Analysis of *che-2*,
a gene that is essential for the formation of sensory cilia in *C.elegans*

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INTRODUCTION

Possible Roles of Sensory Cilia

Many sensory neurons such as vertebrate olfactory receptor neurons, cone and rod cells (photoreceptor neurons), and hair cells (mechanoreceptor neurons) have cilia at the tip. These sensory cilia have a special arrangement of microtubules, like motile cilia. Because sensory cilia contain transduction components such as various chemo-, photo-, and mechano-receptors, G proteins, adenylate cyclases, ion channels, etc. (Pace et al., 1985; Nakamura, 1987; Buck and Axel, 1991; Menco et al., 1992), it is generally assumed that they are the primary sites of transduction where environmental stimuli are converted into neuronal signals known as receptor potentials. Actually, some experiments on olfactory neurons have shown that cilia are important for sensing odors ; no neurons lacking cilia respond to odors, and maximal responses are evoked by pulses apply to cilia (Kurahashi et al., 1989; Lowe and Gold, 1991).

Recently, studies on photoreceptor neurons of *Drosophila* revealed the importance of spatial organization of signaling components in a rhabdomere, a miclovillar structure specialized for light sensation (Montell, 1998). The central player InaD, which contains five PDZ domains for molecular interaction, functions as a multivalent adapter that brings together several components of the phototransduction cascade into a macromolecular complex (Tsunoda et al., 1997). Such a macromolecular complex must facilitate the effective transduction and regulation of the cascade. Although rhabdomeres are not cilia but microvilli, it is possible that similar molecules play a role in making supramolecular complexes and organizing the spatial arrangement of macromolecules in sensory cilia. Actually InaD homologue is found in *C.elegans* genome database, in spite of the absence of rhabdomere-like structure in *C.elegans*

While signal transduction in sensory neurons has been studied to some extent, the way sensory cilia are formed during development and the spatial organization of signal components in the cilia remain to be studied.

Sensory Cilia of *C. elegans*

C. elegans is a good model organism for studying the structure, function, and development of the nervous system. Of the 118 classes of neurons in *C. elegans* hermaphrodites, 24 classes (60 neurons) have cilia (White et al., 1986). Among them, sensory neurons in amphids, a pair of lateral sensilla in the head, were studied well concerning their functions, and observed in detail by electron microscopy (White et al., 1986; Lewis and Hodgkin, 1977; Albert and Riddle, 1983; Perkins et al., 1986) (Fig.1). Each amphid comprises the ciliated dendrites of 12 sensory neurons plus two support cells called sheath and socket cells. The sheath and socket cells form a cylindrical channel open to the outside environment. The length of each single cilium is about 7.5μm in adults (Ward et al., 1975). As shown in Figure1, the amphid cilia are classified morphologically into several types : for instance, the cilia for osmo-sensation (ASH) and chemo-sensation of soluble attractants (ASE) have a single process, the cilia for chemo-sensation of volatile attractants (AWC, AWA) have a fan-like or a branched filamentous shape, and the cilium for thermo-sensation (AFD) has a complex membrane structure with many finger-like protrusions.

Signal Transduciton in the Sensory Cilia of *C. elegans*

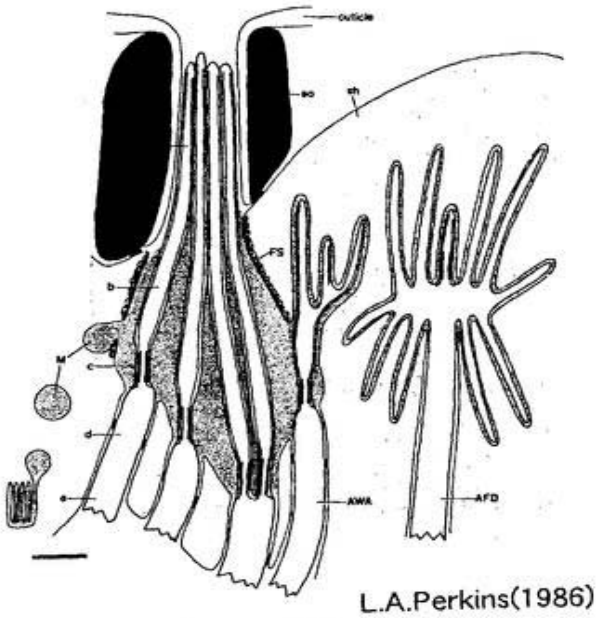
Many signal transduction components have been found in the sensory cilia of *C. elegans*. Many candidates of chemo-receptors, namely, divergent seven transmembrane receptors (Troemel et al., 1995) and receptor-type guanylyl cyclases (Yu et al., 1997), were investigated and found to be localized at cilia. Some G-proteins, which may be coupled with the seven transmembrane receptors, were also studied. Mutations in a G-protein α subunit, ODR-3, result in defects in olfaction and mechanosensation, while antibodies against ODR-3 stained only the cilia of some amphid sensory neurons (Roayaie et al., 1998). Many other G-proteins whose function is unknown yet, are localized at cilia (R.H.A.Plasterk, personal communication). Furthermore, a cyclic nucleotide-gated channel TAX-2/TAX-4 has been identified by analyzing mutants defective in chemotaxis and thermotaxis (Coburn and Bargmann, 1996; Komatsu et al.,

Fig1. Amphid, the main sensory system, of C.elegans

A

the head region of C.elegans

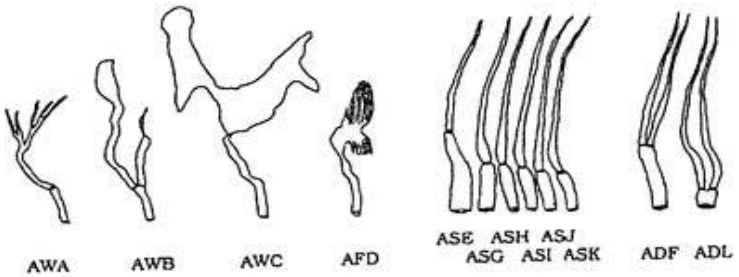
B



L.A.Perkins(1986)

the amphid opening

C



the morphology of each cilium

1996). The TAX-2/TAX-4 channel resembles the vertebrate visual and olfactory channels, which implicates that cGMP and/or cAMP are important second messengers in sensory system in *C. elegans* as well as in vertebrates. The ODR-3 Gα-protein may regulate this channel through production or degradation of cyclic nucleotides. The TAX-2/TAX-4 channel is also a component of sensory cilia.

Cilium-defective Mutants in *C. elegans*

As listed in Figure2, mutants that are abnormal in the cilium structure of *C. elegans* have been isolated and mapped to 29 genes (Lewis and Hodgkin, 1977; Perkins et al., 1986; Zwaal et al., 1997; Roayaie et al., 1998; Starich et al., 1995). Although they were isolated by various phenotypes such as chemotaxis, osmotic avoidance, dauer larva formation and male mating, structural abnormalities in their cilia were investigated in detail by electron microscopy (with the exception *dyf-1* to *dyf-13* mutants, which are characterized by the dye-filling assay mentioned below but not by electron microscopy). Most of them also showed abnormality in the dye-filling assay. The dye-filling assay is a simple method for detecting structural abnormalities in the cilia of some chemosensory neurons, which is performed by soaking worms in solutions of fluorescent dyes, such as FITC, DiO and DiI, and observing the worms under a fluorescence microscope (Hedgecock et al., 1985; Perkins et al., 1986; Starich et al., 1995). In wild-type animals, the dyes can penetrate into eight classes of amphid and phasmid chemosensory neurons (amphid neurons ADL, ASH, ASI, ASJ, ASK, AWB and phasmid neurons PHA, PHB for DiO and DiI; the same but ADF instead of AWB for FITC), which are visualized by fluorescence microscopy. In contrast, in many mutants that have abnormality in the cilium structure, those neurons are not stained by the dyes (Dyf phenotype). While the dye-filling assay is very useful, the mechanism of dye-filling is not clear and cannot be explained by the exposure of cilia to the outside environment, because not all the neurons whose cilium is exposed to outside can take up the dye.

Genes required for the formation of the cilium structure have been revealed by cloning some of the genes in Figure2. Of these genes, *osm-3* and *che-3* encode a

sensory cilium defective /dye-filling defective mutants

Gene	Gene product	Homolog in Chlamydomonas flagella	cilium structure
WT			
daf-19	RFX-type transcriptional factor		
che-2	novel protein		
che-13	?		
osm-1	novel protein	+	
osm-5	?		
osm-6	novel protein	+	
che-3	dynein	+	
osm-3	kinesin	+	
che-11	novel protein (weak similarity to *osm-1*)	+	irregular contours, filled with ground material, etc.
daf-10	novel protein (weak similarity to *osm-1*)		
che-10	?		
che-12	?		abnormal socket cell & sheath cell
che-14	PATCHED receptor family		
daf-6	?		
dyf-1~dyf-13	?		
gpa-3(gf)	G protein		N.D
odr-3	G protein		

kinesin (Shakir et al., 1993) and a cytosolic dynein heavy chain isotype (C. J. de Vries, S. R. Wicks, H. G. A. M. Van Luenen, and R. H. A. Plasterk, personal communication), respectively. Both proteins are known to act in axonal transport. They probably transport materials necessary for sensory cilium formation and the vehicle for the materials. Another cilium structure gene, *osm-6,* encodes a protein that has homology to the predicted mammalian protein NGD5 (Collet et al., 1998), whose mRNA is decreased upon long-term treatment of a neuroblastoma-glioma cell line with the delta-opioid agonist (Wick et al., 1995), suggesting its involvement in signal transduciton. *osm-1*, *che-11* and *daf-10* encode novel proteins containing regions of weak similarity to each other (S.Stone, A.Davies and J.Shaw, personal communication). In addition, OSM-6, OSM-1 and CHE-11 have significant similarity to the components of the flagella of *Chlamydomonas* that are transported by a kinesin (Cole et al., 1998; S.Stone, A.Davies and J.Shaw, personal communication) (see below). Furthermore, it was reported that some mutants in the α subunits of G proteins, namely, a loss-of-function mutant in *odr-3* and a gain-of-function mutant in *gpa-3*, show defects in sensory cilium formation or dye-filling (Zwaal et al., 1997; Roayaie et al., 1998). These Gα proteins are expressed in sensory neurons, and especially the ODR-3 protein is shown to be localized at cilia.

For the moment, the problem of the formation and maintenance of sensory cilia looks complicated, but both transport that involves microtubules and signal transduction that requires an appropriate level of some Gα proteins seem necessary for the correct cilium formation. However, how they act in cilium morphogenesis remains to be studied. The analysis of genes required for the normal cilium structure should help to elucidate the mechanism.

Similarity between Sensory Cilia of *C. elegans* and Flagella of *Chlamydomonas*

Recently, striking similarities were revealed between the sensory cilia of *C. elegans* and the motile cilia (flagella) of *Chlamydomonas reinhardtii.* In the latter organism, a

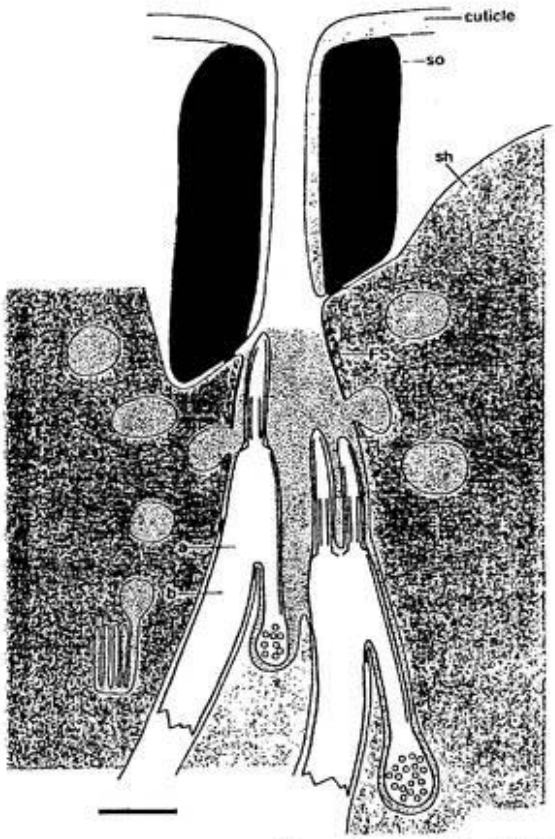
kinesin-dependent anterograde intraflagellar transport (IFT) of particles is required for the formation and maintenance of flagella (Kozminski et al., 1993, 1995). These IFT particles are composed of 15 polypeptides, and three of them have similarity in the amino acid sequence to the *C. elegans osm-6*, *osm-1* and *che-11* gene products, respectively (Cole et al., 1998; S.Stone, A.Davies and J.Shaw, personal communication). Moreover, the retrograde transport of IFT particles by dynein is necessary for correct flagellar formation (Pazour et al., 1998). Thus, the formation, maintenance and function of sensory cilia and motile cilia/flagella may be achieved by a general mechanism common to many organisms.

che-2 Is One of the Cilium-defective Mutants

The *C. elegans* mutant *che-2* is one of the cilium defective mutants showing the Dyf phenotype. Various *che-2* mutants were isolated by different selection methods. Three alleles, *m127*, *mn395*,and *sa133*, were isolated by defects in osmotic avoidance behavior, dye-filling into some neurons, and suppression of the dauer-constitutive phenotype of daf-11, respectively (Starich al.,1995). The allele *e1033* was isolated by male impotency (Hodgkin,1974), and found to show abnormality also in chemotaxis, dauer formation, and the structure of many sensory cilia (Lewis and Hodgkin, 1977)(Fig.3). The sensory cilia of *che-2* (*e1033*)mutants, with a possible exception of those of AFD neurons, are abnormally short, lacking the middle and distal segment (6 to 7μm) and consisting only of the proximal segment (1μm) (Lewis and Hodgkin, 1977; Perkins et al., 1986), which corresponds to the transition zone of the *Chlamydomonas* flagella. In *che-2* mutants, microtubules assemble normally in the proximal segment, but they cannot extend anteriorly. Instead, assembly of microtubules is found also in an abnormal posterior projection adjacent to the defective cilium. It was speculated that the abnormal posterior projection is formed either by the degeneration of the cilium once extended correctly (Lewis and Hodgkin, 1977) or by the accumulation of the components of the cilium that cannot extend correctly. In either case, the *che-2* product may be required for the stability or assembly of microtubules in the middle and distal

Fig. 3 previous studies on *che-2* mutants

alelle	screening	other known defects	
e1033	male activity	chemotaxis, dauer formation morpholgy of cilia	Lewis and Hodgkin(1977)
mn395	dye-filling	--	Starich et al(1995)
m127	dauer formation	--	Starich et al(1995)
sa133	suppressor of *daf-11*(dauer-constitutive mutant)	--	Starich et al(1995)



Lewis and Hodgkin (1977) and Perkins et al(1986)

segment of the sensory cilium, and these defects of cilium morphology may result in the abnormality of the general behaviors. In this paper I report the cloning and expression of *che-2* gene as well as phenotypic characterization of *che-2* mutants. I also report that *che-2* gene acts cell-autonomously, and that the expression of *che-2* at the larval or even the adult stage is sufficient for the formation of sensory cilia, although they are formed in a late embryonic stage during normal development.

EXPERIMENTAL PROCEDURES

Strains: Wild-type animals were *C. elegans* variety Bristol, strain N2. Nematodes were grown at 20°C using standard methods described by Brenner (1974) unless otherwise noted. The alleles of *che-2* used in this study were *e1033* (Lewis and Hodgkin, 1977), *sa133, m127* and *mn395* (Starich et al., 1995).

Cloning of *che-2*: The *che-2(e1033)* mutation had previously been mapped (Avery, 1993; Jongeward et al., 1995) very close to *egl-17 X,* which has been cloned (Burdine et al., 1997). Fifteen overlapping cosmids from this region (about 400 kb) of the genome were introduced separately into *che-2(e1033)* animals, and resulting transgenic strains were tested for the ability of dye-filling (Starich et al., 1995; M. Koelle, personal communication). The cosmid F38G1 could rescue the defect of dye-filling in *che-2(e1033)*, and so could the 6.7-kb *Eco*T14I fragment of F38G1. The *C. elegans* DNA Sequence Consortium (Wilson et al., 1994; Coulson, 1996) determined the DNA sequence of this region and predicted one gene F38G1.1. A partial-length cDNA clone (yk486h11) corresponding to this predicted gene was isolated by the *C. elegans* cDNA project (Y. Kohara et al., personal communication). I obtained the missing 5' part by reverse transcription-PCR with a *C. elegans* splice leader sequence SL1 as the 5' primer, and the missing 3' part by the 3'RACE method. The full-length cDNA thus obtained rescued the *che-2* mutant phenotypes, when it was expressed under heterologous promoters, as described in the Results section.

DNA Sequence Determination of *che-2* Mutations: The entire *che-2* coding region of each allele was amplified by PCR using Expand High Fidelity PCR System (Boehringer Mannheim) with the primers F38-5-seq (5'-CCTCAGAGATTTGGGCTAATC-3') and F38-3-seq (5'-ATTGTTTCCTTTAGCTTTTTCCGC-3'). All the 14 exons in the PCR products were sequenced with gene-specific oligonucleotide primers. The results were confirmed by sequencing the products of at least three independent PCR reactions.

Assay of Dye-Filling: For the characterization of allele phenotypes, I conducted the FITC-filling assay as described by Hedgecock et al. (1985). DiO and DiI-filling assays were performed according to M.Koelle and B.Sawin (personal communication): Stock solution (2mg/ml in dimethyl formamide) was diluted 1: 200 in M9 buffer, and worms were incubated in this solution for 2 hours at 20°C. For the rescue experiment by genomic fragment and the analysis of heat shock induction, DiI-filling assay was performed as described above. For the analysis of rescue by *sra6*::CHE-2, I checked the staining of cells after 30min incubation in more dilute DiI solution (1μg/ml). Longer incubation and higher concentration of dye caused staining of excess cells.

Behavioral Assays: Chemotaxis assays were performed according to the population chemotaxis assay method by Bargmann et al. (1991a, 1993). The concentration of NaCl was 0.4M, and benzaldehyde was diluted to 10^{-2}. The chemotaxis index was calculated as [(number of animals at attractant)-(number of animals at control)] / (total number of animals).

Osmotic avoidance assay was based on the method by Culotti and Russell (1978) with minor modifications. I placed 50 to 200 animals within a high osmotic annular ring (1.5cm in diameter) made of 60μl of 4M NaCl on a plate. The osmotic avoidance index was defined as the fraction of animals that remained inside the ring after 30min. Besides the animals that escaped from the ring, those which died in the high osmotic region were also regarded as non-avoiders.

Male mating ability was tested as follows. An L4 male to be tested was placed on a 3.5cm mating plate with three L4 *unc-31(e169)* hermaphrodites, which were lethargic and seemed relatively easy partners to mate (Liu and Sternberg, 1995). The plates were kept at 20°C for 3 to 4 days and checked for the progeny. If there were non-Unc hermaphrodites and males among the F1 progeny, I considered that the male could mate. The males of *che-2* were obtained by crossing *che-2* hermaphrodites to N2 males, since *che-2* is X-linked.

Visualization and Analysis of Cilium Morphology: *sra-6*::GFP and *gcy-10*::GFP were constructed according to the method by Troemel et al. (1995) and Yu et al. (1997), respectively. *gpa-9*::GFP was a gift from G.Jansen and R.H.A.Plasterk. To see the cilium morphology I introduced these constructs into the wild type and *che-2* mutant animals by microinjection.

Germline Transformation: I performed germline transformation experiments by a standard microinjection method (Mello et al., 1991). For GFP expression studies, the DNA concentrations were 90 μg/ml for the GFP fusion constructs and 10μg/ml for the injection marker plasmid pRF4, which contains the semidominant mutation *rol-6(su1006)*, with the exception of pCHE-2::GFP3 (5 μg/ml for the GFP fusion construct and 95μg/ml for pRF4). For rescue experiments, the DNA concentrations were 5 to 20 μg/ml for the DNA to be tested and 10 to 95 μg/ml for the injection markers. As the markers I used *myo-3*:: GFP (A. Fire et al., personal communication), *sra-6*:: GFP (Troemel et al., 1995) and H20::GFP (T. Ishihara et al., unpublished results), which give expression of GFP in body wall muscles, in a subset of neurons, and in almost all neurons, respectively. Transgenic F1 animals, which showed the Rol phenotype or the GFP expression of the markers, were picked, from which transformed lines were selected and kept as lines carrying an extrachromosomal array.

Constructs for *che-2* Expression Experiments: pCHE-2::GFP1 was made by ligating the 7.6-kb *Sal*I-*Eco*RV fragment of cosmid F38G1 to the *Sal*I-*Sma*I site of the GFP expression vector pPD95.75 (A. Fire, S. Xu, J. Ahnn, and G. Seydoux, personal communication).

To make pCHE-2::GFP2, the *che-2* cDNA was inserted first into pBluescript. The cDNA without the 3' untranslated region and polyA tail was amplified from the plasmid clone using the primers F38-3-a (5'-AGACTTTCGGTGTCAGCCAG-3') and F38-3-GFP (5'-GGGAAGCTTTTGTTTCCTTTAGCTTTTTCCGC-3'). The F38-3-GFP primer was designed to replace the C-terminal amino acid Asp with Lys, followed by a *Hin*dIII site. The 3' region of the *che-2* cDNA in pBluescript was replaced with this PCR product. Then, the *Xba*I- *Hin*cII fragment containing all the coding region was

excised from this plasmid and ligated into the *Xba*I-*Sma*I site of the pPD95.69 GFP vector (gift from A. Fire), resulting in the addition of excess 6 amino acids between CHE-2 and GFP. Finally, the 2.2-kb *Sal*I-*Eco*RV fragment containing most of the 5' region of the *che-2* cDNA was replaced with the 5.4-kb *Eco*T14I-*Eco*RV fragment of the cosmid F38G1, which contained a 650-bp 5' non-coding region and most of the *che-2* coding region in the genome. The construct pCHE-2::GFP2 contains the original *che-2* promoter region and all the *che-2* coding sequence (except the C-terminal amino acid codon), which is connected to the GFP cDNA in frame through a linker amino acid sequence.

pCHE-2::GFP3 was made by removing the *Kpn*I cassette containing NLS from pCHE-2::GFP2. This construct is expected to show the original intracellular localization of CHE-2.

Expression of *che-2* under the Control of the *sra-6* Promoter: To express *che-2* protein only in a subset of sensory neurons, I used the *sra-6* promoter. A *sra-6* promoter region, consisting of a 3.5-kb 5' upstream sequence and the coding sequence for the first 8 amino acids, was amplified by PCR, and ligated to *che-2* cDNA so that the 5' upstream sequence is followed by the full-length *che-2* cDNA. This construct, *sra-6*::CHE-2, together with the *sra-6*::GFP construct as the injection marker, was used for the transformation of *che-2(e1033)* animals.

Induction of *che-2* Expression by Heat Shock Promoter: To make an *hsp::che-2* fusion gene, *che-2* cDNA was inserted into the pPD49.78 vector (gift from A. Fire), which contains a *C. elegans* heat shock promoter hsp16-2. This promoter gives strong expression in neural and hypodermal cells after heat shock (A. Fire, S.White-Harrison and D.Dixon, personal communication). The construct, pHSP::CHE-2, was injected into *che-2(e1033)* animals together with H20::GFP or *sra-6*::GFP as the injection marker. For a control experiment I also injected the pPD49.78 vector into *che-2(e1033)* animals together with *sra-6*::GFP as the injection marker. The animals carrying an extrachromosomal array containing pHSP::CHE-2 (or pPD49.78) and the injection marker, were heat-shocked at 30°C for 6 hours. Dye-filling assays were

performed at various times after the heat-shock. Since in these assays the animals were soaked in DiI (10μg/ml) for 2 hours before observation, time zero means that the animals were observed 2 hours after the end of the heat-shock treatment. DiI is taken up by 16 sensory neurons (pairs of ASK, ADL, ASI, ASH, ASJ, PHA, PHB, and AWB). However, since I did not count the dye-filling of ASI and AWB neurons, which gives only faint fluorescence, the maximum number of cells in dye-filling was 12 in this study. For the observation of cilium extension, I checked the morphology of cilia before and after heat-shock. The heat-shock treatment was operated as same as the dye-filling rescue mentioned above. The morphology of cilia was classified into 5 groups : "long" means 6μm> (WT-like) cilia, "medium" is 3~6μm cilia, "short" is 0~3μm cilia, "posterior projection" means cilia with posterior projections regardless of their length, and "curved" means bend cilia.

Expression of CHE-2/GFP fusion protein in mammalian culture cell lines: For expression in mammalian culture cells, pche-2::gfp3 was modified. The *C.elegans* intron between the che-2 and gfp was removed (by removing PpuMI cassette, A.Fire, personal communication), and the fragment containing this fusion gene was ligated under CAG promoter or CMV promoter in the PCXN2 (H.Niwa et al., 1991) or pcDNA3 (Invitorgen) expression vector respectively. These constructs introduced into the NIH3T3 and NG108 by using LIPOFECTAMINE PLUS Reagent (GIBCO). The fixation of cells were performed 24hr after the start of transfection. Cells were fixed in 3.7% paraformaldehyde for 20min, and permeabilized in 0.4% Triton-X100 for 1hr. Then the cells were incubated with anti-αtubulin antibody, DM1A (SIGMA). After rinse, incubation with the Cy3-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Lab, 115-165-146) was done. All preparations were performed at room temperature.

RESULTS

Characterization of Various *che-2* Mutants

Various *che-2* mutants were isolated by different selection methods. The allele *e1033* was isolated by male impotency (Hodgkin, 1974), and found to show abnormality also in chemotaxis, dauer formation, and the structure of many sensory cilia (Lewis and Hodgkin, 1977). Subsequently, three other alleles, *m127*, *mn395*, and *sa133*, were isolated by defects in osmotic avoidance behavior, dye-filling into some sensory neurons, and suppression of the dauer-constitutive phenotype of *daf-11*, respectively (Starich et al., 1995). In this study I investigated five phenotypes of these alleles, namely, cilium morphology, dye-filling, osmotic avoidance, chemotaxis, and male mating ability. Although some of them have been studied for some alleles, I repeated such assays to allow direct comparison between different alleles. Since I found that *m127* and *sa133* have an identical mutation as shown below, I analyzed only *m127* as a representative allele of this group.

First, I investigated the morphology of sensory cilia, using *sra-6*::GFP, which gives strong GFP expression in ASH and ASI ciliated sensory neurons as well as PVQ interneurons (Troemel et al., 1995). When this construct was introduced into the wild type (N2) strain, the cilia together with the processes and cell bodies of ASH and ASI neurons were visualized well by GFP fluorescence (Figure 4A, left). In contrast, 81% of *che-2(e1033)* animals carrying this construct showed abnormally short cilia, often with an abnormal posterior projection (Figure 4A, right, and 4B). These observations are consistent with the analysis by electron microscopy (Lewis and Hodgkin, 1977; Perkins et al., 1986). Among other alleles of *che-2*, *m127* showed similar morphological defects as *e1033*: 73% of the animals showed deformed cilia. On the other hand, *mn395* showed milder defects: as many as 65% of the cilia had a normal shape (Figure 4B).

Second, I studied the ability of dye-filling. The fluorescent dyes FITC and DiO were taken up by twelve amphid and four phasmid sensory neurons in N2, but by none

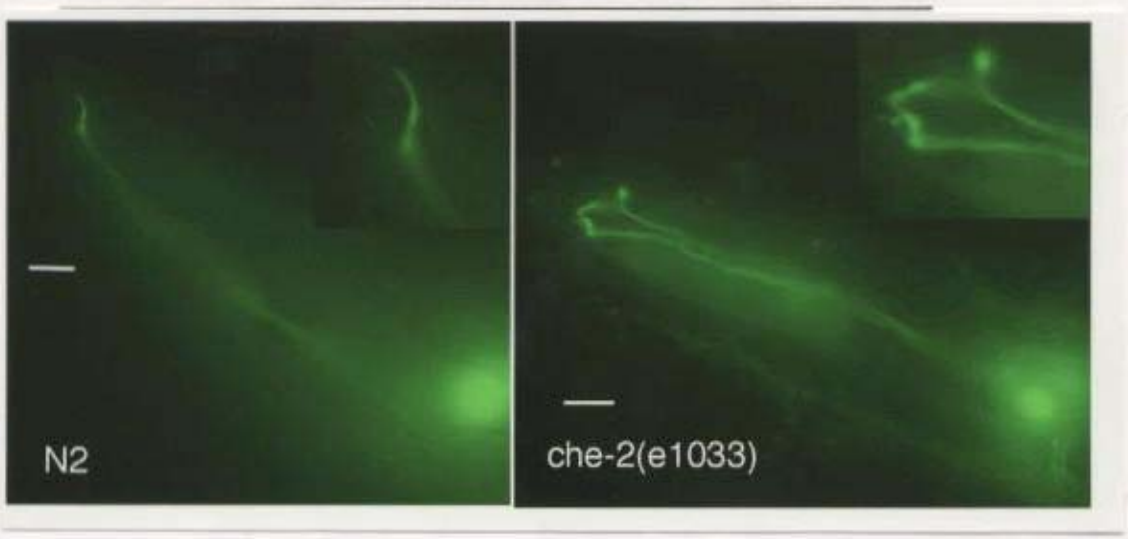


Fig.4 (A) Cilium morphology of the wild-type (left) and *che-2 (e1033)* mutant (right) animal. ASH and ASI neurons in the head of adult animals are visualized by *sra-6*::GFP. Anterior is to the left. The bright spheres on the right are the cell bodies, from which dendrites extend to the left, ending in cilia. Normal cilia are seen in the wild-type animal, while short cilia with (upper) and without (lower) an abnormal posterior projection are seen in the *che-2(e1033)* mutant. The bars indicate 10 μm.

of the neurons in all the three *che-2* alleles, *e1033, m127, mn395*, as reported (Hedgecock et al., 1985; Perkins et al., 1986; Starich et al., 1995). The same results were obtained with the fluorescent dye DiI for N2, *e1033* and *m127*, but not for *mn395*. The *che-2(mn395)* animals could often take up DiI (10μg/ml). In particular, ASK and ASH and ASJ neurons of *mn395* were frequently stained clearly with DiI.

Third, the osmotic avoidance behavior (Culotti and Russell, 1978) of each allele was analyzed. N2 worms avoided high osmotic pressure, while *e1033* and *m127* mutants did not (Figure 4C), as reported for *e1033* (Perkins et al., 1986). In contrast, *mn395* showed milder defects: 86% of the worms could avoid high osmotic strength. This result is consistent with the milder defects in the cilium morphology of ASH neurons mentioned above, since osmotic avoidance behavior is known to be mediated by ASH neurons (Bargmann et al., 1990).

Fourth, chemotactic behavior to NaCl and to benzaldehyde was analyzed for each allele (Figure 4D). All the alleles had a severe defect in chemotaxis to the water-soluble attractant NaCl, as reported by Perkins et al. (1986) for *e1033*. They also showed a defect in chemotaxis to the volatile attractant benzaldehyde, as reported by Bargmann et al. (1993) for *e1033*.

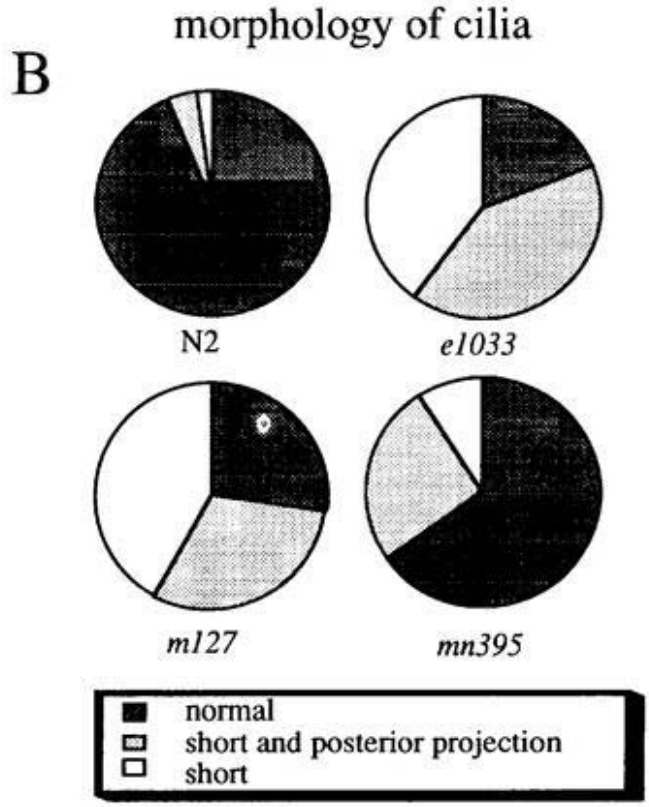
Finally, I assayed the male mating activity of each allele (Figure 4E). Males of all the three *che-2* alleles showed no or little mating activity, consistent with the report by Hodgkin (1983) for *e1033*.

In summary, *e1033* and *m127* showed severe defects in all the assays, whereas *mn395* showed milder phenotypes except for chemotaxis and male mating activity.

Formation of Deformed Cilia in the *che-2(e1033)* Mutant

Taking advantage of GFP, I analyzed the morphology of cilia during development. As mentioned above, the amphid sensory neurons of the *che-2(e1033)* mutant have an abnormal posterior projection at the tip instead of the normal cilium extending anteriorly (Lewis and Hodgkin, 1977; Perkins et al., 1986). The posterior projection was suspected to be produced by the degeneration of cilia once extended (Lewis and

characterization
of che-2 alleles



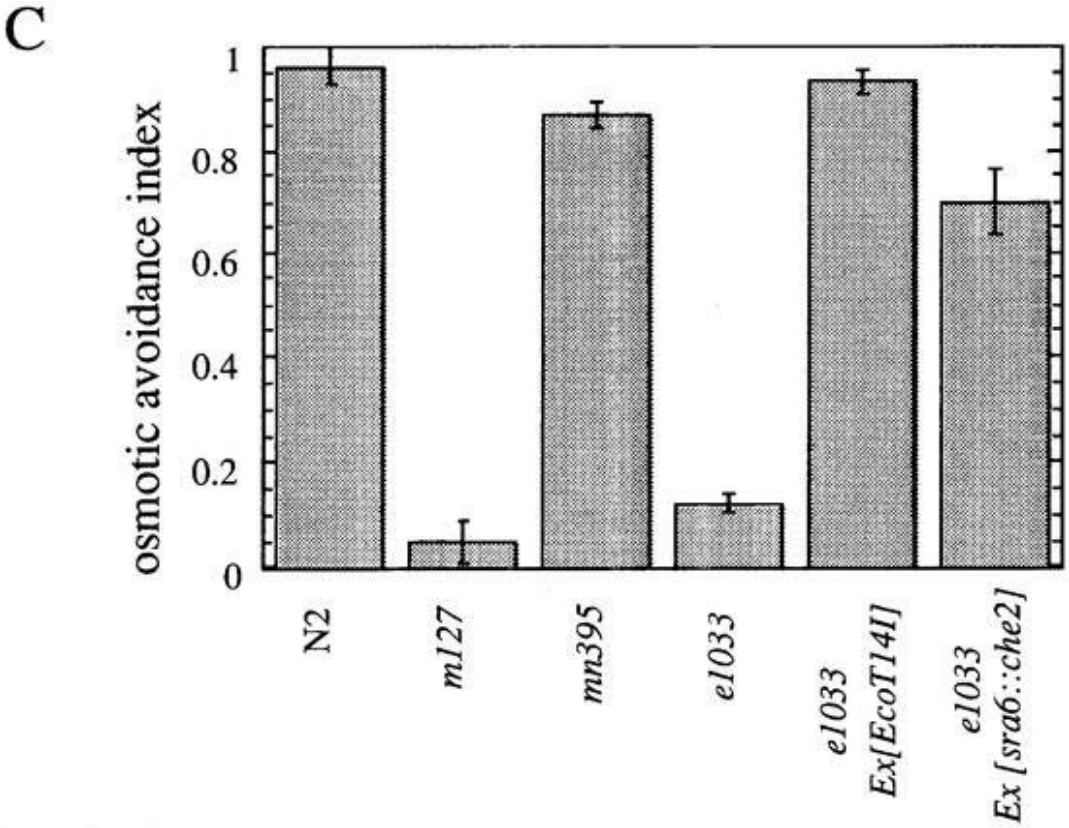


Fig.4 (B) Cilium morphology statistics of the ASH and ASI neurons in wild-type (N2) and various *che-2* alleles. About 100 cilia in L3 and L4 larvae and in adults were observed for each allele. (C) Osmotic-avoidance response of N2, various *che-2* alleles and *che-2(e1033)* strains carrying a wild-type *che-2* gene (*Eco*T14I fragment) or a *che-2* gene under the control of the *sra-6* promoter (*sra-6*::CHE-2). Each data point represents at least three independent assays. Error bars indicate the standard error of the mean (SEM).

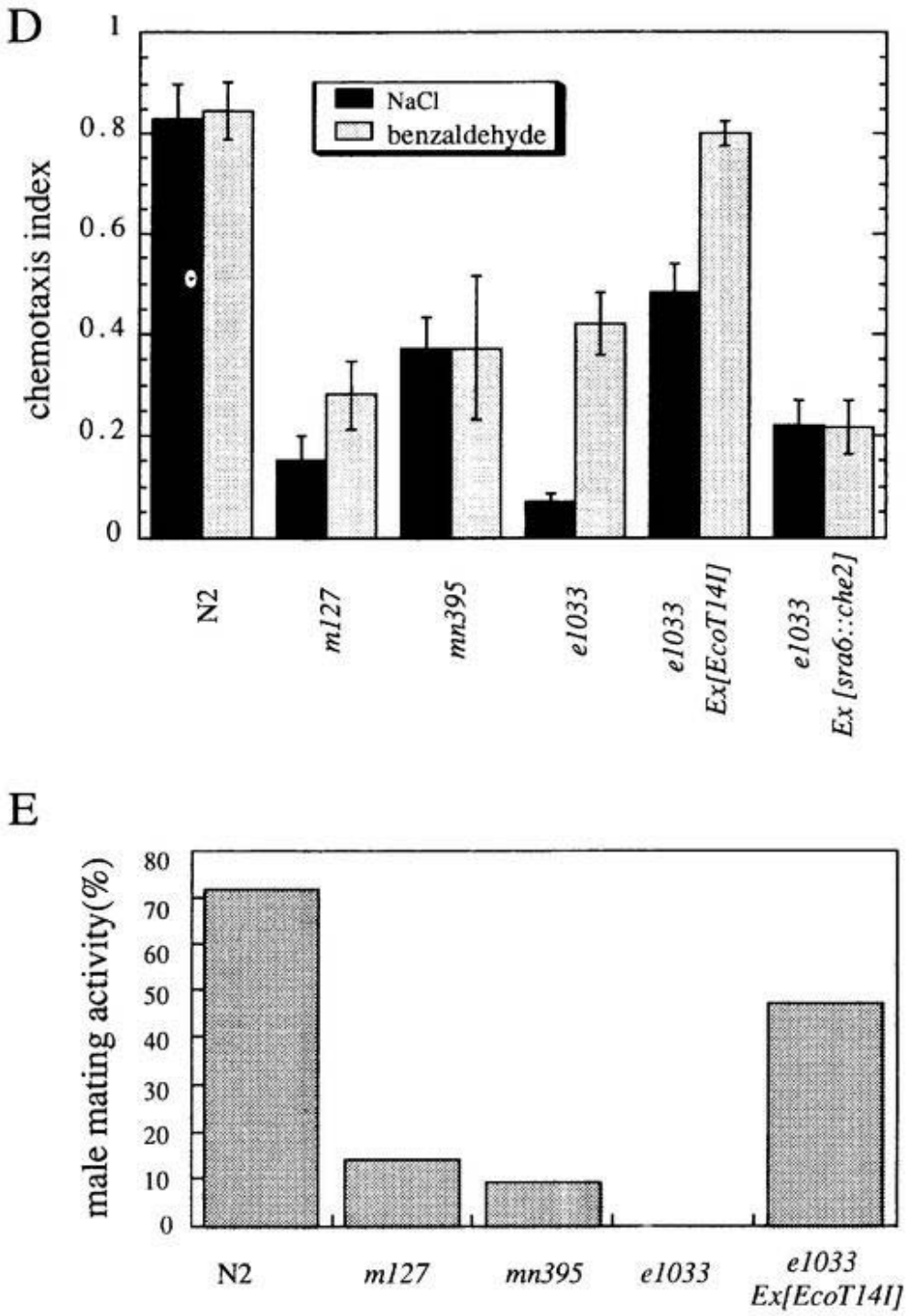


Fig.4 (D) Chemotaxis to NaCl and to benzaldehyde of the same strains as (C). Each data point represents at least four independent assays. Error bars indicate SEM. (E) Male mating activity of the same strains as (C), excluding *che-2(e1033) Ex[sra-6::che-2]*. The percentages of males that have mating activity are shown. Fifteen to twenty independent assays were conducted for each strain.

Hodgkin, 1977). Hence I traced the cilium morphology of ASH and ASI neurons using strains carrying *sra-6*::GFP. Unfortunately, I could not observe the beginning of the extension of the cilia, because the fluorescence of *sra-6*::GFP was too weak to visualize the cilium morphology until the midpoint of the three-fold stage of embryos, when the cilia had extended already to over 3μm in N2 animals (Table 1). I divided the latter half of the three-fold stage further into two substages, using the mouth morphology as an index: the embryo has a mouth plug with a straight-sided cylinder of the buccal cavity in the first substage (650 to 770min after the first cell division), and the mouth plug has already fallen away in the second substage (770 to 800min after the first cell division) (Sulston et al., 1983). I also checked the length of cilia of young L1 animals within 2 hours after hatching.

As shown in Table 1, the amphid sensory cilia of N2 extended rapidly during the latter half of the three-fold stage. On the other hand, the *che-2(e1033)* mutant did not extend their cilia, but began to make the posterior projection during this period. At the first substage the mutant cilia seldom had the projection, although they were shorter than the N2 cilia at the same stage. At the second substage, 21% of the mutant cilia had the posterior projection, while about half of cilia had the projection at the early L1 stage. I therefore conclude that the *che-2* mutant never forms normal sensory cilia even transiently during development, and that the abnormal posterior projection of the *che-2* amphid sensory neurons is made due to the inability of cilium formation, not by the degeneration of cilia once formed correctly.

che-2 Encodes a Novel Protein with WD40 Repeats

The *che-2* gene was cloned by the rescue of the dye-filling abnormal phenotype of the *che-2(e1033)* mutant (See Materials and Methods for the details). The cosmid F38G1 as well as its 6.7-Kb *Eco*T14I subclone could rescue the mutant phenotype (Figure 5). I confirmed that the subclone could rescue other defects of *che-2(e1033)*, namely, osmotic avoidance (Figure 4C), chemotaxis to NaCl and benzaldehyde (Figure 4D) and male mating activity (Figure 4E). I obtained the full-length cDNA using an EST

TABLE 1.

Elongation of cilia and emergence of abnormal posterior projections during development

developmental stage	length of cilia (mm)				deformed cilia with a posterior projection
	1.5-3	3-5	5-7	7-	
N2 Ex[*sra-6*::GFP]					
1st substage	0%	72%	27%	0%	0%
2nd substage	0%	17%	83%	0%	0%
after hatching	0%	15%	62%	23%	0%
che-2(e1033) Ex[*sra-6*::GFP]					
1st substage	92%	8%	0%	0%	5%
2nd substage	71%	29%	0%	0%	21%
after hatching	90%	10%	0%	0%	47%

The cilium morphology of wild-type (N2) and *che-2(e1033)* mutant animals was observed at the 1st and 2nd substages in the latter half of the three-fold stage and just after hatching. At each stage, 30 to 50 cilia of *che-2(e1033)* and over 15 cilia of N2 were observed.

clone (Kohara et al., personal communication) and by RT-PCR. The cDNA consisted of 2375 bases excluding poly(A), began with the *C. elegans trans*-splice leader sequence SL1 (Krause and Hirsh, 1987), and ended with a poly(A) stretch. The *che-2* gene had 14 exons that encoded a predicted protein of 760 amino acids (Figure 6 and 8).

A hydropathy profile (Kyte and Doolittle, 1982) predicted that the protein has neither a signal peptide nor a transmembrane domain. A search of protein databases revealed that it has similarity to the WD40 protein family (Neer et al., 1994), such as TFIID and G protein β subunits. I found four WD40 motifs in the N-terminal region of CHE-2, from the amino acid 100 through 297 (Figure 6 and 7). The 1st and 2nd repeats match the WD-repeats consensus regular expression defined by Neer et al. (1994), with each one mismatch. The third repeat has three mismatches, and the fourth repeats has four mismatches. This CHE-2 protein were assigned to belong to WD-repeat family, since WD proteins were defined as having at least one unit that matches the expression with zero or one mismatch, and at least one other unit that has three or fewer mismatches. Although the WD40 protein family is divided into subfamilies (Neer et al., 1994; Voorn and Ploegh, 1992), CHE-2 does not belong to any of them.

The function of WD40 motifs is not so clear and maybe diverse (Neer et al., 1994), but many WD proteins form multiprotein complexes, often interacting with other proteins through the WD-repeat region. For example, the WD40 motifs of a G protein β subunit assist the assembly of a ternary complex of a G protein-coupled receptor, Gα, and Gβ/γ (Clapham and Neer, 1993; Neer, 1995; Sondek et al, 1996; Lambright et al, 1996; Gaudet et al, 1996). Hence, the WD40 repeats of CHE-2 protein may be involved in the formation of a complex needed for sensory cilium formation.

The non-WD40 region of CHE-2 did not show significant similarity to any amino acid sequence predicted from the DNA databases, except the conceptual translation product of the human EST clone zs23c07. This clone (EMBL/GenBank/DDBJ accession no. AA262097) was isolated from a human cDNA library prepared from tonsillar cells enriched for germinal center of B cells by flow sorting in the Cancer Genome Anatomy Project of National Cancer Institute. Although it is only a partial-

cloning of che-2

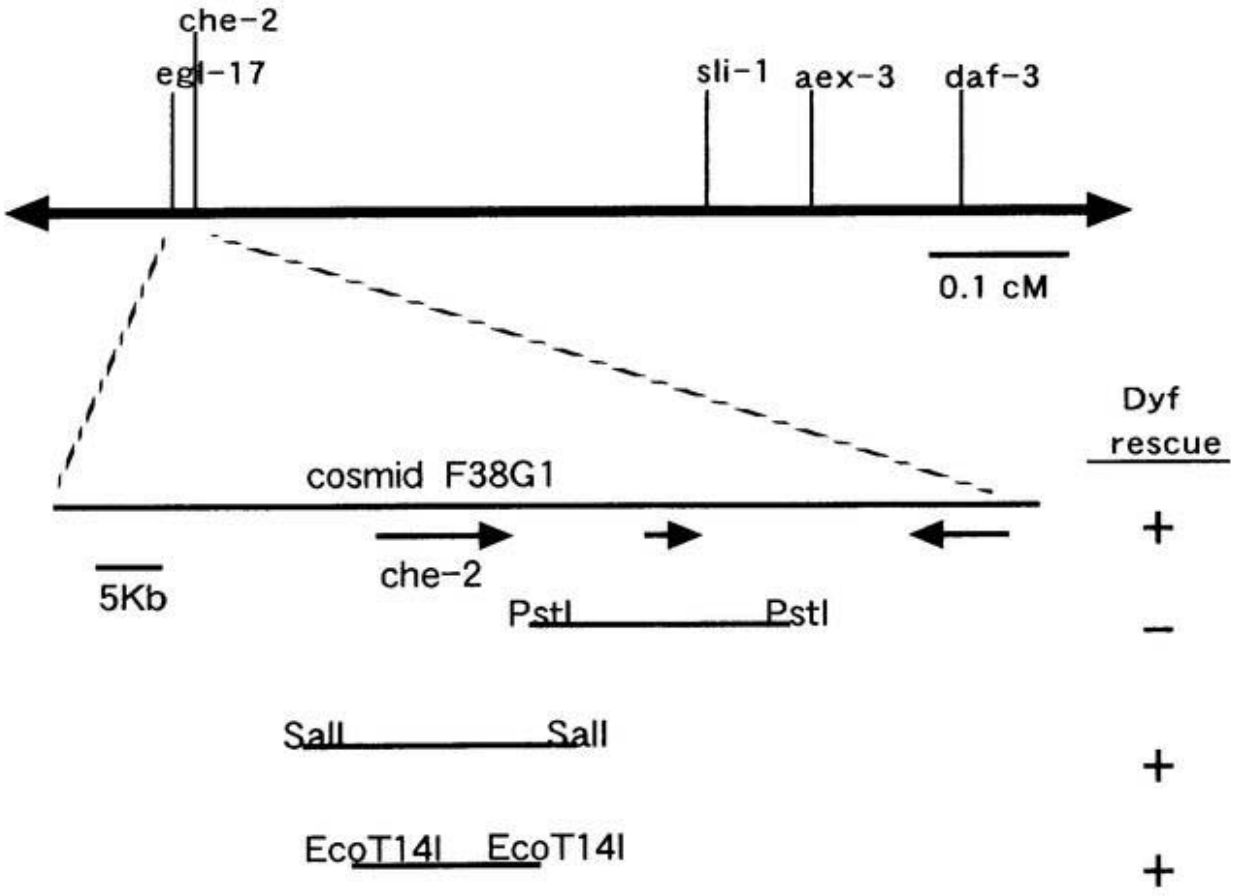


Fig.5 Cloning of *che-2* gene. Genetic and physical map of the *che-2* region. The arrows below the cosmid F38G1 indicate genes predicted by the *C. elegans* genome project.

length cDNA, similarity to CHE-2 is found in all the sequenced regions of zs23c07: the third and the fourth WD40 repeats (54% (46/85) identity) and a non-WD40 72 amino acid stretch (368 - 439) (39% (28/72) identity). It is possible that the cDNA encodes a human ortholog of CHE-2.

DNA Alterations in *che-2* Mutants

The coding regions of the four known *che-2* mutant alleles (*e1033*, *mn395*, *m127*, *sa133*) were sequenced to identify the mutations responsible for their defects. In all the mutants, G to A transitions were found, which is consistent with the fact that all the mutations were induced with ethyl methanesulfonate (Anderson, 1995).

Three of them had nonsense-mutations almost at the same position: the mutation *e1033* altered the codon 601 from glutamine-encoding to a TAA stop codon, while the mutations *m127* and *sa133* were associated with the same change of the codon 599, from tryptophan-encoding to a TAG stop codon (Figure 6 and 7). Those three mutants seem to produce truncated forms of CHE-2 protein lacking about 160 amino acids in the C-terminal region.

In the remaining allele*mn395,* the codon 126 was changed from glycine- to glutamate-encoding (Figure 6 and 7). This substitution is in the first WD40 repeat of CHE-2. Although this allele shows weaker defects than the other alleles in various assay as mentioned above, the phenotypes still suggest the importance of WD40 repeats in the functions of CHE-2. CHE-2 might interact with other components of cilia to extend it, through WD40 motif.

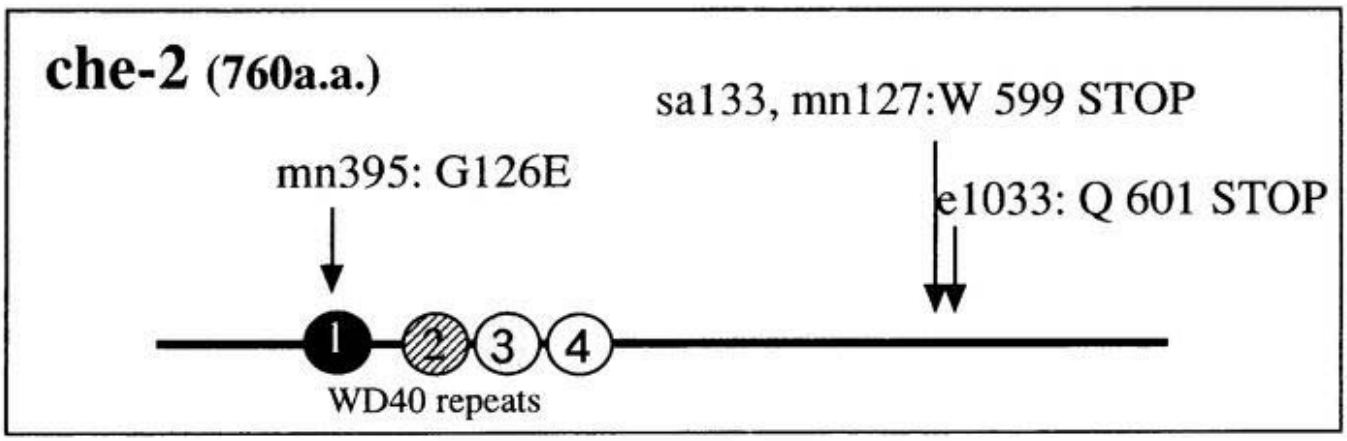
che-2 Is Expressed in Most Ciliated Sensory Neurons

To identify cells in which *che-2* is expressed, I used two GFP reporter genes containing a nuclear localization signal, pCHE-2::GFP1 and pCHE-2::GFP2 (Figure 2B). The former had a 2.9-kb 5' upstream sequence and only part of the exons of *che-2* gene, whereas the latter had a 650-bp 5' upstream sequence and all the coding sequence.

```
1     GGTTTAATTACCCAAGTTTGAG AATGAAATTAAAACTATCGGCGTCTCGGAAAACACGCCAC
1                             M  K  L  K  L  S  A  S  R  K  T  R  H

63    ACAGAAATGGTTTGTGGGGTGGGATGGATAGGCACCGAGGCCATATTGTCGGCAGCCGAT
14     T  E  M  V  C  G  V  G  W  I  G  T  E  A  I  L  S  A  A  D

123   GATCATGTTTTTCTGCTAACAAATACCGCCACAAATGAATCTCAACAAATTTTGAATATG
34     D  H  V  F  L  L  T  N  T  A  T  N  E  S  Q  Q  I  L  N  M

183   CCGGAAACTTTCTTCCCCACGAGTCTTCATATTTTTCCACGTTCCCAAACTAAAGGTGGT
54     P  E  T  F  F  P  T  S  L  H  I  F  P  R  S  Q  T  K  G  G

243   CAAAATGATGTGTTTGCAGTTTCAACTTCTGATGGAAAGATAAACATACTTTCGCGAAAC
74     Q  N  D  V  F  A  V  S  T  S  D  G  K  I  N  I  L  S  R  N

303   GGAAAAGTTGAAAACATGGTAGATGCTCACAACGGAGCAGCTCTCTGTGCTCGATGGAAT
94     G  K  V  E  N  M  V  D  A  H  N  G  A  A  L  C  A  R  W  N

363   TCTGATGGGACTGGATTGTTGTCAAGCGGAGAAGATGGGTTTGTGAAAATGTGGTCGCGG
114    S  D  G  T  G  L  L  S  S  G  E  D  G  F  V  K  M  W  S  R
                                                ▲ mn395
423   AGTGGAATGCTGCGTTCAGTGCTGGCTCAATTTGCAACAGCCGTTTACTGTGTGGCATGG
134    S  G  M  L  R  S  V  L  A  Q  F  A  T  A  V  Y  C  V  A  W

483   GACTCTACGTCTTCCAATGTTTTGTACTGTAACGCTGACCATTGTTATATCAAATCGTTG
154    D  S  T  S  S  N  V  L  Y  C  N  A  D  H  C  Y  I  K  S  L

543   AAAATGCAAGTTGCTCCGATAAAATGGAAAGCCCATGATGGAATCATTTTGTGTTGCGAC
174    K  M  Q  V  A  P  I  K  W  K  A  H  D  G  I  I  L  C  C  D

603   TGGAATCCAACTTCGGATTTGATAGTGACTGGCGGGGAAGATTTGAAGTTCAAGGTTTGG
194    W  N  P  T  S  D  L  I  V  T  G  G  E  D  L  K  F  K  V  W

663   GACGGATTTGGTCAGATCCTGTTCAATTCTTCTGTGCACGACTATCCTATAACAAGCATT
214    D  G  F  G  Q  I  L  F  N  S  S  V  H  D  Y  P  I  T  S  I

723   TCGTGGAACACTGATGGAACTCTGTTTGCTGTTGGATCTCATAATATTCTAAGACTATGT
234    S  W  N  T  D  G  T  L  F  A  V  G  S  H  N  I  L  R  L  C

783   GATAAGTCTGGATGGTCTCATTCTTTGGAGAAAATGAATGCTGGATCTGTGATGGCACTC
254    D  K  S  G  W  S  H  S  L  E  K  M  N  A  G  S  V  M  A  L

843   AGTTGGTCACCTGATGGAACTCAATTAGCCGTTGGCACTGCTGCTGGGCTTGTCTTCCAT
274    S  W  S  P  D  G  T  Q  L  A  V  G  T  A  A  G  L  V  F  H

903   GCACATATTATTGATAAGAGACTGACTTATGAAGAATTTGAAATTGTTCAAACCCAGAAA
294    A  H  I  I  D  K  R  L  T  Y  E  E  F  E  I  V  Q  T  Q  K

963   ACCGTTATAGAGGTTCGAGACGTCAGCAGTGAAGTTTCCCGAGAAACTCTCGAAACGAAA
314    T  V  I  E  V  R  D  V  S  S  E  V  S  R  E  T  L  E  T  K

1023  GAAAGAATATCGAAAATCGCGATTCTATATAAGTATCTGATTGTGGTCACATCATCCCAT
334    E  R  I  S  K  I  A  I  L  Y  K  Y  L  I  V  V  T  S  S  H

1083  ATCTATATTTACAGCTCCAAAAACTGGAATACTCCCACTATGATCGAGTACAATGAGAGA
354    I  Y  I  Y  S  S  K  N  W  N  T  P  T  M  I  E  Y  N  E  R

1143  ACTGTGAATATCATTGTGCAATGTGAAAAGATATTTCTTGTGTCCGATGGAATGACAATA
374    T  V  N  I  I  V  Q  C  E  K  I  F  L  V  S  D  G  M  T  I

1203  ACTATATTCACTTATGAGGGAAGAAAACTTATAAACCTAAACCCACCGGGTCAAGTAATG
394    T  I  F  T  Y  E  G  R  K  L  I  N  L  N  P  P  G  Q  V  M

1263  GCTCTACTAGACGAAAGAAAGATCGACCTGGCGAATGATACACTTGTGGTCCGAGATCGT
414    A  L  L  D  E  R  K  I  D  L  A  N  D  T  L  V  V  R  D  R

1323  GCCGACAATAAAGTCTTGCACTTTTTCGATCCAACAACAGGAAAAGCACAGGGTGATGGA
434    A  D  N  K  V  L  H  F  F  D  P  T  T  G  K  A  Q  G  D  G

1383  AATTTGAAACATGAGCATGATATTGTAGAGCTGACTGTGAATCAGTGCGGACCACTCAAT
454    N  L  K  H  E  H  D  I  V  E  L  T  V  N  Q  C  G  P  L  N

1443  GATAGAAATGTTGCCTTCCGTGATCAAATCGGTGCAGTTCACATAGCTATGGTGAAGACT
474    D  R  N  V  A  F  R  D  Q  I  G  A  V  H  I  A  M  V  K  T

1503  TTCGGTGTCAGCCAGAGGATGGTAAAAATAGGATCACTAGTTGAACAGCTTGTCTTCAAC
494    F  G  V  S  Q  R  M  V  K  I  G  S  L  V  E  Q  L  V  F  N

1563  GATGTCACAAATATGCTGTGTGGAATTTCGGAAGGAAAGATTGCTGTTTGGCCTCTTCCA
514    D  V  T  N  M  L  C  G  I  S  E  G  K  I  A  V  W  P  L  P

1623  AACGTTGCCTTCCACGACAGAAATCTTCTACAGAAATCATTAATACAGAAGAACATCGGC
534    N  V  A  F  H  D  R  N  L  L  Q  K  S  L  I  Q  K  N  I  G

1683  AGCGTCGGAAAATTTCCACAGTTGGCTAATTTTGCCGGAAATACAATAGTTATCAGGAAA
554    S  V  G  K  F  P  Q  L  A  N  F  A  G  N  T  I  V  I  R  K

1743  TCAGATGGATGTTTACTGCCAACTGGGATTCTTCCGTTCTATGGAACACTCATCACAATG
574    S  D  G  C  L  L  P  T  G  I  L  P  F  Y  G  T  L  I  T  M

1803  GCATCTCAATCAAAATGGGATCAAGCAATACGACTATGCAGAAGTATTGGAAATGACACA
594    A  S  Q  S  K  W  D  Q  A  I  R  L  C  R  S  I  G  N  D  T
   mn127/sa133 ▲        ▲ e1033
1863  ATGTGGGCGACTTTTGCTGGATTAGCTGTTCTCCACAAAAATATGATCGTTATGGAAATT
614    M  W  A  T  F  A  G  L  A  V  L  H  K  N  M  I  V  M  E  I

1923  GCCTATGCAGCATTGGAGGATGATGAAAAAGTTTCCCTGATCAACGAAATCAAAGACAAA
634    A  Y  A  A  L  E  D  D  E  K  V  S  L  I  N  E  I  K  D  K

1983  ACCGATAAAGAGACTCGTCAAGCTATGCAAGTGGTATTAACTGGAAAGCTCGCAGATGCT
654    T  D  K  E  T  R  Q  A  M  Q  V  V  L  T  G  K  L  A  D  A

2043  GATGTCTTGCTAGAAAGAAGTGGACTAAGCTTTCGCTCGCTTATGTTGAATATACAAATG
674    D  V  L  L  E  R  S  G  L  S  F  R  S  L  M  L  N  I  Q  M

2103  TTCAAGTGGAAAAGAGCACTCGAACTTGGCTTGAAAAATAAACAGTGGCTCGAAATTGTA
694    F  K  W  K  R  A  L  E  L  G  L  K  N  K  Q  W  L  E  I  V

2163  ATGGGATATCGTGAGAAATACTTGAAGAACTGTGGACAAAAAGAGACAGACCCTCTTTTT
714    M  G  Y  R  E  K  Y  L  K  N  C  G  Q  K  E  T  D  P  L  F

2223  TTAAAGCACATGTCAGAGGTCGAAATCGACTGGGTGCATATACGAGAGCTGATTGCCGCG
734    L  K  H  M  S  E  V  E  I  D  W  V  H  I  R  E  L  I  A  A

2283  GAAAAAGCTAAAGGAAACAATTGACTCCATTTTTGTATTTGTTTGATGTACCCATTCGAA
754    E  K  A  K  G  N  N  *

2343  TTGTTATAATTTACTCAAAAAATTTCTAATTTCAAAAAAAAAAAAA
```

Fig.6 cDNA and protein sequence of *che-2*. The SL1 *trans*-splice leader sequence at the 5' end is boxed. Four WD40 repeats are underlined. The residues marked with arrows are changed in *che-2* mutants. The sequence has been submitted to EMBL/GenBank/DDBJ database, accession number AJ011523.

Fig.7


che-2 (760a.a.)
sa133, mn127:W 599 STOP
mn395: G126E
e1033: Q 601 STOP
1
2
3
4
WD40 repeats

They showed an essentially identical expression pattern. I found that pCHE-2::GFP2 can rescue the dye-filling defect of *che-2(e1033)*. This is not strange, because in worms carrying this construct, GFP is present not only in the nuclei of amphid neurons, but also in the cilia (Figure 9A).

The *che-2*::GFP transgenics first expressed GFP in some head neurons between the comma and 1.5-fold stage of embryos (Figure 9B). The number of cells expressing GFP increased, as the development proceeded to the adult stage. The expression at the adult stage (Figure 9A) was detected in many ciliated sensory neurons as follows: all the amphid sensory neurons except AFD (AWA, AWB, AWC, ASE, ADF, ASG, ASH, ASI, ASJ, ASK, and ADL), phasmid neurons PHA and PHB, all the inner labial neurons (IL1 and IL2), all the outer labial neurons (OLQ and OLL), CEP, PDE, FLP, PQR, and unidentified 3 neurons (perhaps AQR and ADEL/R). Thus, *che-2* seems to be expressed in all the ciliated sensory neurons of *C. elegans* except BAG and AFD. The expression pattern is consistent with the report showing that most sensory cilia, with a possible exception of AFD, are abnormal in *che-2* mutants (Lewis and Hodgkin (1977), considering that CHE-2 acts cell-autonomously (see below). Since AFD neurons are known to act as a temperature sensor (Mori and Ohshima, 1995), the expression pattern is also consistent with the report showing that *che-2* mutants show no defect in thermotaxis (Perkins et al., 1986). AFD neurons have only a rudimentary cilium even in wild type animals (Perkins et al., 1986). The formation of the AFD and BAG cilia may be performed by a different mechanism that does not require CHE-2.

Since *che-2(e1033)* males show defects in mating (Lewis and Hodgkin, 1977), I observed GFP expression pattern in N2 males tails (Figure 9C), where there are many male-specific sensory neurons required for mating behavior. Although I could not identify all the GFP-expressing neurons, I could see that GFP was expressed at least in all the rays, which have ciliated sensory neurons (Sulston et al., 1980). Spicules also contain ciliated sensory neurons (Sulston et al., 1980), but I could not confirm the expression in spicules, due to strong autofluorescence.

Fig.8 GFP expression constructs

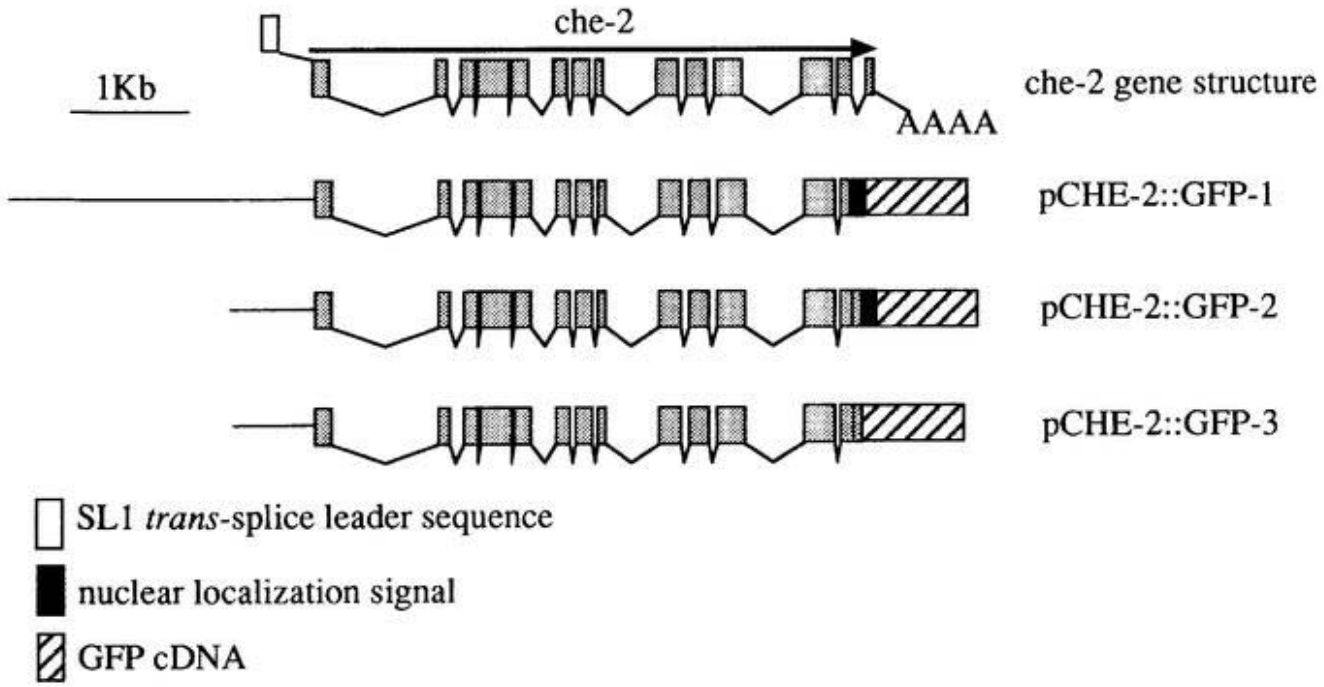


Fig.8 The structure of *che-2* gene and *che-2*::GFP fusion constructs in this study. *che-2* gene has 14 exons (heavily dotted box).

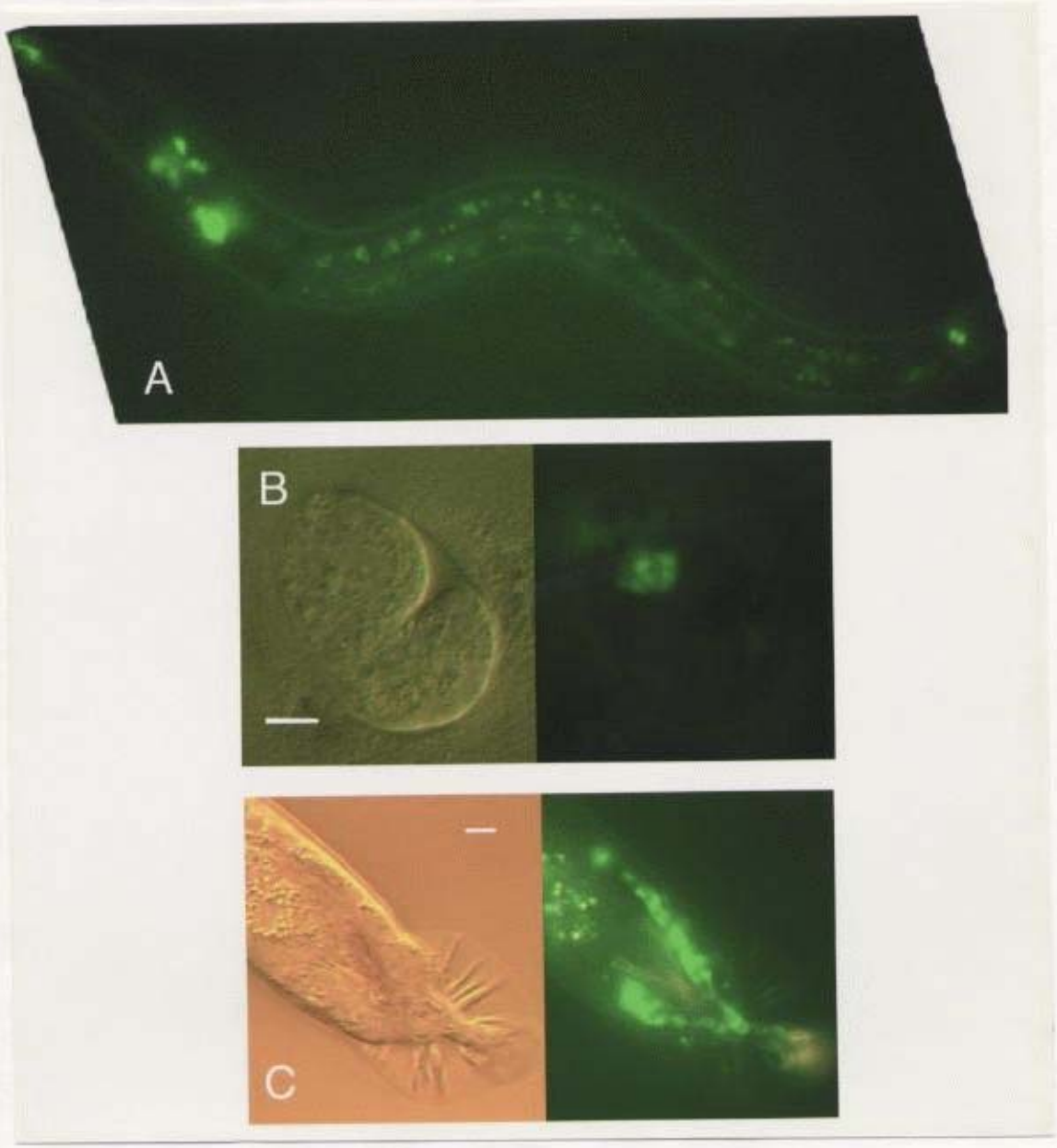


Fig.9 Expression of *che-2*::GFP fusion genes. (A) Expression pCHE-2::GFP2 in the L1 hermaphrodite. Almost all the ciliated sensory neurons show fluorescence. (B) Expression of pCHE-2::GFP2 in a mid-stage embryo. Some head neurons show fluorescence. (C) Expression of pCHE-2::GFP2 in the adult male tail. Ray neurons show fluorescence. The bars indicate 10 μm.

CHE-2 Is Localized in Sensory Cilia

To know the subcellular localization of the *che-2* product, I used pCHE-2::GFP3, which was made from pCHE-2::GFP2 by removing the nuclear localization signal. This construct also rescued the dye-filling defect of *che-2 (e1033)*. Most of the GFP produced from this construct was localized in cilia. This could be seen clearly in amphid sensory neurons (Figure 10), since their cilia are fasciculated (Perkins et al., 1986). The processes and cell bodies showed only faint fluorescence, and the nuclei were not fluorescent. These results suggest that the *che-2* product probably acts in sensory cilia.

che-2 Acts Cell-autonomously

To determine if *che-2* acts cell-autonomously, I expressed *che-2* in a subset (ASH and ASI) of amphid neurons in the *che-2 (e1033)* mutant, by using the *sra-6* promoter (*sra-6*::CHE-2) (Troemel et al., 1995). The use of *sra-6*::GFP as the injection marker enabled us to check whether the cilia of ASH and ASI neurons have abnormal shape and whether the extra chromosomal array of *sra-6*::CHE-2 and *sra-6*::GFP, which is lost at a certain probability, was still present in ASH and ASI neurons.

As mentioned above, the *che-2 (e1033)* mutant does not show dye-filling in any neuron. However, the *che-2 (e1033)* strain carrying *sra-6*::CHE-2 took up DiI specifically in ASH and ASI neurons (Figure 11A). Moreover, the morphology of the cilia of these neurons became almost normal, which I checked by using *sra-6*::GFP (Figure 11B). Since incubation in DiI solution for a long time caused penetrance of DiI into other neurons such as ASK, ASJ, and AWB neurons and further into sheath cells (see Materials and Methods). I therefore checked the morphology of AWB and ASJ cilia using *gcy-10*::GFP (which is expressed in AWB and AWC neurons) and *gpa-9*::GFP (which is expressed in ASJ neurons), respectively. The results showed that while ASH cilia recovered their normal morphology, ASJ cilia remained short (Figure 11B), and AWB cilia remained to be deformed (data not shown). Furthermore, this strain (*che-2(e1033); Ex[sra-6*::CHE-2*]*) showed an almost normal response to high osmotic strength (Figure 4C), which is mediated by ASH neurons (Bargmann et al., 1990), but

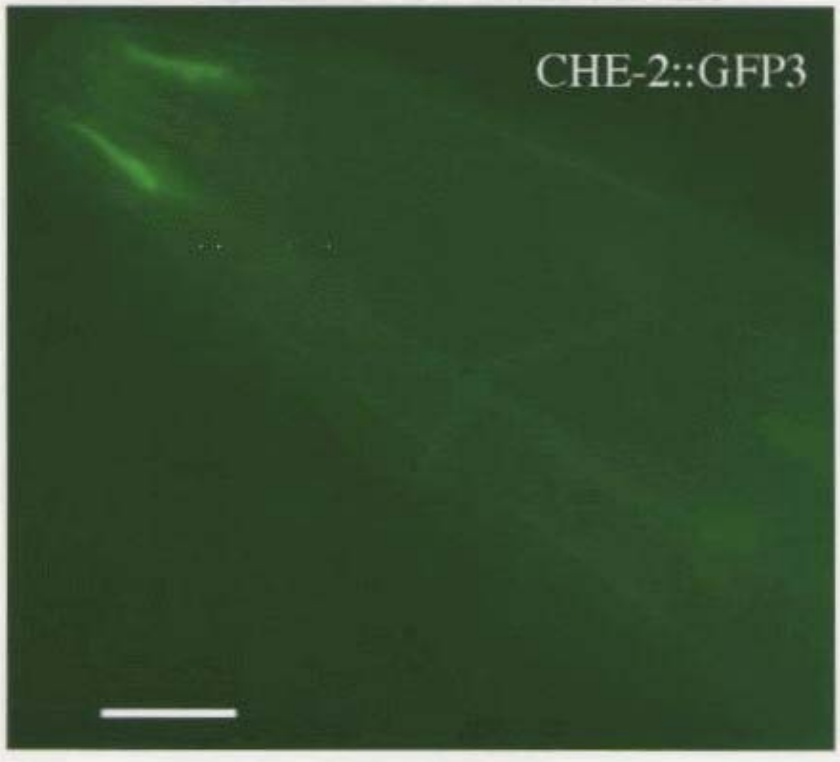
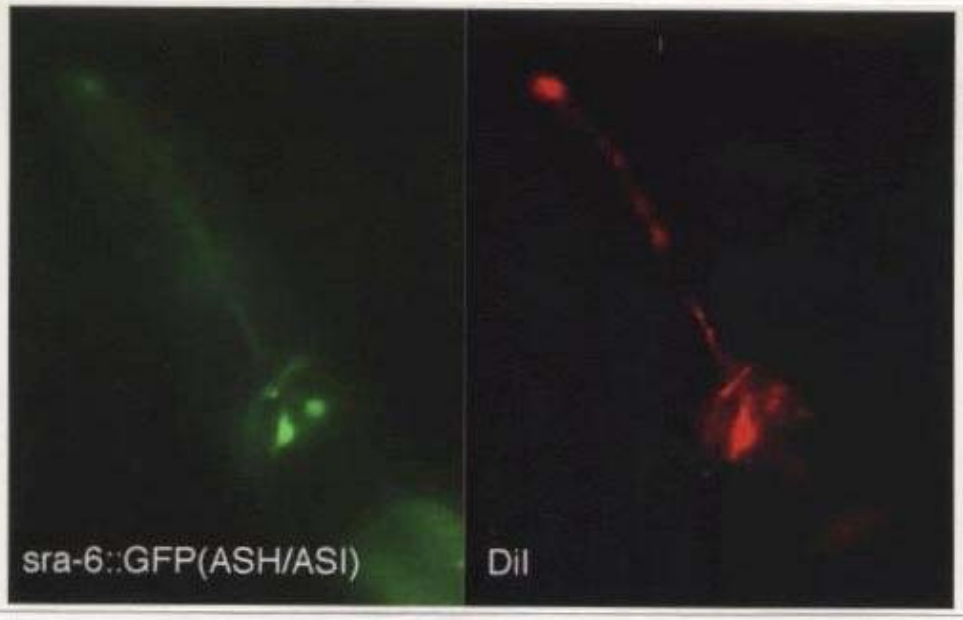


Fig.10 Localization of pCHE-2::GFP3 in the amphid neurons of an L3 hermaphrodite. The cilia show strong fluorescence, while the processes and cell bodies show faint fluorescence.

A



B

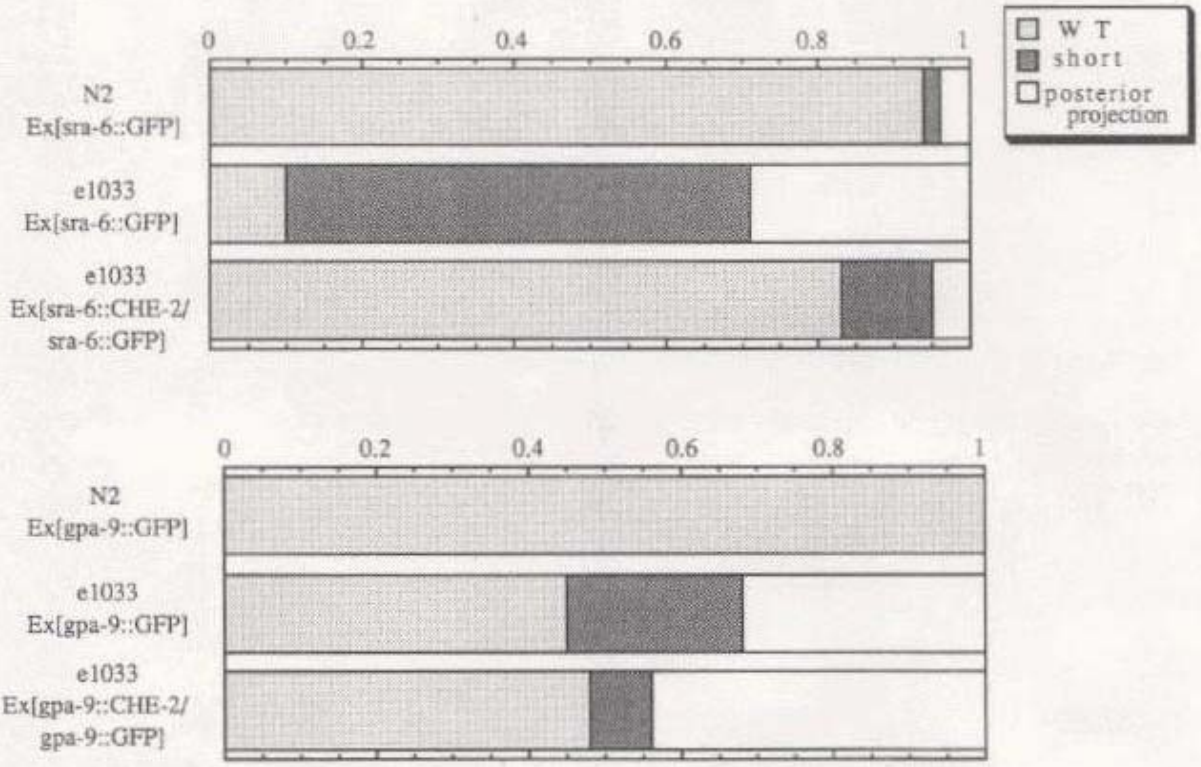


Fig.11 (A)Dye-filling of *che-2(e1033)* animals carrying *sra-6*::CHE-2 and *sra-6*::GFP. The animal expresses *sra-6*::GFP only in ASH and ASI neurons (left) and take up DiI in the same neurons (right). (B)The statistics of cilium morphology of the ASH and ASI cilia (visualizedf by sra-6::GFP), and ASJ cilia (visualized by gpa-9::GFP). The morphological defects of ASH and ASI cilia is rescued by expressing sra-6::CHE-2, while the ASJ cilia is not rescued. Each data point was obtained with 20 to 30 animals.

abnormal responses to NaCl and to benzaldehyde (Figure 4D), which are mediated mainly by ASE and AWC neurons, respectively (Bargmann and Horvitz, 1991a; Bargmann et al., 1993). I therefore conclude that the cilium morphology and functions of only the cells that expressed *che-2* were restored, i. e., *che-2* acts cell-autonomously.

che-2 Is Required for the Maintenance As Well As the Formation of Sensory Cilia

To know the developmental stage of *che-2* expression sufficient for cilium formation, I used a heat shock promoter to drive expression of *che-2* cDNA (pHSP::CHE-2) and analyzed the rescue of the dye-filling phenotype of *che-2(e1033)*. Without heat shock, the animals showed no dye-filling at all. I performed heat shock treatment (30°C, 6 hours) of the animals at various stages: embryos, L1 to L2 larvae, and adults. The heat-shock treatment at embryos rescued the defect of dye-filling (Figure 12). The percentage of animals that showed dye-filling at least in one neuron, had a maximum (over 80%) at 12 hours after the heat-shock (Figure 12A). The rescue continued at least for 80 hours after the heat shock. Surprisingly, the animals restored the ability of dye-filling, even if they were heat-shocked at larval or adult stages. In these cases, the percentage of animals that showed dye-filling at least in one neuron had a maximum (over 70%) also at 12 hours after the heat shock, and the effect continued at least for 48 hours.

Then I investigated the type and number of neurons that showed dye-filling after the heat-shock, for each animal. There were no neurons that preferentially took up DiI after the heat shock, among the 6 types of neurons (ASK, ADL, ASH, ASJ, PHA, and PHB) that showed strong dye-filling in wild-type animals. As shown Figure 12B, the average number (per animal) of the neurons that showed dye-filling reached a maximum at 12 to 24 hours after the heat shock. The number gradually decreased after the maximum. The result shows that the neurons that once restored the ability of dye-filling by the heat shock induction of *che-2*, lost the ability again. Hence, the *che-2* product

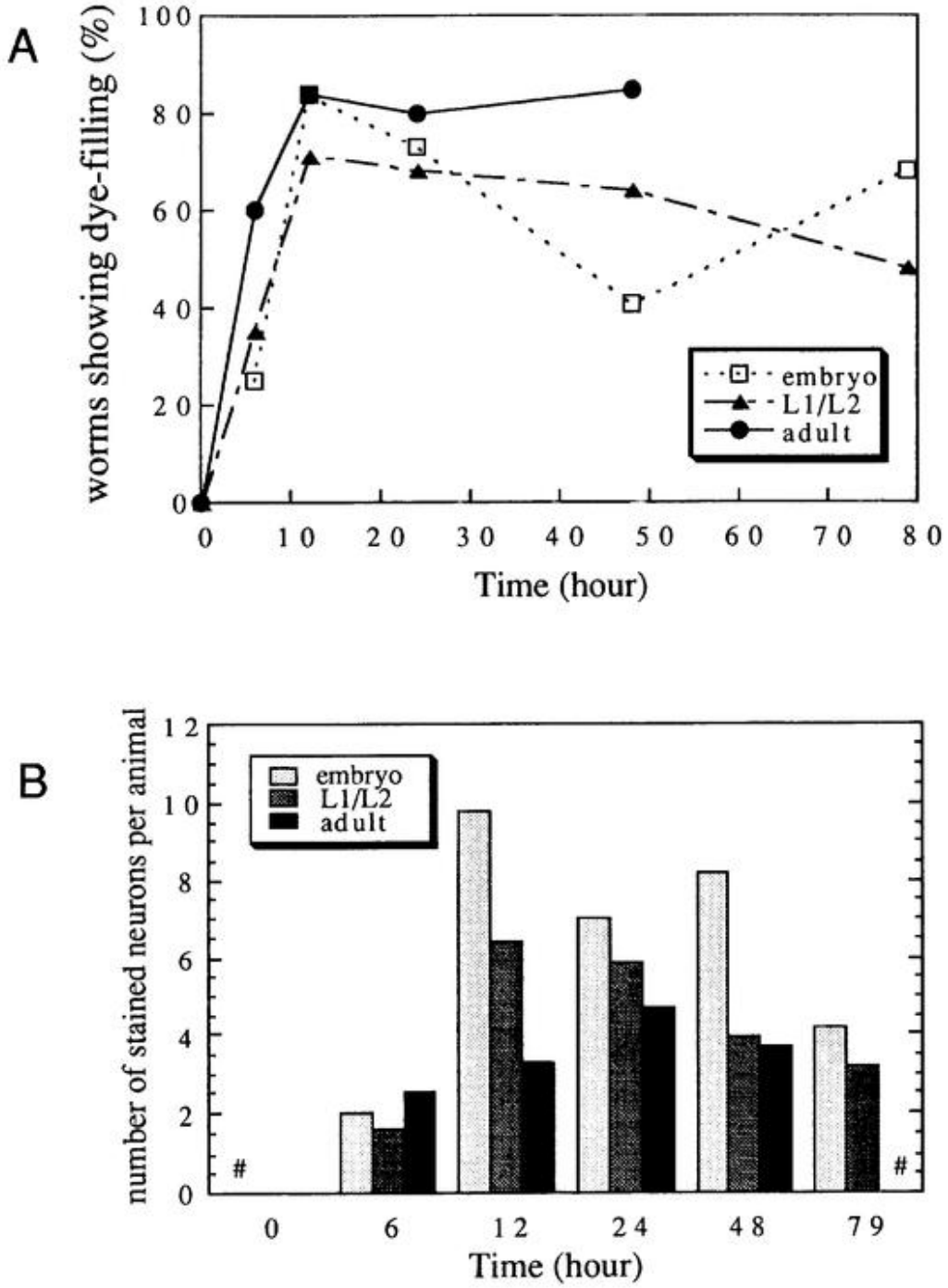


Fig.12 The dye-filling of *che-2(e1033)* animals carrying an *hsp::che-2* fusion gene (pHSP::CHE-2) after heat shock treatment. (A) The percentage of animals that showed dye-filling at least in one neuron. (B) The average number (per animal) of neurons that showed dye-filling. Heat shock was performed either at embryos, L1/L2 larvae, or young adults. Each data point was obtained with 20 to 30 animals. The data points at 0 hour of the animals heat-shocked at embryos and at 79 hours of the animals heat-shocked at young adults are missing (#), because most animals remained unhatched and could not be stained with DiI, and because the animals looked unhealthy, respectively. The *che-2(e1033)* mutant carrying only the vector did not show dye-filling at any time points (data not shown).

must be required not only for the formation but also for the maintenance of sensory cilia.

Since the recovery of dye-filling does not necessarily mean the recovery of cilium structure, I also checked the morphology of cilia before and after heat shock. For this purpose I used a *che-2(e1033)* strain carrying *sra-6*::GFP and pHSP::CHE-2 as an extrachromosomal array. Like the dye-filling experiment above, animals were heatshocked at embryos, L1 to L2 larvae or young adults. As shown in Figure13, the cilia of ASH and ASI neurons, as visualized with *sra-6*::GFP, extended after the heat shock in all the cases. The peak of highest recovery was just after hatching when the animals were heatshocked at embryos, and 12 hours after the heat shock when the animals were heatshocked at larvae or young adults. Degeneration of cilia long after the heat shock was also found at least for animals heatshocked at embryos and adults.

To detect the extension of cilia more precisely, I compared the cilium morphology of the same animals just before and 15 hours after the heat shock of young adults. As shown in Figure14, this experiment clearly showed that cilia can extend by heat-shock induction of *che-2* even at the adult stage. Furthermore, I noticed that some cilia extended in a bent form rather than the normal straight form (Figure14, the lowest example). Since such bent cilia showed abnormality in dye-filling (data not shown), it seems that they do not extend along the pore made by the socket cell. Thus, there must be a mechanism that enables cilia to extend straight into the pore in normal development, and the mechanism often does not work in the enforced, heterochronic extension of cilia at the adult stage.

Genetical Interaction of che-2 with Other Genes

Many mutants that show dye-filling defect and/or abnormal morphology of cilia are known in *C. elegans*. And many components of cilia such as chemo-receptors and nucleotide gated channels have been reported. Does CHE-2 protein cooperate with the products of other cilium structure genes in the formation of cilia? Does CHE-2 protein interact with signal transduction molecules localized at cilia, possibly through the WD40

cilia morphology is rescued by heat shock induction of CHE-2

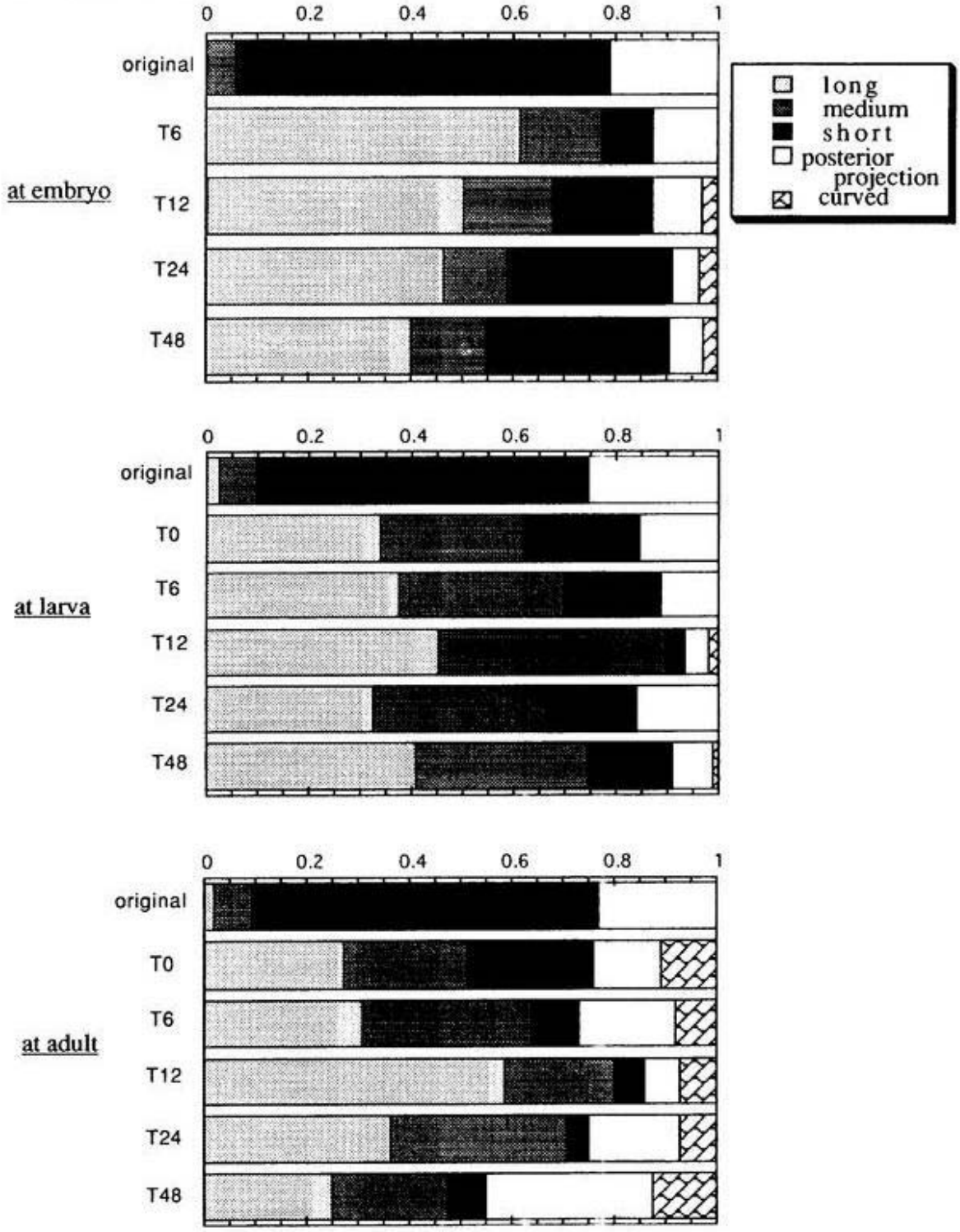


Fig.13 The extension of cilia in *che-2(e1033)* animals carrying *hsp::che-2* (pHSP::CHE-2) and *sra-6*::GFP, after heat shock treatment. Heat shock was performed either at embryos, L1/L2 larvae, or young adults. The graphs show cilium morphology statistics of ASH and ASI neurons of 20 to 30 animals at various points after heat-shock induction. "Tx" means data at x hours after the heat shock. The graphs lack T0 point for heat shock at embryos, because many embryos did not hatch yet. "original" means the statistics of cilium morphology before the heat-shock induction, except that for the embryonic stage induction it means the cilium morphology of the T6 control, which was not heat-shocked.

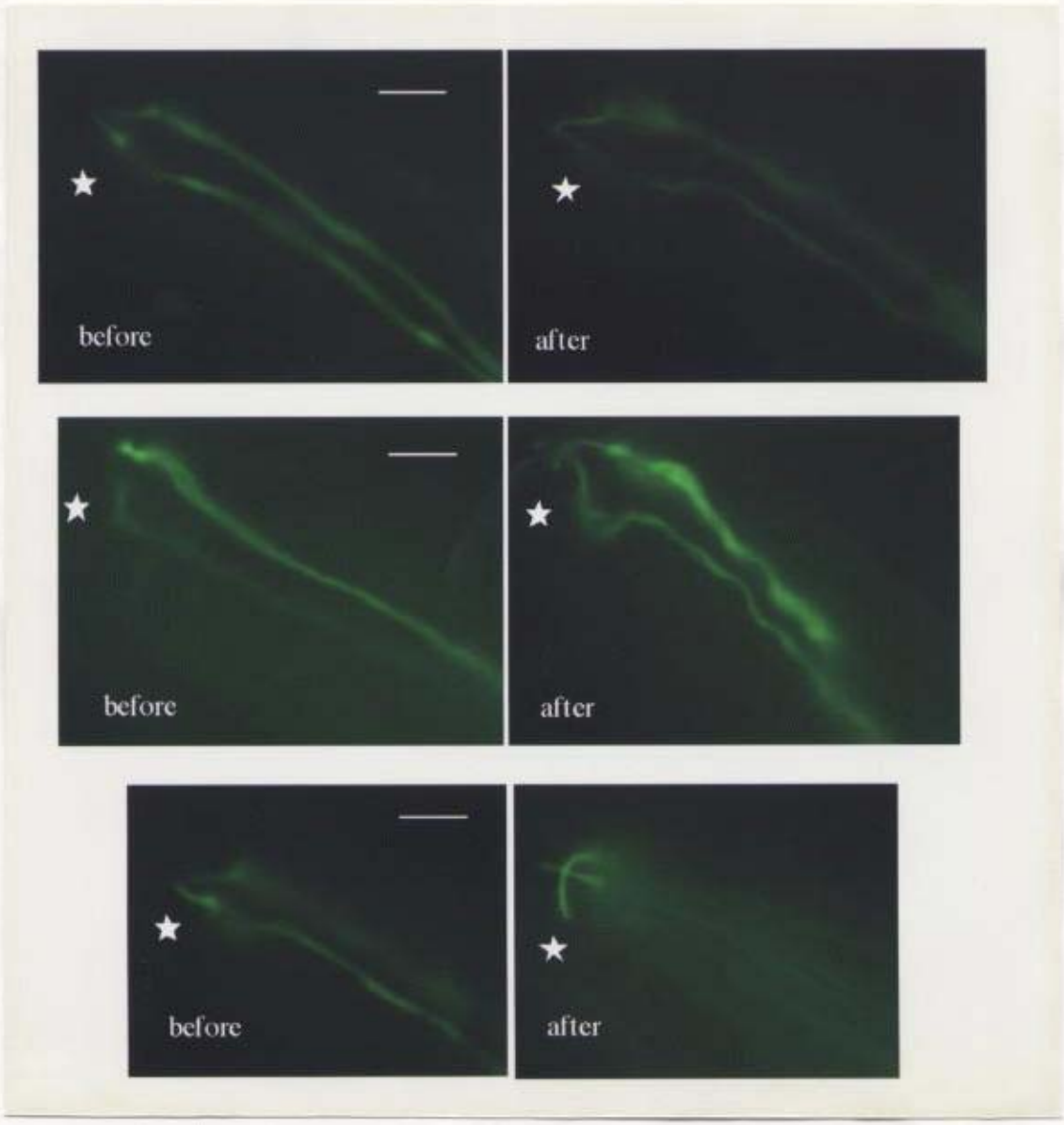


Fig.14 Three examples of cilia extension. The figures compare the cilia of the same *che-2(e1033)* animals carrying *hsp::che-2* (pHSP::CHE-2) and *sra-6*::GFP before and after heat shock. In the bottom figure it can be seen that the cilia not only extended, but also bent. Scale bars indicate 5μm

repeats? To answer to these questions, I studied the interaction of *che-2* with other genes.

#ODR-10::GFP expression in *che-2* mutants

ODR-10 is the diacetyl receptor of *C. elegans*, which is expressed in AWA chemosensory neurons and localized at cilia in wild type animals (Sengupta et al., 1996). I tested whether ODR-10 could be localized also at cilia in *che-2* mutants, using two alleles of *che-2* (*e1033, mn395*) and *odr-10*::GFP as an extrachromosomal array. If CHE-2 protein works in anchoring membrane-bound components to the cytoskeleton of cilia, ODR-10 protein might not be localized at cilia in *che-2* mutants The result showed that ODR-10::GFP was localized at the cilia in the *che-2* mutants (Figure 15) like in wild type animals, although the cilia of AWA neurons in the *che-2* mutants were slightly deformed. The result also indicates that CHE-2 activity is not necessary for the expression of ODR-10. The expression of another chemo-receptor, however, was observed to be downregulated in *che-2* mutant (C.Bargmann, personal communication).

#CHE-2::GFP3 expression in *osm-3* and *che-3* mutants

osm-3 and *che-3* encode *C. elegans* kinesin and dynein, respectively (Shakir et al., 1993; C. J. de Vries, S. R. Wicks, H. G. A. M. Van Luenen, and R. H. A. Plasterk, personal communication), and their mutants show the cilium-defective phenotype. Hence I suspected that they may play a role in the transport of the components of sensory cilia in dendrites. To test this hypothesis, I investigated the localization of the product of pCHE-2::GFP3 in the *osm-3* and *che-3* mutant backgrounds. If the hypothesis were true, the pCHE-2::GFP3 product would not be transported to the base of cilia and instead accumulated in cell bodies. In *osm-3* mutant, the GFP looks slightly down-regulated and rather diffused. However the most of CHE-2::GFP is also localized at the base of cilila (figure 16) in the mutants, although the cilia of *osm-3* are short as observed before (Perkins et al., 1986). In *che-3* mutant, the localization of

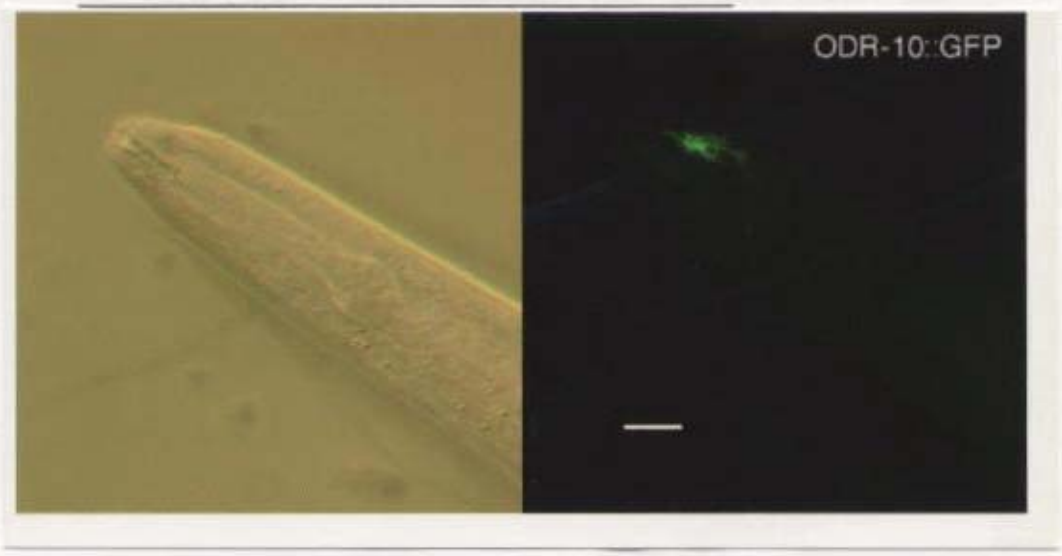


Fig.15 Localization of ODR-10::GFP in a *che-2(e1033)* mutant animal. ODR-10::GFP is localized at the cilium of AWA neurons in *che-2* mutants, like in wild type animals.

CHE-2::GFP at cilia is more obvious, although those are bulb-shaped as also reported before (Perkins et al., 1986). Thus, CHE-3 and OSM-3 may be involved in intraciliary transport rather than transport in dendrites. This is consistent with the report that c-CHE-3 is localized at cilia (C.J.de Vries, S.R.Wicks, H.G.A.M.Van Luenen, and R.H.A.Plasterk, personal communication). It also supports the parallelism between the sensory cilia of *C. elegans* and the flagella of *Chlamydomonas*, because the *Chlamydomonas* OSM-3 homolog (FLA10 protein) acts in intraflagellar transport (Kozminski et al., 1993, 1995, Cole et al., 1998).

#Interaction of CHE-2 with GPA-3

gpa-3 encodes a G-protein α subunit, which is expressed in chemosensory neurons (Zwaal et al., 1997). Loss-of-function mutants in *gpa-3* show responses to dauer-inducing pheromone. On the other hand, an activated form of this gene results in dauer formation under non-dauer-forming conditions and abnormality in dye-filling (Zwaal et al., 1997). One possibility is that CHE-2 is a component of a signal transduction system that inactivates GPA-3 to allow normal extension of cilia, and that the lack of CHE-2 results in abnormal activation of GPA-3, causing a defect in cilium formation. I tested this possibility by investigating the phenotype of the double mutant *gpa-3(lf); che-2*. If the cilium-defective phenotype of *che-2* mutants were caused by abnormal activation of GPA-3, the *gpa-3(lf)* mutation could suppress the defect of *che-2*. However, the double mutant still showed a dye-filling defective phenotype.

Next, I tested another possibility, i. e., the activated form of GPA-3 might show a dye-filling defect by blocking the activity of CHE-2. For this purpose, I introduced pCHE-2::GFP3 into a *gpa-3(gf)* mutant. Because the product of pCHE-2::GFP3 has CHE-2 activity, I expected that the overexpression of pCHE-2::GFP3 as an extrachromosomal array could rescue the *gpa-3(gf)* phenotype. Furthermore, the fluorescence of the pCHE-2::GFP3 product would be reduced, if the activated form of GPA-3 block the CHE-2 activity by inhibiting the transcription of *che-2*. However, I could not observe the rescue of the *gpa-3(gf)* phenotype, nor the reduction of

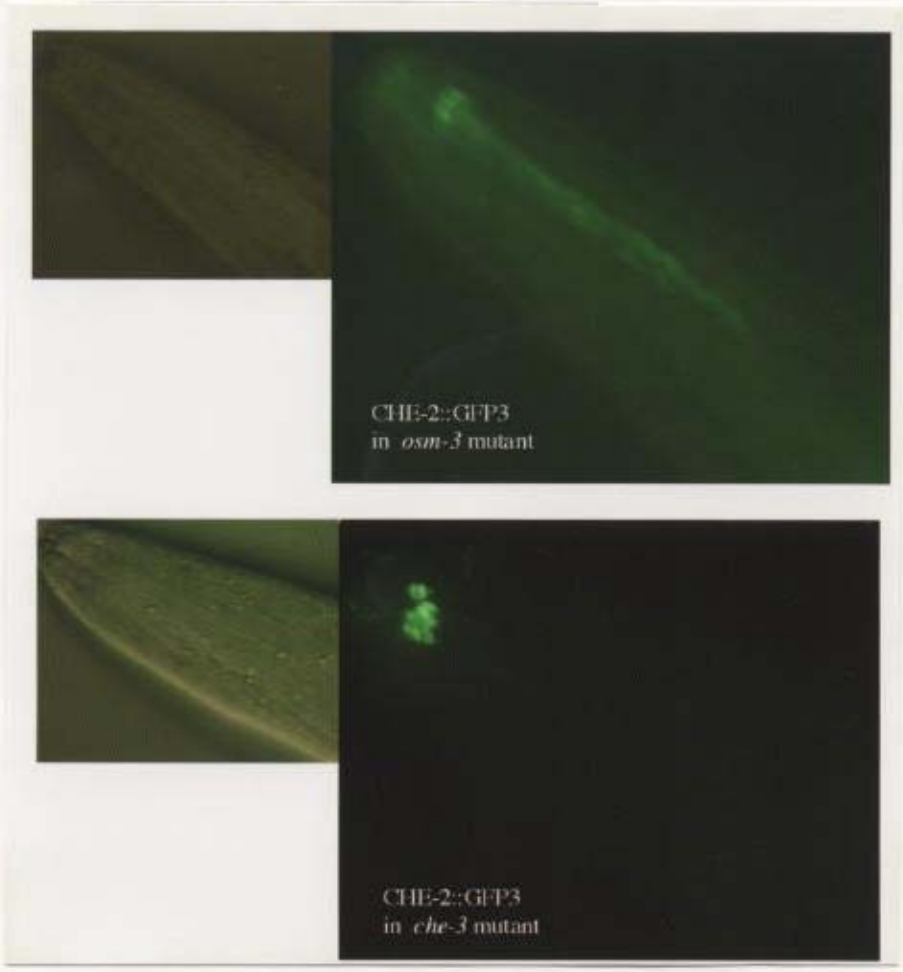


Fig.16 The expression of CHE-2::GFP3 (pCHE-2::GFP3) in the *osm-3(p802)* and *che-3(e1124)* mutants. In the *osm-3* mutant (top), GFP looks slightly down-regulated and rather diffuse. Nevertheless, most GFP is localized at the base of cilia. The cilia in *osm-3* are short . In the *che-3* mutant (bottom), the localization of GFP at cilia is more obvious. The cilia in *che-3* are bulb-shaped.

fluorescence of the pCHE-2::GFP3 product. Hence I conclude that the lack of CHE-2 and the activation of GPA-3 cause defects in cilium morphology through two separate pathways.

Is CHE-2 Associated with Microtubules?

If CHE-2 protein is a structural component of cilia and/or a component of a transport system in cilia, it might be associated with a microtubule, the main cytoskeletal component of cilia. To test this possibility, I expressed the *che-2* product in mammalian culture cell lines and investigated its localization. I used mammalian culture cells, because the cilia of *C. elegans* is too small to investigate detailed subcellular localization by a light microscopic method.

First, I made two constructs for the expression. In these constructs, *che-2* cDNA was ligated to the GFP cDNA in frame, and the fusion gene was put under mammalian general promoters, CMV and CAG promoters, respectively. I confirmed that the GFP fusion protein retained the *che-2* activity, by expressing it under the *sra-6* promoter in a *che-2* mutant of *C. elegans* and checking dye-filling ability.

Next, these constructs were transfected into NIH3T3 and NG108 culture cell lines by a lipofection method. NIH3T3 was chosen because it is suited for the observation of mictrotubules, while NG108, a hybridoma of neuroblastoma and glioma, was chosen because it resembles neurons. When the DNA was tranfected transiently, dead cells were accumulated after 48 hours. I therefore stopped the culture 24 hours after the transfection and fixed the samples. After fixation, I incubated the samples with an alpha-tubulin primary antibody (DM1A), followed by incubation with a Cy3-conjugated secondary antibody.

As shown Figure 17, however, I could not find any colocalization of CHE-2::GFP with microtubules in NIH3T3 cells, nor any localization of CHE-2::GFP in the neuronal processes of NG108 cells. In both cell lines, CHE-2::GFP was distributed uniformly in cytosol. The results mean that CHE-2 is not associated with mammalian microtubules, but they do not necessarily exclude the possibility that CHE-2 may be associated with

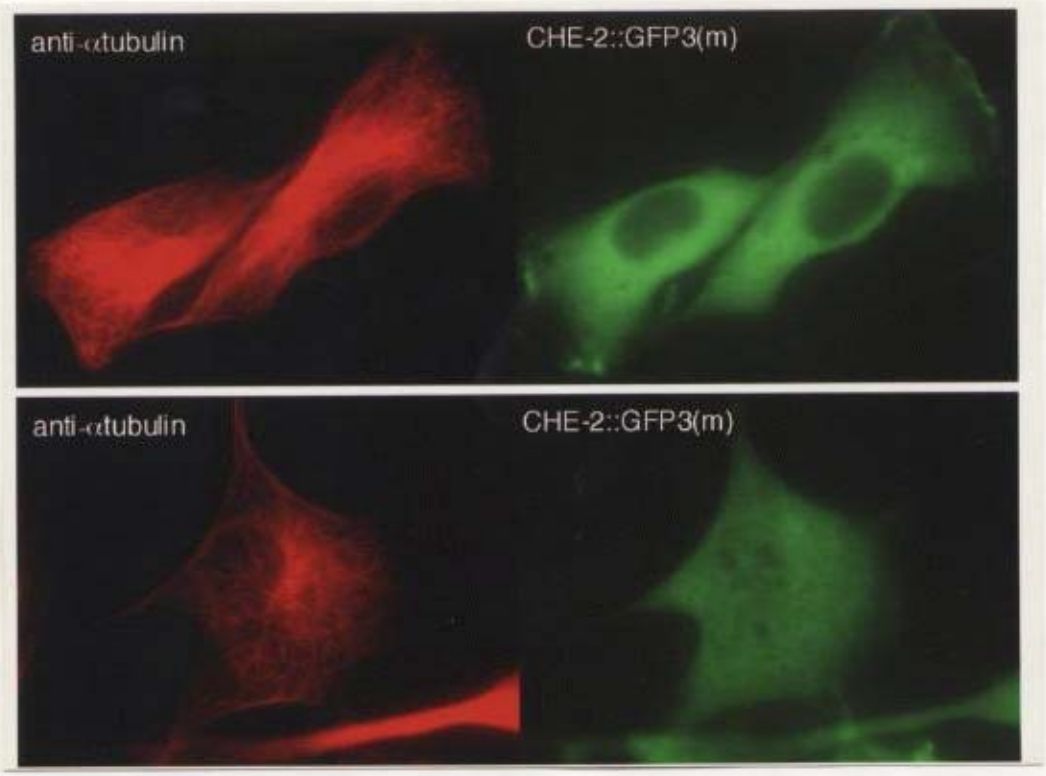


Fig.17 Localization of CHE-2::GFP3(m) in NIH3T3 cell lines. The red color shows the location of anti-α tubulin. The green color shows the location of CHE-2::GFP3(m). While the filamentous structure of microtubules is clearly seen (red), the CHE-2::GFP fusion protein (green) is diffused in the cytosol of NIH3T3 cells.

C. elegans microtubules. Thus I could not derive any conclusion from these experiments.

DISCUSSION

che-2 Encodes a New Member of the WD40 Protein Family

The data presented here reveal that *che-2* encodes a new member of the WD40 protein family. Proteins of this family seem to act in diverse functions, such as vesicle traffic, gene transcription and cytoskeletal assembly (Neer et al., 1994). Nevertheless, it is known that many of them form multiprotein complexes by interacting with other proteins through the WD40 repeat region (Neer et al., 1994; J.Sondek et al., 1996).

In *che-2 (mn395)*, a glycine residue in the first WD40 repeat (Gly126) is changed to a glutamate residue. The result shows that the WD40 repeat in CHE-2 plays an important role in its function. WD40 repeats is known to be involved in the protein-protein interaction and the formation of multiprotein complexes. It may means that the protein-protein interaction through WD40 motif is also necessary for the CHE-2 function.

Three other alleles (*e1033, sa133, m127*) had nonsense mutations almost at the same position in the C-terminal region. These mutations had severe defects in all the phenotypes. Hence, the C-terminal region of CHE-2 must play an important role in the function. Conservation of a part of the non-WD40 sequence between *che-2* and zs23c07 supports this argument.

The Function of CHE-2 Protein

Many investigations, including this study, provide the following data for speculating the function of CHE-2 protein. First, the *che-2(e1033)* mutant lacks a major part of the cilium in many sensory neurons, and instead has an abnormal projection extending posteriorly at the base of the abnormally short cilium (Lewis and Hodgkin, 1977; Perkins et al., 1986). I found that the posterior projections in *che-2* are made

roughly at the time when cilia are formed in wild-type animals. Moreover, I never detected normal cilia in *che-2* before the formation of the posterior projections or any time. These observations show that *che-2* is required for the extension of cilia. They also suggest that the posterior projections are formed probably by the accumulation of materials that cannot be transported into cilia. It also denies the hypothesis suggested by Lewis and Hodgkin (1977) that the abnormal posterior projection is made by the degeneration of a cilium once extended normally. Second, CHE-2 protein is expressed in almost all the ciliated sensory neurons and localized in cilia (this study). Third, *che-2* gene acts cell-autonomously (this study). Fourth, I found that in the *che-2* background, the expression of *che-2* either at the embryonic, larval, or adult stage is sufficient for cilium formation, as detected by the dye-filling assay and by cilium morphology. The time required for the cilium formation (about 10hrs) is almost independent of the stage of *che-2* expression. These results show that a process specific to a special developmental stage is not involved in the formation of normal cilia, possibly except for the mechanism that allows straight extension of cilia into the socket cell pore. I also found that the dye-filling ability was gradually lost again after turning off the *che-2* expression. This result indicates that the *che-2* product is needed not only for the formation but also for the maintenance of cilia, and that continuous turnover of CHE-2 takes place in cilia.

On the basis on these data, I propose three possibilities for the function of CHE-2. One is that CHE-2 is a structural component of sensory cilia, such as some microtubule-associated proteins. CHE-2 plays a role in the assembly of microtubules and stabilize them as an accessory structure. However, no accessory structure that can be a candidate of CHE-2 has been found by electron microscopy in the sensory cilia of *C. elegans*. The cilium structure consists of nine outer doublet microtubules and a variable number of central microtubules, and structures attached to the microtubules, such as the dynein or nexin arms and radial spokes, are apparently absent (Perkins et al., 1986).

A second possibility is that the *che-2* product may be a molecule that acts in the transport of the cilium precursor proteins in cilia, probably in cooperation with the OSM-3 kinesin and the CHE-3 dynein, which are essential for the formation of sensory cilia. In the *Chlamydomonas* flagella, it was reported that the protein components are transported by a kinesin to the distal tip (Cole et al., 1998) where most of the assembly of the microtubular axoneme takes place (Johnson and Rosenbaum 1992), while a dynein plays a role in the retrograde transport (Pazour et al., 1998). Both the kinesin and the dynein are essential for the formation and maintenance of flagella. On the other hand, it is known that the OSM-3 kinesin and the CHE-3 dynein of *C. elegans* are expressed in the ciliated chemosensory neurons open to the external environment and all the ciliated sensory neurons, respectively (Tabish et al., 1995; C. J. de Vries, S. R. Wicks, and R. H. A. Plasterk, personal communication). CHE-3 is shown to be localized in the cilia. Therefore, OSM-3 in chemosensory cilia and CHE-3 in all sensory cilia seem to play a role similar to the *Chlamydomonas* flagellar kinesin and dynein, respectively, while another kinesin may be used for this purpose in non-chemosensory cilia. However, mutants in *osm-3* and *che-3* have apparently weaker morphological phenotypes than those in *che-2*. Mutants in *che-2* have very short cilia consisting only of the proximal segment. In contrast, mutants in *che-3* have truncated cilia having enlarged, bulb-shaped endings filled with dark ground material (Lewis and Hodgkin, 1977), while mutants in *osm-3* have both the proximal and the middle segment (Perkins et al., 1986).

A third possibility is that CHE-2 may be a component of a signal transduction system. Recently, some G protein mutants (*gpa-3(gf)* and *odr-3(lf)*) were shown to have defects in sensory cilium formation, as mentioned in the Introduction. These G proteins act in the signal transduction of pheromone-sensation (Zwaal et al., 1997) and olfaction as well as osmosensation and mechanosensation (Roayaie et al., 1998), respectively, in amphid sensory neurons. Therefore, these signal transduction systems may regulate cilium structure indirectly. Another piece of evidence that connects cilium

structure and signal transduction arose from studies on another cilium structure gene, *osm-6*. This gene encodes a novel protein with a motif that might interact with SH3 domains (Collet et al., 1998), which often plays a role in signal transduction (Raymond et al., 1996). Those results suggest that signal transduction is required for the correct cilium formation. Some WD-repeat proteins such as Gβ subunit are known to act in signal transduction. CHE-2 may participate in the signal transduction required for the cilium formation, together with ODR-3, GPA-3 or OSM-6.

Controlled Expression of *che-2* Can Be Used for Determining the Functional Identity of Ciliated Sensory Neurons

Since all the neurons in *C. elegans* can be identified in living animals, the functions of each type of neurons have been investigated by killing them with a laser beam and by examining the functional abnormalities of the resulting animal (Chalfie et al., 1985; Avery and Horvitz, 1987). The functions of many sensory neurons, such as touch response, osmotic avoidance, chemotaxis, etc., have been determined by this method (Chalfie et al., 1985; Bargmann and Horvitz, 1991a,b; Kaplan and Horvitz, 1993; Mori and Ohshima, 1995). However, this method has a drawback. It requires laborious work and is not suited for experiments in which many neurons have to be killed or in which a large number of animals are needed for the functional assay.

In this study I expressed *che-2* in a *che-2* mutant under the control of the *sra-6* promoter, which gives expression in ASH and ASI neurons. The cells expressing *che-2* were confirmed by a *sra-6*::GFP fusion gene in the same extrachromosomal array as the *sra-6::che-2* fusion gene. The animals showed dye-filling only in ASH and ASI neurons but not in other 12 sensory neurons that show dye-filling in wild-type animals. Furthermore, they restored the ability of osmotic avoidance, which is mediated by ASH neurons, but not the ability of chemotaxis to NaCl or benzaldehyde, which is mediated at least mainly by cells other than ASH and ASI. The results show that *che-2* acts cell-

autonomously for ASH and ASI, and that the same is probably true for other *che-2*-expressing cells.

This experiment provides the basis of an alternative method for determining which subset of neurons are sufficient for a certain function, provided that the function is impaired in *che-2* mutants. Recent studies have revealed many promoters that positively regulate expression in various neurons (Troemel et al., 1995; Yu et al., 1997). Using these promoters, *che-2* can be expressed in various subsets of sensory neurons and test the animals for various *che-2* functions, as I did with the *sra-6* promoter. If the animals are normal for a certain *che-2* function, the neurons that express *che-2* in these animals must be sufficient for the function, because *che-2* seems to act cell-autonomously. This method has an advantage over the laser-killing method. To show which neurons are sufficient for a certain function, it is enough to use a promoter for a limited number of neurons, instead of killing many neurons.

A General Mechanism for the Formation of Cilia and Flagella May Exist.

Two pieces of evidence suggest that there may be a general mechanism in the formation of sensory cilia and motile cilia/flagella probably in most eucaryotes. First, the cDNA represented by the EST clone zs23c07 may encode a human ortholog of CHE-2, although it was isolated from tonsillar cells, which apparently have no cilia or flagella. Second, there are homologs of OSM-6 and OSM-1 in the IFT particles of *Chlamydomonas reinhardtii* (Cole et al., 1998), while mutants in *osm-6* and *osm-1* show essentially the same cilium-defective phenotype as *che-2* mutants (Perkins et al., 1986). However, the function of zs23c07 and the role of IFT particles remain to be investigated. Studies using various biological organisms, especially those on the *C. elegans* cilium structure genes and on the *Chlamydomonas* IFT particle components, will benefit each other in elucidating the general mechanisms for the formation of sensory cilia and motile cilia/flagella.

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