitutive dauer formation, or Daf-c phenotype). Therefore, these two pathways are thought either to enhance normal development or to inhibit

dauer formation. *daf-7* is expressed specifically in the ASI chemosensory neuron pair and its expression is regulated by environmental signals (Ren et al., 1996; Schackwitz et al., 1996), which suggests that ASI links the sensation of the environmental signals and the TGF-β pathway.

In this report, a *C. elegans* RTK gene *kin-8* was identified that encodes a member of an orphan RTK subfamily including human and *Drosophila* RORs (Masiakowski and Carroll, 1992; Wilson et al., 1993). In addition, a *kin-8* knock-out animal was produced. The phenotypes of the mutant, partially constitutive dauer formation, reduction of *daf-7* gene expression and detachment of sensory processes of ASI from the amphid provide the first genetic evidence suggesting neurodevelopmental and possible neurophysiological functions of this RTK subfamily.

MATERIALS AND METHODS

Strains and genetics

Worms were cultivated on NGM agar plates seeded with OP50 strain of *E. coli* at 20°C except when indicated (Brenner, 1974). The following strains were used in this work: wild-type *C. elegans* variety Bristol strain (N2), MT3126 *mut-2(r459)* I; *dpy-19(n1347)* III, SP637 *unc-4(e120) mnDf68/mnC1 dpy-10(e128) unc-52(e444)* II and CB3775 *dpy-20(e2017)* IV, DR40 *daf-1(m40)* IV, GR1311 *daf-3(mgDf90)* X.

Cloning and structural analysis of *kin-8* gene and cDNAs

Cloning and molecular manipulations of DNA and RNA were performed essentially according to Sambrook et al. (1989). 3.9×10^4 plaques of an EMBL4 genomic library constructed from *Mbo*I partial digests of *C. elegans* N2 DNA (a gift from C. Link) were screened with 0.75 kb *Eco*RI-*Pvu*II fragment of *v-ros* oncogene (Neckameyer and Wang, 1985) under low stringency conditions. Nylon membrane filters (Biodyne A) were prehybridized at 42°C for 3 hours in 20% formamide, 6× SSC, 5× Denhardt's, 0.2% SDS, 500 µg/ml salmon sperm DNA. Then about 0.5 µg ^{32}P-labeled probe DNA was added and hybridization was performed under the same conditions for about 24 hours. Thereafter, the filters were washed at 42°C for 3 hours with 3× SSC, 0.1% SDS, and exposed to X-ray film (Kodak X-OMAT AR) for 24-40 hours with an intensifying screen. A gene in one clone, NGros04, was mapped on cosmids C25D8 and D2013, and named *kin-8* by A. Coulson and J. Sulston (MRC, England). About 7×10^5 plaques from an oligo dT primed cDNA ZAP library (Barstead and Waterston, 1989) were screened with an insert DNA of NGros04. Seventeen clones were obtained. Among them, Cros26 had 2.3 kb of insert cDNA toward the 3′ end. By screening a ZAP-II phage library of cDNA reverse transcribed from N2 mRNA by using an oligo DNA primer PK8-5 (5′-agaattcataagttctggga, antisence to the 7th exon), a clone 8C115-4-1, which had a 5′ end of the cDNA extended to the initiation methione in the first exon, was obtained (corresponding to the type 1 mRNA). By 5′-RACE method using primers 5′-gtcgccgacacggtatt and 5′-gttctgatacagcgaca (antisense to the 5th exon), 0.6 kb and 0.55 kb products were obtained. The former contained a type 1 cDNA, SL1 sequence followed by aaATGTCT... (sequences from the exon 1 to exon 5). The latter contained a type 2 cDNA, SL1 sequence followed by atttctttcgttaactttcattttgtctcag (3′ end of intron 1 of the type 1 transcript) and aATGGAG...(a sequence from the exon 2 to the exon 5 of the type 1 transcript). The sequences of about 15 kb genomic DNA covering the entire coding region of the *kin-8* and the cDNAs mentioned above were determined.

Construction and expression of *gfp* fusion constructs

Two kinds of transcriptional gfp fusion constructs, pK8ETB-gfpE and pK8I1-gfpE were constructed by inserting a 1st promoter region (a 5.6 kb *Eco*T22I-*Nae*I fragment derived from C14B2 cosmid and a DNA from *Nae*I into the nucleotide just before the first initiation codon plus a *Bam*HI site produced by PCR) and a 2nd promoter region (a 4.8 kb *Eco*T22I-*Sac*I fragment derived from C25D8 and a DNA from *Sac*I to the nucleotide just before the 2nd initiation codon plus *Bam*HI site produced by PCR) between the *Pst*I and *Bam*HI sites of a GFP expression vector pPD95.77gfpE, respectively. pPD95.77gfpE was made from pPD95.77 (A. Fire, J. Ahnn, G. Seydoux and S. Xu, personal communication) by introducing F64L and S65T mutations in GFP (T. Ishihara, personal communication). The sequences derived from the PCR products were confirmed to have no mutations. A translational GFP fusion, p95.75-K8RV1 was constructed by ligating a 12.7 kb *Eco*RV fragment (from the 983th nucleotide of the first intron to the first nucleotide of the codon for Y896) derived from C25D8 into the *Sma*I site of a GFP expression vector pPD95.75 in frame (A. Fire, J. Ahnn, G. Seydoux and S. Xu, personal communication).

Transgenic lines were produced by the method of Mello et al. (1991). 50 ng/µl of pK8ETB-gfpE, 50 ng/µl of pK8I1-gfpE or 20 ng/µl of p95.75-K8RV1 was injected along with 50 ng/µl of pMH86 *dpy-20(+)* marker into *dpy-20* animals. GFP expression was observed in transgenic animals using fluorescence microscopy. At least two transgenic lines for each expression construct were obtained and they showed the same expression pattern. Neurons that express GFP were identified by monitoring the cell body positions in mainly L1 and L2-L4 animals using Normarski optics and by the characteristic axon morphology for several neurons. The position of the cells in *C. elegans* has been described by Sulston et. al. (1983) and White et. al. (1986). Transgenic animals each carrying *ttx-3p::gfp*, *unc-86p::gfp* or *daf-7p::gfp* were observed as references for the identification of the AIY, AIZ and ASI neurons, respectively (Hobert et al., 1997; Baumeister et al., 1996; Ren et al., 1996).

Isolation of a *kin-8* knock-out animal

kin-8(ks52) was generated by the insertion and imprecise excision of the transposon *Tc1* (Zwaal et al., 1993). *Tc1* and *kin-8* specific primers (PK8-9: 5′-aagaagcatcctaatca and PK8-10: 5′-gaatcaccatcctctga) were used to screen 384 frozen stocks of the mutator strain MT3126 by PCR. One strain with a *Tc1* insertion within the 7th exon of *kin-8*, FK162 *kin-8(ks51)* was isolated. 524 cultuers of FK162 on 6 cm or 3.5 cm NGM plates were screened for imprecise excision of the *Tc1* by PCR using sets of *kin-8*-specific primers (PK8-12: 5′-tgcggtgaaattgtcac, PK8-13: 5′-gaaaggatcacatcact, PK8-9, and PK8-10) that are about 3 kb apart from each other in the intact genome across the *Tc1* insertion site. One line of animals with a 1008 bp deletion *(ks52)* was isolated. The animal was back-crossed with wild-type N2 animal three times, then the FK163 *kin-8(ks52)* strain was established. The *Tc1* insertion site and the deletion sites were determined by sequencing the PCR products.

Dauer formation assay

In the experiments of Fig. 4 and Table 2, one to a hundred adult hermaphrodites were placed and allowed to lay eggs for one day usually or for about half a day for the heat induction experiments, on a 6 cm NGM agar plate with *E. coli* OP50. The agar plate was made as follows. About 20 µl of an over-night culture of OP50 in LB broth was spread over about one third of the surface of the plate with a pipette, then the plates were left in a plastic container at a room temperature for 2 days (new lawn) or more than 2 weeks (old lawn). After the parental animals were removed, plates were incubated either for 2 days at 25°C, for 3 days at 20°C or for 4 days at 15°C. Then, F_1 progeny were counted and evaluated to be either dauer or non-dauer (L3 to adult). The heat shock was given immediately for induction in embryos at 33°C for one hour, or 13-14 hours after removing the parents, for induction in the L1 stage. *kin-8/mnDf68* animals were produced as F_1 progeny of *unc-4 mnDf68/mnCI* hermaphrodites mated with *kin-8/+* males. About 200 F_1 animals were

sampled and their genotypes were examined by single-worm PCR (Williams et al., 1992). One quarter of adult or L4 animals and almost all of dauer larvae were *kin-8/mnDf68* hemizygotes.

Rescue experiments

pK8-22.3 (see Fig. 1) is a 22.3 kb *Eco*T22I-NheI genomic DNA of *kin-8* subcloned in pBluescript SK(+) (Stratagene), which was constructed from restriction fragments of cosmids C25D8 and D2013 via a series of subcloning. A kinase-negative kin-8 gene clone, pK8-22.3-K-M was produced by PCR and a series of subcloning, which is the same as pK8-22.3 except for the substitutions of lysines 624 and 625 for methionine in the subdomain II of the kinase domain. The region derived from the PCR product was examined by DNA sequencing. 30 ng/μl of pK8-22.3 or pK8-22.3-K-M was injected along with 30 ng/μl of pK8I1-gfpE (as a marker) and 60 ng/μl of pBluescript SK(+) (as a carrier) into FK163 *kin-8(ks52)*. For pK8-22.3, nine transformant lines with an extrachromosomal array and one line of spontaneous chromosome-integrated transformant FK167 *kin-8(ks52); ksIs1[pK8-22.3, pK8I1-gfpE, pSK(+)]* was obtained.

daf-7::gfp expression

The pdaf7-gfpE transcriptional fusion was constructed by subcloning a *daf-7* promoter region (Ren et al., 1996), 3.1 kb DNA from *Xba*I, to the nucleotide just before the initiation codon plus a *Bam*HI site produced by PCR, into pPD95.77gfpE. By injecting 50 ng/μl of pdaf7-gfpE along with 50 ng/μl of a marker plasmid, pRF4, carrying a dominant *rol-6(su1006)* mutant gene, into a wild-type N2, a roller (Rol) transformant, FK168, carrying an extrachromosomal array *ksEx26[pdaf7-gfpE, pRF4]* was obtained. The males of heterozygous *kin-8(ks52)/+*, produced by crossing FK163 hermaphrodites with N2 males, were crossed with a FK168 Rol hermaphrodite. Ten F_1 Rol animals were then selected and cultured individually. After allowing them to lay eggs for 3 days, the F_1 animals were examined by PCR for *kin-8(ks52)/+*. About 30 F_2 Rol hermaphrodites from a single F_1 hermaphrodite *kin-8(ks52)/+; ksEx26* were picked and cultured individually. By examining the F_2 and F_3 by PCR, three lines of *kin-8(ks52)/kin-8(ks52); ksEx26* (named C81-2, 7 and 15) and three lines of *+/+; ksEx26* (named C81-1c, 5c and 9c) were selected. Five young adult Rol hermaphrodites of each C81 line were cultured on a 2-day-old or a 2-week-old plate for 3 days at 20°C. About a hundred of the F_1 Rol animals at L2 stage were selected randomly and scored for the intensity of fluorescence of GFP under a Leica MZ-APO stereomicroscope equipped with a fluorescence apparatus.

cDNA expression constructs and DiI staining

daf-7 cDNA was produced by RT-PCR. pK8ETB-daf7c was made by replacing the *gfp* region with the *daf-7* cDNA in pK8ETB-gfpE. pdaf7p-K8c was made by replacing the gfp region with a full length cDNA for the type1 *kin-8* mRNA in pdaf7-gfpE. phs-K8c and pmec7-K8c were constructed by inserting the *kin-8* cDNA under the *hsp16-2* heat shock promoter in pPD49.78 and the *mec-7* promoter in pPD52.102, respectively (Mello and Fire, 1995). pK8ETB-daf7c (50 ng/μl) was injected with pK8I1-gfpE (50 ng/μl), as a marker, into FK163 *kin-8(ks52)* or DR40 *daf-1(m40)* to obtain the transformants shown in Table 2A. pdaf7p-K8c, phs-K8c and pmec7-K8c (33 ng/μl) were injected with two markers, pmec7-gfp and pdaf7-gfpE (33 ng/μl each), into FK163, respectively. The transformants were used in the experiments shown in Table 2C and Fig. 8. In Fig.8, animals were placed in M9 buffer with10 ng/ml DiI (Molecular Probes) for 2-3 hours at room temperature, then washed with M9 buffer and allowed to crawl on a bacterial lawn for several hours. Then, the animals were examined under the fluorescence microscope for DiI uptake in ASI. The *gfp* expression from pdaf7p-gfpE was useful for identification of ASI. Heat induction was performed in the same way as in the experiments shown in Table 2C.

RESULTS

kin-8 encodes a receptor tyrosine kinase

A *C. elegans* genomic library was screened with a 0.75 kb *Eco*RI-*Pvu*II fragment of the oncogene *v-ros* encoding the tyrosine kinase domain (Neckameyer and Wang, 1985). One of the six classes of hybridizing genes obtained after such screening was named *kin-8*, the 8th kinase gene in *C. elegans*. Genomic and cDNA sequences of *kin-8* revealed that it produced two types of transcripts (types I and II), both of which were trans-spliced to a splicing leader SL1 (Huang and Hirsh, 1989) (Fig. 1). The type II transcript lacks the first exon encoding 26 amino acid residues (see Fig. 2). Since SL1 is thought to be trans-spliced to an acceptor site near the 5′ end of a primary transcript (Conrad et al., 1991, 1993), two different promoter regions for the type I and type II transcripts (1st and 2nd promoters in Fig. 1) are predicted. The deduced protein products of *kin-8* exhibit the canonical features of receptor tyrosine kinases (RTKs), including a large extracellular domain, a transmembrane region and a cytoplasmic domain containing 12 subdomains that characterize a functional tyrosine kinase (Hanks et al., 1988). Among all known RTKs, KIN-8 is most highly related to ROR-

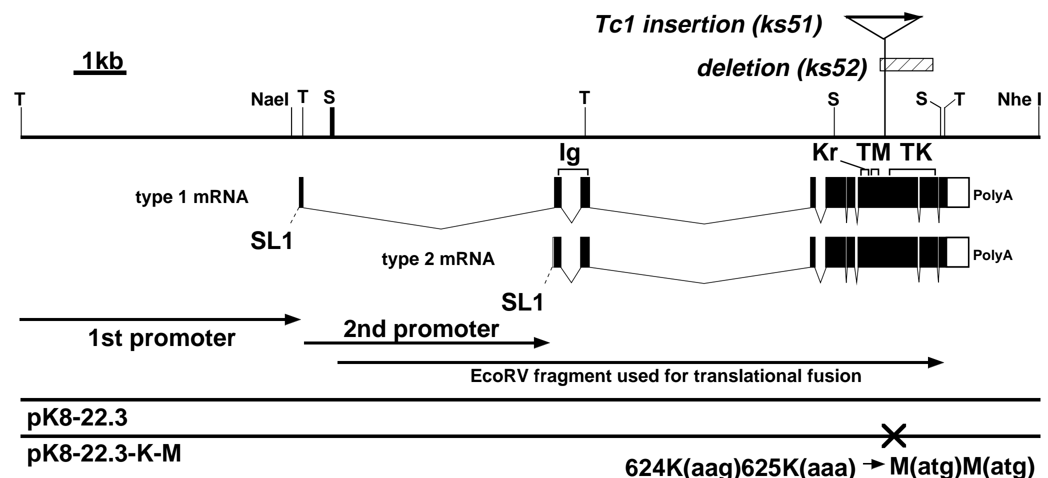

Fig. 1. Genomic organization of the *kin-8* gene. The top line represents 22.3 kb of the *kin-8* genomic region. The *Tc1 insertion* site of *ks51* and the deleted region in *ks52* (a hatched box) are indicated above the line. Solid boxes are coding exons and open boxes are non-coding exons of the *kin-8* gene. The three arrows indicate the regions used for the expression analysis. (T) *Eco*T22I; (S) *Sal*I; (Ig) immunogloblin-like domain; (Kr) kringle domain; (TM) transmembrane domain; (TK) tyrosine kinase domain; (SL1) splice-leader 1.

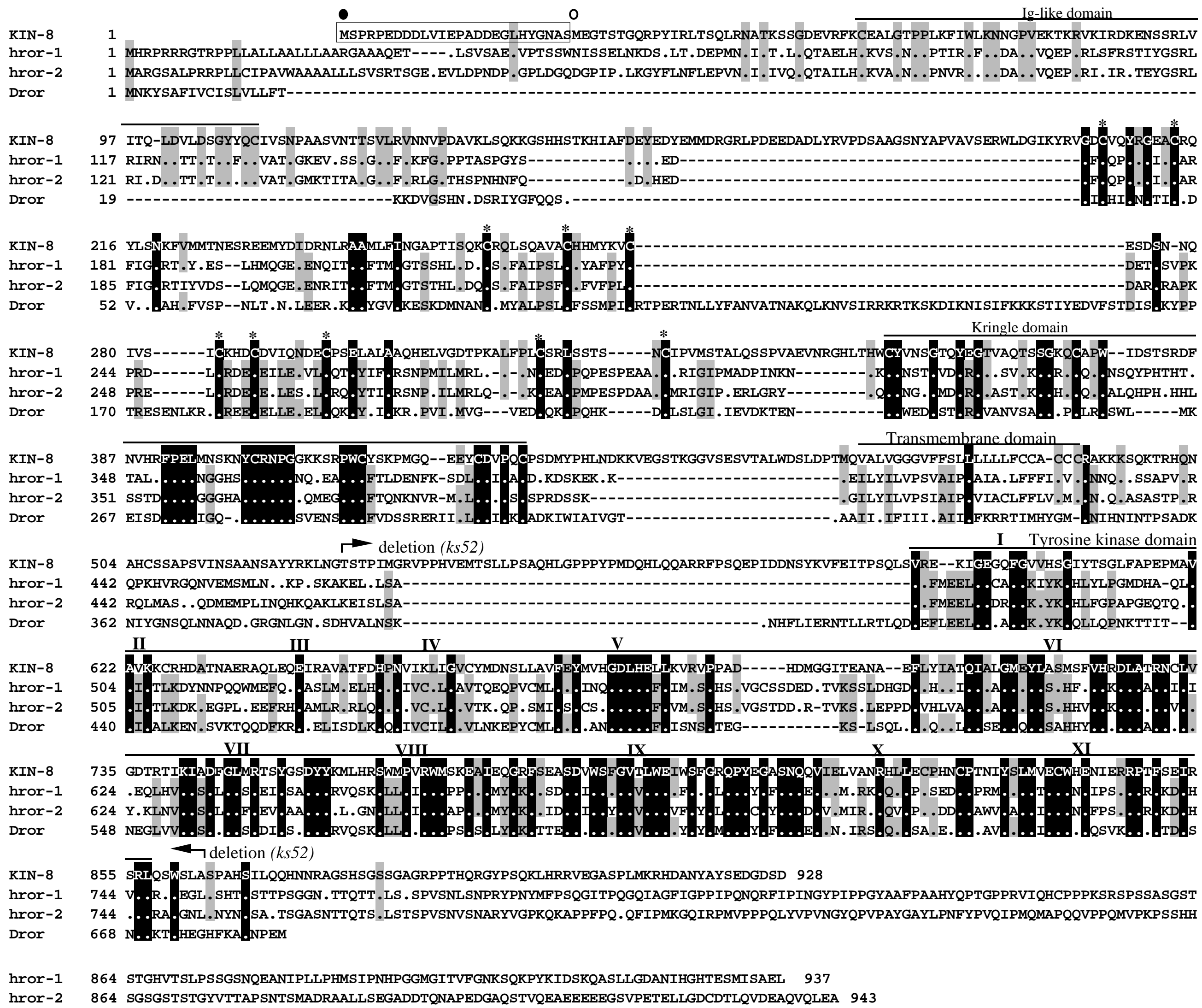

Fig. 2. The deduced amino acid sequence of the entire KIN-8 protein and comparison to those of other RORs: (hror-1 and hror-2) human ror-1 and 2; (Dror) *Drosophila* ror. Several structural features are overlined. The asterisks indicate conserved cysteines in the cysteine rich region between the Ig and kringle domains. The residues shared by all and three members are in black and shaded boxes, respectively. Dots signify the residues identical to those of KIN-8. Dashes show the spaces for alignment. The solid circle and open circle indicate the first methionines of the type 1 and type 2 products, respectively. The difference of the two types of proteins at the N terminus are boxed.

1 and ROR-2, the products of the genes cloned from a human neuroblastoma cell line (Masiakowski and Carroll, 1992) and Dror of *Drosophila* (Wilson et al., 1993) (Fig. 2). The kinase domain of KIN-8 is most similar to those of RORs, and 45.9%, 47.4% and 44.7% of the amino acids are identical to those of ROR-1, 2 and Dror, respectively, while it shows 43.3% identities to those of human MusK (Valenzuela et al., 1995), 43.1% to *Torpedo* RTK (Jennings et al., 1993), 41.3% to mouse trkB (Klein et al., 1989), 40.5% to human insulin receptor (Ebina et al., 1985; Ullrich et al., 1985), 32.1% to human EGF receptor and 26.9% to mouse PDGF receptor (Yarden et al., 1986). The extracellular domains of KIN-8 and RORs share a structural organization comprising an immunoglobulin (Ig)-like domain, a cysteine rich region with ten conserved cysteine residues and a kringle domain, which is a specific character for these RTKs (Masiakowski and Carroll, 1992; Wilson et al., 1993). Therefore, both KIN-8 and RORs form a novel subfamily of RTK. Any function in vivo of this subfamily member is unknown, although protein kinase activity was shown to be associated with the tyrosine kinase domain of ROR-2 in vitro (Masiakowski and Carroll, 1992) and selective neural expression in the embryos was observed for *Dror* (Wilson et al., 1993).

The cellular specificity of *kin-8* expression was analyzed in transgenic animals expressing a green fluorescent protein (GFP) reporter gene (Chalfie et al., 1994), under the control of each of the two *kin-8* promoters, which are regions upstream of the first exon (first promoter) or the second exon (second promoter). Fig. 3A,B shows photographs and Table 1 summarizes the results. The *gfp* expression was observed in many neurons, pharyngeal muscles, muscles in the head, and sometimes in body wall muscles, intestine, seam cells and

distal tip cells. The expression patterns did not appear to change through the larva to adult stages. Embryonic expression was also observed. Since these *gfp* fusions lack the introns and the 3′ untranslated region, they might be lacking potential regulatory sequences. In that case, the *gfp* expression patterns may not precisely represent those of the endogenous *kin-8* gene. Also, expression of a translational fusion construct for the type 2 KIN-8 protein, 33 C-terminal amino acid residues of which were replaced with GFP (Fig.1), was examined. The expression pattern was similar to that of the second promoter fusion, but the KIN-8::GFP fusion protein was localized in the axons and plasma membranes (Fig. 3C,D).

Isolation of a *kin-8* deletion mutant

To examine the *kin-8* function in vivo, a presumed loss-of-function mutant animal was identified by using a transposon-based PCR-sib selection method (Zwaal et al., 1993). A mutant with a *Tc1* insertion in the 7th exon of the endogenous *kin-8* gene was obtained (Fig. 1). Then, a deletion mutation *kin-8 (ks52)* was generated by an imprecise *Tc1* excision. This deletion eliminated all *kin-8* sequences encoding the tyrosine kinase catalytic domain (Fig. 2). A homozygous strain FK163, carrying the *kin-8 (ks52)* deletion was established by back crossing to a wild-type N2 strain three times and this was then used in the analysis described below. In spite of the broad expression of the *kin-8::gfp* fusions, the homozygote of *kin-8 (ks52)* deletion is viable and moves almost normally: approximately 10% of animals show locomotion waves with an amplitude of about 1.5 times as large as that of wild type and a few percent of animals are sometimes coiling into a spiral. Transgenic animals in which *mec-4(d)* degenerin was expressed by the first and second promoters of *kin-8* showed a weak Unc phenotype (slow movement) and an Unc phenotype (slow and kinked), respectively (data not shown). The *kin-8::gfp* transcriptional fusion constructs were introduced into *kin-8 (ks52)* animals, but the expression patterns appeared to be the same as those in wild-type animals (data not shown). These results suggest that *kin-8* is not essential for the gross development and survival of the animals, except in the development of the gonad and the posterior half of the body that are described later.

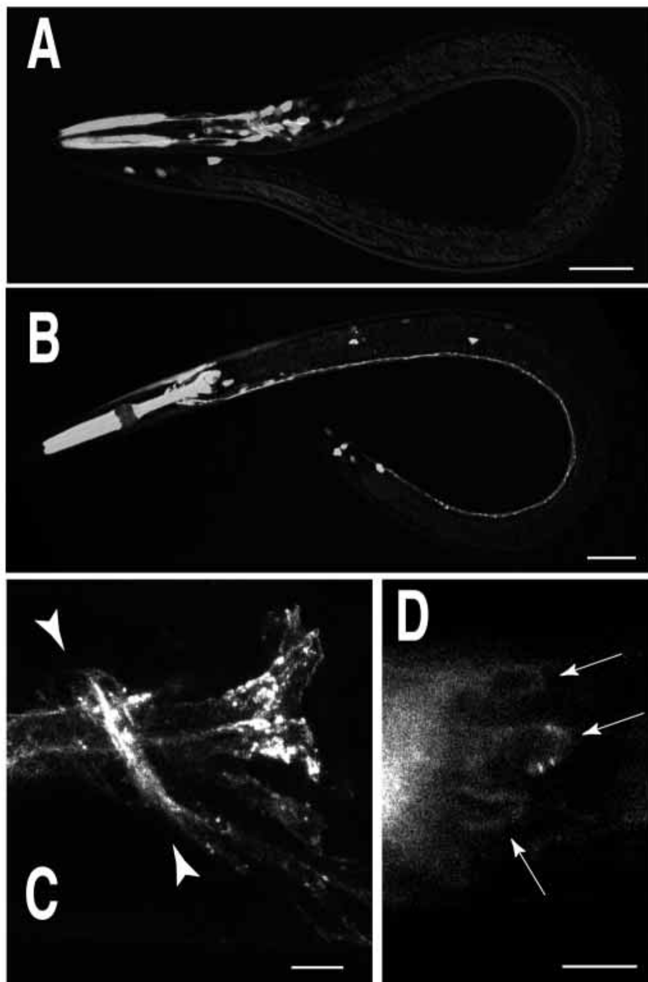

Fig. 3. The expression patterns of *kin-8::gfp* fusions. Confocal projection images of an L1 animal expressing the *kin-8* first promoter*::gfp* fusion gene (A), and an L3 animal expressing the *kin-8* second promoter*::gfp* fusion gene (B). (C,D) An L4 animal expressing the KIN-8::GFP fusion protein; A confocal projection image (C). The arrowheads indicate axons in the nerve ring. A fluorescent micrograph (D). The arrows indicate cell membranes of neural cell bodies. The confocal images were obtained with a Zeiss LSM410 confocal laser scanning microscope. Bars, 25 µm in A and B, 5 µm in C and D.

The *kin-8* deletion mutation causes a Daf-c phenotype

The homozygous *kin-8* deletion strain produced dauer larvae in a small fraction of the F_1 progeny, when they were cultivated on a new (such as 2 days old) lawn of *E. coli* as food (Fig. 4, diamonds). However, when they were cultivated on an older (such as 15 days old) lawn, about half of the population became dauer larvae, even though abundant *E. coli* food remained (Fig. 4 open circles). The production of dauer larvae seemed to be influenced to some extent by the population density. A wild-type strain, N2, did not form dauer larvae on an old or a new

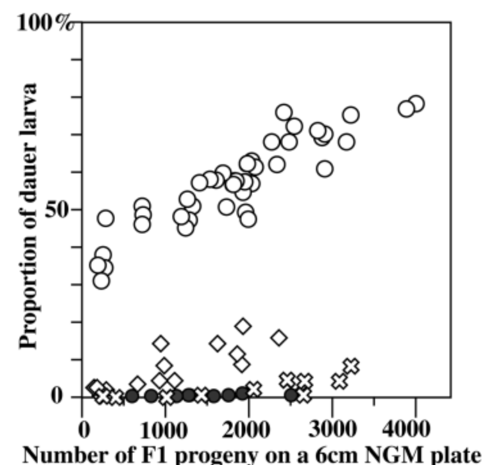

Fig. 4. Daf-c phenotype of the *kin-8* deletion mutant. Each point represents the score of a paticular plate. They were cultivated at 20°C on more than 2-week (15-18 days)-old *E. coli* lawns or on new (2-day-old) lawns. Open circles, gray circles, and crosses show *kin-8(ks52)*, *kin-8(ks52); ksIs1[kin-8(+)]* and N2, respectively, on old lawns. Diamonds show *kin-8(ks52)* on new lawns.

Table 1. Expression pattern of kin-8p::gfp transcriptional fusion genes

1st promoter
Chemosensory neurons in amphid: ADL, ASI, ASH, ASK"
Sensory neuron?: AUA
Interneurons: AIM, ADA, DVC, RIM, RIV, RMG, URX, AVE, PVT"
About 10 neurons anterior to the nerve ring in the ring ganglion
About 2 neurons in the ventral ganglion
Four muscles in the head
2nd promoter
Chemosensory neurons in amphid: ASH
Other sensory neurons: ADE, FLP"
Touch receptor neurons: AVM, ALM, PVM, PLM"
Amphid interneurons: AIY, AIZ"
Other interneurons: RIC, RMG, RIS, DVA, AVA, AVE, PVC, AVK, PVQ"
Interneurons?: ALN, BDU, SDQ"
Ring motor/inter neurons: RMD, RMDV
Ring motor neurons: RMED, RMEV
Five neurons out of the following six, RIV, AVH, AVB, AVJ, AVD, AIN
About seven neurons in retrovesicular ganglion
Pharyngeal muscles in procorpus and isthmus
M4 and several pharyngeal neurons
A part of intestine and a few body wall muscles near the head (weak)
Distal tip cells (sometimes and weak)
A few ventral motor neurons and seam cells (rarely and weak)

lawn of *E. coli* under the same conditions (Fig. 4, crosses). This Daf-c (constitutive dauer formation) phenotype of the *kin-8* deletion mutant was completely rescued by the introduction of pK8-22.3, a *kin-8* wild-type gene clone (Fig. 4, gray circles). *kin-8/+* animals did not show a Daf-c phenotype, and the Daf-c phenotype was not enhanced when *kin-8(ks52)* was placed in *trans* to the *mnDf68* deficiency deleting a chromosomal *kin-8* region (Sigurdson et al., 1984), suggesting that the *kin-8(ks52)* mutation is a null allele with regard to dauer formation (Table 2A). *kin-8(ks52)/mnDf68* animals also appeared to be the same as *kin-8(ks52)* in other phenotypes. To examine whether the kinase activity is required for KIN-8 function, a putative kinase negative clone, pK8-22.3-K-M (Fig. 1) was constructed, in which the invariant lysine critical for kinase activity in the subdomain II (Hanks et al., 1988) was substituted by methione. Surprisingly, the kinase negative clone rescued the Daf-c phenotype (Table 2A), indicating that the kinase activity is not required for KIN-8 function in dauer formation. The transformants carrying the kinase-negative *kin-8* were Unc (kinked and slow movement) in more than 80% (line no. 1 and no. 2) or about 10% (line no. 3) of animals.

The reason why the age of *E. coli* lawn influences the dauer larva formation in the *kin-8* mutant is unknown. The accumulation of some metabolite of *E. coli*, which is sensed

Table. 2 Dauer formation in various strains

Genotype		Average proportion of dauers	Total worms in a plate			
			average	min.	max.	*n**
A wild type		3%	1782.7	254	3226	12
kin-8 (ks52)		65%	1776.1	341	3220	40
kin-8; ksIs1[kin-8(+)]		2%	1771.5	304	3226	14
kin-8/+		0%	1129.0	756	1438	3
kin-8/mnDf68		34%	825.8	549	1236	5
kin-8; *Ex[kin-8(kinase-negative)]*						
line 1		1%	1182.4	716	2034	5
line 2		0.4%	1230.4	834	1824	5
line 3		2%	1141.3	660	2062	3
kin-8; Ex[daf-7cDNA]						
line 1		0%	1429.3	1265	1641	4
line 2~5		0%	977.5	636	1200	4
kin-8; daf-3(mgDf90)		3%	1769.0	1282	2288	7
daf-1(m40)	25°C	100%	180.2	92	230	6
daf-1; Ex[daf-7cDNA]						
line 1~4	25°C	99%	146.9	57	224	7
B *kin-8 (ks52)*	15°C	32%	316.4	68	656	7
kin-8 (ks52)	20°C	47%	450.6	173	867	7
kin-8 (ks52)	25°C	43%	348.7	113	795	7
C *kin-8; Ex[daf7p::kin-8cDNA]*						
line 1		73%	2661.3	1666	3670	8
line 2		72%	3450.7	2530	3986	4
kin-8; Ex[mec7p::kin-8cDNA]						
line 1		70%	2115.7	1714	2820	6
line 2		60%	2419.3	1510	3178	6
kin-8; Ex[hsp16p::kin-8cDNA]						
line 1		68%	1432.3	276	3192	20
line 2		64%	1150.8	310	2706	17
line 1	hs1	59%	2112.0	611	3088	3
line 2	hs1	57%	1812.0	820	2612	3
line 1	hs2	62%	3069.0	1762	4836	3
line 2	hs2	67%	2822.0	1638	4318	3

Animals were cultured on old lawns (about 15 days old) in a 6 cm NGM plate at 20°C if not indicated otherwise. Heat shock was given at 33°C for one hour in embryo (hs1) or L1 (hs2) stages. *n**: total number of plates used for the assay.

by *C. elegans* as a signal of an unfavorable environment, might enhance dauer formation. Being transferred to new plates, about 90% of *kin-8(ks52)* dauers and 100% of wild-type dauers recovered to a normal developmental cycle within a day. The Daf-c phenotype of *kin-8* did not show any clear temperature dependency, though the dauer production slightly decreased at 15°C (Table 2B).

DAF-7 TGF-β expression is reduced in the *kin-8* deletion mutant

Among the cells in which the *kin-8p::gfp* fusion genes were expressed, ASI seemed to join *kin-8* function and Daf-c phenotype of the deletion mutant, because ASI is the only pair of neurons expressing *daf-7* which encodes a TGF-β family protein, and because loss-of-function mutations of *daf-7* cause Daf-c phenotype (Ren et al., 1996; Schackwitz et al., 1996). Therefore, we examined whether elimination of *kin-8* activity affects *daf-7* expression. An extrachromosomal array, *ksEX26*, containing a *gfp* reporter gene under the control of the *daf-7* promoter (*daf-7p::gfp*) and *rol-6(d)* marker gene, was made by germline transformation (Mello et al., 1991) in a wild-type animal. Then, from a heterozygote *kin-8(ks52)/+; ksEx26* made by genetic crossing with *kin-8(ks52)*, three lines of *kin-8(ks52)/kin-8(ks52); ksEx26* and three lines of *+/+; ksEx26* were derived. The expression of *daf-7p::gfp* was greatly reduced in the *kin-8(ks52)* lines; only up to 10% of the animals were categorized as 'strong' expressors, 80-90% (on old *E. coli*) or about 70% (on fresh *E. coli*) as 'invisible', and the rest as 'faint' or 'weak' expressors. In contrast, more than 80-90% of the animals were categorized as 'strong' expressors in *kin-8(+)* lines (Fig. 5). Judging from the photographs showing the expression of the *daf-7p::gfp*, the relative brightness of the fluorescence in those categories was estimated to be about 1/30 for 'weak', about 1/120 for 'faint', and about 1/240 or less for 'invisible' as compared to that for 'strong' (Fig. 6). These results indicate that the elimination of *kin-8* function causes a reduction in the *daf-7* transcription.

This reduction in the *daf-7* activity seems to explain the partial Daf-c phenotype of the *kin-8* mutant, since the elimination of the *daf-7* activity causes a complete Daf-c phenotype. Dauer formation in only about half the *kin-8* animals seems to reflect an incomplete elimination of *daf-7* expression. The *daf-7* expression appeared to improve slightly when the animals were fed on 2-day-old *E. coli* compared to those fed on 2-week-old *E. coli*. However, this difference in *daf-7* expression appears to be too small to explain the increase in dauer formation depending on the age of food. Information on the age (non freshness) of *E. coli* food is probably processed in a separate pathway which enhances dauer formation.

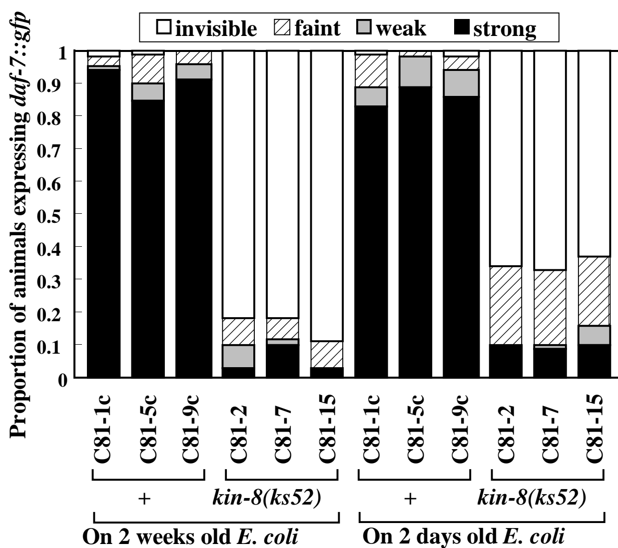

Fig. 5. The *daf-7* gene expression is reduced in the *kin-8* deletion mutant. About a hundred L2 roller animals from each line carrying *ksEx26[daf-7::gfp, rol-6(d)]* were randomly selected. The fluorescence of GFP was scored and classified into four grades, under a stereomicroscope equipped with a fluorescence apparatus.

kin-8 Daf-c phenotype is suppressed by *daf-7* cDNA expression or a *daf-3* mutation

To confirm that the *kin-8* Daf-c phenotype is due to the reduced *daf-7* expression, two experiments were performed. First, *daf-7* cDNA was expressed by the first promoter of *kin-8* in ASI and other cells (see Table. 1). The *daf-7* cDNA expression suppressed the *kin-8* Daf-c phenotype completely, but not Daf-c phenotype of *daf-1* (Table 2A). Second, a *daf-3* null mutation (deletion) was introduced into the *kin-8* mutant. *daf-3* encodes a Smad protein working in the signaling pathway downstream of DAF-1/DAF-4 receptors for DAF-7, and *daf-3* mutations are known to suppress the Daf-c phenotype of *daf-7* mutant (Patterson et al., 1997). The *daf-3* null mutation suppressed the Daf-c phenotype of *kin-8* nearly completely (Table 2A). These results indicate that the *kin-8* Daf-c phenotype results from the reduction of *daf-7* expression and that *kin-8* functions upstream of *daf-7* and *daf-3* in a genetic pathway for the dauer formation.

The *kin-8* mutant is dye-filling defective in ASI sensory neurons

In the wild-type animal, a lipophilic dye DiI is taken up by six classes of amphid sensory neurons, ASI, ADL, ASK, ASH, ASJ and AWB (Fig. 7A,B) and two classes of phasmid neurons (PHA and PHB) through the amphid or phasmid channels (small pores in the cuticle) and the sensory cilia exposed to the external environment. In the *kin-8* mutant, DiI uptake into the ASI neurons was not observed in almost all the individuals (Figs 7C-E, and Fig. 8), while dye uptake was observed in other neurons of the same individuals. Sometimes, uptake was not observed in certain other cells, which were likely to be AWB or ASH. Although *daf7p*::*gfp* expression allowed observation of the morphology of ASI in a fraction of the *kin-8* mutant animals, the sensory process of ASI was detached from the amphid channel in these animals (Fig. 7G-J). The end of the sensory process appeared to terminate slightly before the amphid channel in most cases (Fig. 7H), but sometimes inbetween the ASI cell body and the amphid (Fig. 7I). Also, cell positions were often slightly abnormal in the *kin-8* mutant: the position of the ASI cell body was often misplaced posteriorly or ventrally (Fig. 7E), and the cell bodies of ASK and ADL were frequently not adjacent (Fig. 7D,E). The sensory processes of ASI as well as the other amphid neurons develop in embryos as follows.

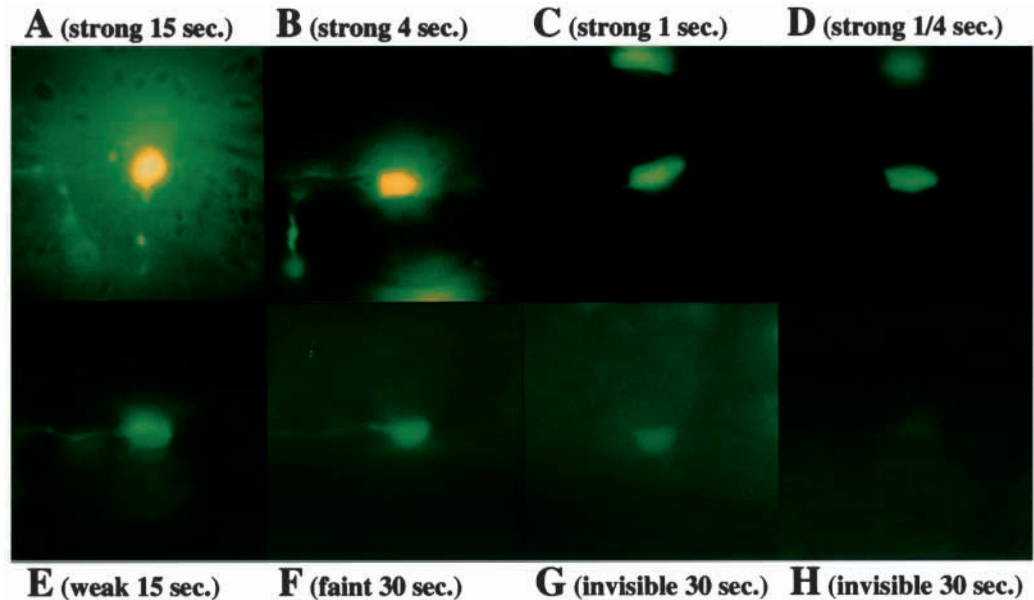

Fig. 6. Examples of the four grades of *daf-7p::gfp* expression in ASI. The photographs were taken at the indicated exposure time using a microscope (Zeiss Axioplan, ×1000) and ISO400 color slide film (Provia 400, Fujifilm). (A-D) 'strong' fluorescence of GFP in ASI neurons of C81-9c *+/+; ksEx26* animals. (E-H) 'weak', 'faint' and 'invisible' fluorescence of GFP in ASI neurons of C81-15 *kin-8(ks52); ksEx26* animals.

ASI moves towards the tip of the head, there the rudiment of the sensillium is formed, then the cell body moves posteriorly again laying down its dendritic process (Sulston et al., 1983). KIN-8 may be involved somewhere in this developmental process.

Expression of *kin-8* cDNA by the *daf-7* promoter in ASI or by a heat shock promoter rescues the dye-filling defect of ASI, but not the Daf-c phenotype

The cellular focus of the *kin-8* gene related to the dye-filling defect (Dyf) or Daf-c phenotypes was expected to be ASI. Three kinds of expression constructs were made in which a full length cDNA corresponding to the type1 mRNA of the *kin-8* gene was put under the control of the *daf-7* promoter (ASI specific), the *hsp16-2* heat shock promoter (broad expression in neural and hypodermal cells), and the *mec-7* promoter (touch cell specific) as a negative control, respectively. These constructs were introduced into the *kin-8* mutant. Recovery of dye-filling was significant in *kin-8* animals carrying the *daf-7* promoter construct or the heat shock construct as compared with that of animals carrying the *mec-7* construct (Fig. 8). Although the fraction of dye-filling recovery by the *daf-7* promoter construct was not very high, this is reasonable because the *daf-7* promoter activity is significantly reduced in the *kin-8* mutant. Slight recovery by the *mec-7* construct may be due to its non-specific expression in ASI. These results support the KIN-8 function in ASI for dye-filling. The recovery by the hsp construct was observed in similar levels either without heat induction or with heat induction, which suggests that the recovery is due to basal expression from the heat shock promoter and that temporary expression of *kin-8* in embryos or L1 larvae may not be sufficient. This implies that KIN-8 is required for keeping the sensory ending structure in the amphid as well as in development of the sensory process of ASI.

In spite of significant recovery of the dye-filling in ASI, the Daf-c phenotype was not suppressed at all by the *daf-7* promoter construct or the heat shock promoter construct even with a heat induction (Table 2C). This indicates that the recovery of the dye-filling in ASI is not sufficient for the rescue of the Daf-c phenotype. In dauer formation, there may be other cellular foci than ASI, or continuous and higher level expression of *kin-8* may be required in ASI or other cells.

Abnormality in the elongation of a gonad and the reduction of posterior body sizes

The *kin-8* deletion mutant shows an abnormality in the migration of a distal tip cell (DTC) and elongation of the gonad, and also a reduction in the body size, although the penetrance of these phenotypes are about 10-20%. These defects were observed almost exclusively in the posterior half of the body. In 1-2% of *kin-8* animals, the posterior gonad was not elongated at all. These phenotypes were rescued by the transgene of the *kin-8* genomic clone. A reduction in the gonad length often accompanied immature and misplaced oocytes, while wild-type adult animals have about 3-6 well developed oocytes arranged in line in the proximal region of the gonad (Kimble and Ward, 1988; Schedl, 1997). This mutant phenotype is probably due to a shorter distance in the mutant between the proximal region of the gonad (where meiosis occurs and sperms and oocytes are developed) and the DTC, which produces a signal preventing the meiosis (Kimble, 1981). In 5-10% of *kin-8* animals, incorrect migration of the posterior DTC was observed. The patterns of the abnormal migration varied greatly. However, in general, these patterns seem to have originated because the DTCs turned to the dorsal side before they reached the normal turning position, and then they recovered the normal migration course. Since *kin-8p::gfp* expression was observed in the DTCs (Table 1), it could be possible that the *kin-8* function in the DTCs is involved in the migration. However, the expression was weak and observed only in a small fraction (~8%) of animals at adult or L4 stages, in which the DTCs have already turned to the normal position (Hedgecock et al., 1987; Antebi et al., 1997).

DISCUSSION

We have shown that an allele of the *kin-8* gene deleting the entire kinase domain causes the Daf-c phenotype via reduction of the *daf-7* expression. How is KIN-8 involved in the control of the *daf-7* expression? *daf-7* is expressed specifically in ASI (Ren et al., 1996; Schackwitz et al., 1996), the *kin-8p::gfp* fusion gene is expressed in ASI, *daf7p::gfp* expression is greatly reduced in the *kin-8* mutant, and the *kin-8* mutant has an ASI-specific defect in dye-filling. These results suggest that KIN-8 has a function in ASI itself for *daf-7* expression, although KIN-8 function may also be required in other cells. There are two possibilities: the first one is that the *kin-8* mutation affects *daf-7* expression as a secondary effect through a developmental defect of ASI or related cells, and the second is that KIN-8 has a separate, physiological function involved in the control of the *daf-7* expression.

In the *kin-8* mutant, ASI seems to be born and differentiates as authentic ASI. This is based on the following three observations: (1) the fluorescence of *daf-7p::gfp* was observed in a cell likely to be ASI in many of the *kin-8* mutant animals under high magnification; (2) the axons and dendrites of those putative ASI were often visible and their morphology looked basically normal (such that they had two processes from the cell body, one goes toward the amphid and the other goes into the nerve ring along the normal path); (3) KIN-8 does not appear to be involved in fundamental development or differentiation of *kin-8*-expressing cells, since the *kin-8* mutant looks almost normal and the expression patterns of the *kin-8p::gfp* constructs in the *kin-8* mutant were similar to those in the wild-type animals (data not shown). Thus, reduction of *daf-7* expression in *kin-8* is not likely to be caused by the complete destruction of ASI. The dye-filling defect in the ASI is probably not a direct cause of the reduced *daf-7* expression and the Daf-c phenotype, because mutants of many genes showing the dye-filling defect (due to such as truncated sensory cilia or occluded amphid openings) were reported to have the opposite, namely dauer defective, phenotype (Perkins et al., 1986). Actually, in *daf-6*, *daf7p::gfp* expression appeared similar to that in the wild type (data not shown). The results of the rescue of Dyf but not Daf-c by the cDNA expression experiments also support this idea. Therefore, the first possibility is thought unlikely. However, there might be fine structural defects not yet identified, such as one in synapse formation in which MuSK, a KIN-8-related mammalian protein has a function (DeChiara et al., 1996). Even if this is the case, it is difficult to think that KIN-8 functions purely in development, since temporal expression of the *kin-8* cDNA by the heat shock promoter in embryos or the L1 stage is not sufficient for the rescue of the Daf-c and for the complete rescue of Dyf phenotypes. It may be possible that KIN-8 is continuously required for producing and/or maintaining such a fine structure.

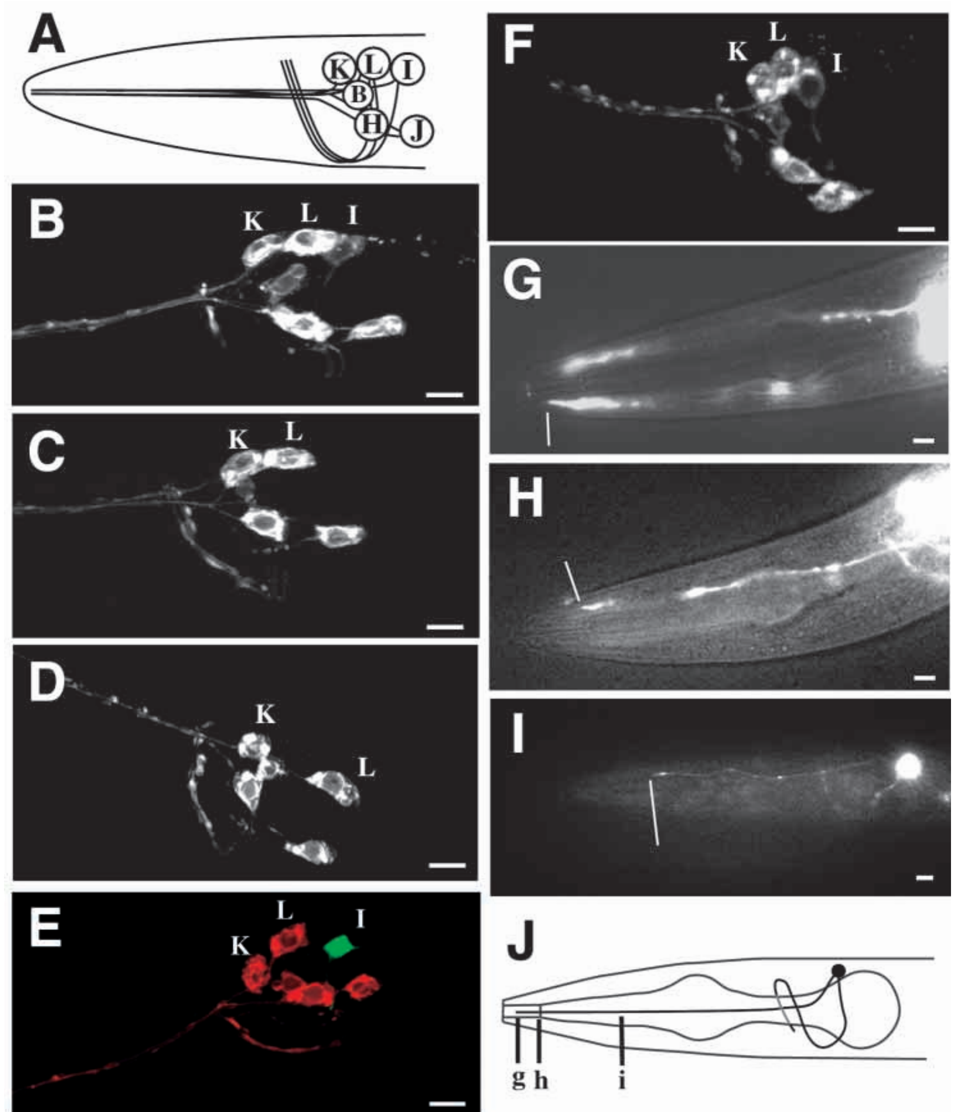

Fig. 7. The kin-8 mutant shows dye-filling defect in ASI. (A) A schematic drawing of a wild-type animal showing cells in the head taking up DiI. K,ASK; L, ADL; I, ASI; B, AWB; H, ASH; J, ASJ. (B-I) The cells in L1 animals taking up DiI or expressing the *daf7p-gfp* reporter were visulalized by fluoresecnt microscopy. (B-F) Confocal projection images; (B) DiI fluorescence in wild-type N2; (C,D) DiI in FK163 *kin-8(ks52)*; (E) *kin-8(ks52); ksEx26[daf7p-gfpE, rol-6(d)]*, DiI in red and GFP in green; (F) DiI in *kin-8(ks52); ksIs1[kin-8(+)]*. K, L, I in these images indicate ASK, ADL and ASI, respectively. (G) DiI in neurons other than ASI, and (H,I) GFP in ASI in the *kin-8(ks52); ksEx26[daf7p-gfpE, rol-6(d)]*; white bars indicate the sensory endings of ASI (H,I) or the other neurons (G). (J) g, h and i show schematically the positions of the endings observed in G,H,I. Bars, 5 μm.

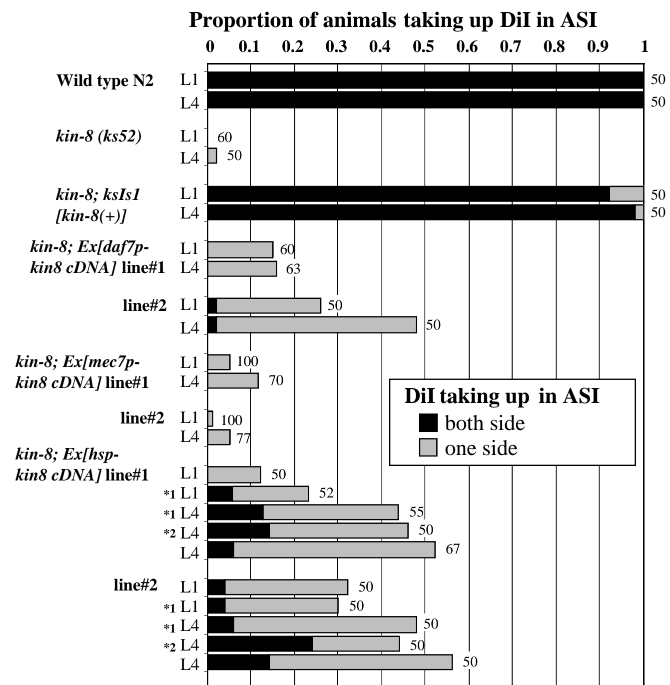

Fig. 8. The dye-filling defect in the *kin-8* mutant is recovered by *kin-8* cDNA expression from heterologous promoters. Bars indicate proportion of animals taking up DiI in ASI. The number on the right is the total number of animals examined in each class. L1 or L4 stage animals were examined. *1 and *2 indicate that they were given one hour heat induction at 33°C in their embryonic and L1 stages, respectively.

If the second possibility is true, four cases should be considered that are combinations of the following two sets of alternatives: KIN-8 is a component in a signal transduction pathway directly controlling *daf-7* expression (A); KIN-8 acts in another pathway which influences or modifies the direct pathway (a) and the activation of KIN-8 is dynamically regulated by the amount of its ligand that probably responds to an environmental condition (B); KIN-8 is constitutively activated (b). Although we do not so far have evidence clearly supporting any one of these four cases, the B cases (AB or aB) seem interesting. Transcription of the *daf-7* gene is controlled by environmental stimuli; the expression is high under non-dauer-inducing conditions (abundant food, a low concentration of dauer pheromone and a low temperature), whereas it is suppressed under dauer-inducing conditions (Ren et al., 1996; Schackwitz et al., 1996), and these stimuli are sensed by sensory neurons in the amphid (Albert et al., 1981; Perkins et al., 1986; Bargmann and Horvitz, 1991; Vowels and Thomas, 1992; Shakir et al., 1993). Therefore, in the B cases, a possible ligand for KIN-8 should be secreted under the non-dauer inducing conditions and not under the dauer inducing conditions. Sensory neurons in the amphid may be plausible candidates producing the KIN-8 ligand, because sensing of the environmental stimuli and secretion of a ligand can be simply linked within a single cell and because secretion of an endocrinologic factor is a fairly common property of neurons (Kupfermann, 1991; Schwartz, 1991). This implies a neuroendocrinological signaling pathway in which a sensory neuron perceives environmental information and transforms it into an amount of the KIN-8 ligand to be secreted. The ligand may be received by KIN-8 in ASI or related cells. The Daf-c phenotype of *kin-8* is not temperature-sensitive and similar to that of *daf-7; ttx-3* double mutant (Hobert et al., 1997), which might imply that environmental temperature information could be transduced to ASI through such a KIN-8 pathway.

As is KIN-8, DAF-1/DAF-4 receptor for DAF-7 TGF-β is expressed in amphid sensory neurons including ASI and many interneurons (Riddle and Albert, 1997; Patterson et al., 1997). DAF-2 insulin receptor-like molecules (Kimura et al., 1997) may also work in neurons, because a downstream factor, DAF-16 fork head transcription factor, is expressed in the neurons, ectoderm, muscles and intestine (Ogg et al., 1997). Although a ligand for DAF-2, a presumed insulin-like molecule has not yet been identified, it might also be produced in a sensory neuron. These arguments lead us to speculate that the neurons sensing environmental signals secrete factors such as DAF-7, a DAF-2 ligand and a KIN-8 ligand, then the information represented by these factors are processed and integrated through paracrine, endocrine or autocrine mechanisms among the neurons expressing the corresponding receptors. This type of signaling may be advantageous in a process such as decision between normal development and dauer formation, in which integration of a variety of environmental information over a period of time is probably required.

The *kin-8* mutant phenotypes in the posterior body part are similar to the phenotypes called withered tails (Wit) in *C. elegans*, which is caused by defects in migration or elongation of the canal-associated-neurons, CANs (Manser and Wood, 1990; Wightman et al., 1996; Forrester and Garriga, 1997; Forrester et al., 1998). There may be such defects in the *kin-8* mutant. The *kin-8* mutant shows some defects in development of ASI sensory process and in migration of DTCs. These results suggest that KIN-8 may be involved in migration of those cells and processes, although specific mechanisms are unknown.

What are the molecular mechanisms of KIN-8 function? We showed that *kin-8(ks52)*, deleted of most of the cytoplasmic region including the kinase domain, is null for dauer formation and a kinase-negative KIN-8 is functional. Given that little or no *kin-8* truncated protein is present in *kin-8(ks52)* mutant, it may be possible that only the extracellular region of KIN-8 is required as a scaffold for other proteins in the wild type. But if *kin-8(ks52)* is null in spite of the presence of truncated KIN-8, the cytoplasmic region of wild-type KIN-8 is thought to have a function. In this case, a protein is expected to be associated with the KIN-8 cytoplasmic region. This presumed associated protein is possibly activated without the kinase activity of KIN-8 when a ligand binds to KIN-8. A possible mechanism is such as that dimerization of KIN-8 upon ligand binding brings the associated proteins in close contact with each other and into activation. The Unc phenotype of kinase-negative KIN-8 suggests that the kinase activity has another role than dauer formation, if the Unc phenotype represents an authentic KIN-8 function.

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