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A novel WD40 protein, CHE-2, acts cell-autonomously in the formation of *C. elegans* sensory cilia

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SUMMARY

To elucidate the mechanism of sensory cilium formation, we analyzed mutants in the *Caenorhabditis elegans che-2* gene. These mutants have extremely short cilia with an abnormal posterior projection, and show defects in behaviors that are mediated by ciliated sensory neurons. The *che-2* gene encodes a new member of the WD40 protein family, suggesting that it acts in protein-protein interaction. Analysis of mutation sites showed that both the amino-terminal WD40 repeats and the carboxyl-terminal non-WD40 domain are necessary for the CHE-2 function. CHE-2-tagged green fluorescent protein is localized at the cilia of almost all the ciliated sensory neurons. Expression of *che-2* in a subset of sensory neurons of a *che-2* mutant by using a heterologous promoter resulted in restoration of the functions and cilium morphology of only the *che-2*-expressing neurons. Thus, *che-2* acts cell-autonomously. This technique can be used in the future for determining the function of each type of *che-2*-expressing sensory neuron. Using green fluorescent protein, we found that the extension of cilia in wild-type animals took place at the late embryonic stage, whereas the cilia of *che-2* mutant animals remained always short during development. Hence, the abnormal posterior projection is due to the inability of cilia to extend, rather than degeneration of cilia once correctly formed. Expression of *che-2* in a *che-2* mutant under a heat shock promoter showed that the extension of cilia, surprisingly, can occur even at the adult stage, and that such cilia can function apparently normally in behavior.

Key words: WD40 repeat, Sensory cilium, *C. elegans*

INTRODUCTION

Many sensory neurons, such as olfactory receptor neurons, cone and rod cells (photoreceptor neurons) and hair cells (mechanoreceptor neurons) in vertebrates, have cilia at their tips. These sensory cilia have a special arrangement of microtubules, like motile cilia. Because the cilia contain chemo-, mechano- or photo-receptors and various signal transduction components such as G proteins, adenylate cyclases, ion channels, etc. (Pace et al., 1985; Nakamura and Gold, 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. Experiments on olfactory neurons showed that sensory cilia are important for sensing odors, because the neurons lacking cilia do not respond to odors and because maximal responses are evoked by pulses applied to the cilia (Kurahashi, 1989; Lowe and Gold, 1991). Recently, studies on *Drosophila* photoreceptor neurons demonstrated that the signaling components of the phototransduction cascade are organized as a functionally important macromolecular complex in the rhabdomere (non-ciliated ending) (Tsunoda et al., 1997; Montell, 1998). However, the way sensory cilia are formed during development and the spatial organization of signal components in sensory cilia remain to be revealed.

C. elegans is a good model organism for studying the structure, function and development of the nervous system. The morphology of sensory cilia has been studied by electron microscopy (Lewis and Hodgkin, 1977; Albert et al., 1981; Perkins et al., 1986; White et al., 1986). Genes required for the formation of cilia have been revealed by isolation of mutants abnormal in the cilium structure (Lewis and Hodgkin, 1977; Perkins et al., 1986; Starich et al., 1995). Of those genes, *osm-3* and *che-3* encode a kinesin heavy chain (Shakir et al., 1993; Tabish et al., 1995) and a cytosolic dynein heavy chain isotype (S. R. Wicks, G. Jansen and R. H. A. Plasterk, personal communication), respectively. In addition, 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 abnormal cilium structure (Zwaal et al., 1997; Roayaie et al., 1998). The analysis of these and other genes required for normal cilium structure should help to elucidate the mechanism of cilium formation.

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., 1995); so is dynein, which plays a role in the retrograde transport (Pazour et al., 1998). The IFT particles are composed of 15 polypeptides, and two of them have similarity in amino acid sequence to the protein products of *C. elegans osm-6* and *osm-1* genes (Cole et al., 1998), which are required for normal cilium structure (Perkins et al., 1986). The formation and maintenance of sensory cilia and motile cilia/flagella, therefore, may be achieved by a general mechanism common to many organisms.

C. elegans mutants of *che-2* have extremely short cilia, often with an abnormal posterior projection in most ciliated sensory neurons (Lewis and Hodgkin, 1977; Perkins et al., 1986). They also show defects in various behaviors mediated by some of these neurons: avoidance of high osmotic pressure (Perkins et al., 1986) (mediated by ASH neurons; Bargmann et al., 1990), chemotaxis to NaCl (Lewis and Hodgkin, 1977; Perkins et al., 1986) (mainly ASE neurons; Bargmann and Horvitz, 1991) and to many odorants (Bargmann et al., 1993) (AWA/AWC neurons; Bargmann et al., 1993), and male mating (Hodgkin, 1983) (male ray neurons etc.; Liu and Sternberg, 1995). In wild-type animals, hydrophobic fluorescent dyes such as FITC, DiO and DiI penetrate into eight types of ciliated neurons (Hedgecock et al., 1985; Starich et al., 1995). However, in *che-2* mutants these dyes cannot penetrate into any neuron (Perkins et al., 1986; Starich et al., 1995). In this paper we characterize the *che-2* gene and its mutants and demonstrate the following: (1) *che-2* encodes a novel WD40 protein; (2) *che-2* is expressed in most ciliated sensory neurons and localized at cilia; (3) *che-2* acts cell-autonomously; (4) forced expression of *che-2* at the larval or even the adult stage is sufficient for the formation of sensory cilia in a *che-2* mutant, although cilia are formed during late embryogenesis in normal development.

MATERIALS AND METHODS

Strains and genetic methods

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), *m127*, *mn395* and *sa133* (Starich et al., 1995), where *m127* and *sa133* have an identical mutation (this study).

We placed the *che-2(e1033)* mutation over the deficiency *meDf6 X* (Villeneuve, 1994), which deletes *che-2* and *dpy-3* but not *unc-2*. To make active males having a *che-2* mutation, we injected a clone of *che-2* gene (6.7-kb *Eco*T14I fragment), together with H20::GFP (which is expressed in almost all neurons), into *che-2(e1033)* hermaphrodites and mated the transformants with N2 males. The males among the progeny were crossed to *meDf6/dpy-3(e27)unc-2(e55)*. F_1 and F_2 non-Dpy non-Unc hermaphrodites that did not show GFP fluorescence were candidate *che-2(e1033)/meDf6* heterozygotes. These were used in single-animal chemotaxis to benzaldehyde assays, and their genotype was determined by the phenotype of their self-progeny. After the determination of the genotype, only the data for *che-2(e1033)/meDf6* were collected (Fig. 1F).

Visualization and analysis of cilium morphology

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

Assay of dye-filling

We performed the FITC-filling assay according to Hedgecock et al. (1985) and the DiO-filling assays according to Starich et al. (1995). The DiI-filling assay in most experiments was performed by incubating worms in the dye solution (10 μg/ml in M9 buffer) for 2 hours at 20°C. However, for the analysis of rescue by *sra-6*::CHE-2, the animals were stained in 1 μg/ml DiI for 30 minutes to avoid the staining of excess cells. Under this condition, DiI stained only ASH and ASI neurons in the *che-2(e1033)* strain carrying *sra-6*::CHE-2. Longer incubation or higher dye concentration caused penetration of DiI into other neurons, ASK, ASJ and AWB, and further into the sheath cells in this strain but not in *che-2(e1033)* without *sra-6*::CHE-2.

Behavioral assays

Population and single-worm chemotaxis assays were performed according to Bargmann and Horvitz (1991) and Bargmann et al. (1993). The concentration of NaCl was 0.4 M, and that of benzaldehyde was 1% (v/v). The chemotaxis index was calculated as (number of animals at attractant – number of animals at control)/(total number of animals). 50-200 animals were used for each population chemotaxis assay.

The assay of osmotic avoidance was based on the method by Culotti and Russell (1978) with minor modifications. We placed 50-200 animals inside a high osmotic annular ring (1.5 cm in diameter) made with 60 μl of 4 M NaCl on a chemotaxis plate. The osmotic avoidance index was defined as the fraction of animals that remained inside the ring after 30 minutes. 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 3.5 cm mating plate with three L4 *unc-31(e169)* hermaphrodites. The plates were kept at 20°C for 3-4 days and checked for progeny. Non-Unc hermaphrodites and males among the F_1 progeny indicated that the male could mate.

For Cu^{2+} avoidance assay, we used 6 cm Petri dishes containing 3 ml of 1.8% agar, 10 mM Hepes-NaOH (pH 7.0), 50 mM NaCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. 10 μl of 100 mM copper acetate was spread along a line that divides the surface of agar into two halves, which we designated A and B. After 3 hours, about 80 washed young adults were placed on A, and allowed to move for 1 hour. The Cu^{2+} avoidance was calculated as (number of animals on A – number of animals on B)/(total number of animals). In the experiment shown in Fig. 4C, we used the progeny of *che-2(e1033)* carrying both *sra-6*::CHE-2 and *sra-6*::GFP as an extrachromosomal array. Since some of the animals lost the extrachromosomal array during culture, we checked GFP fluorescence of the animals after the assay and judged those with and without fluorescence as those with and without *sra-6*::CHE-2, respectively.

Cloning of *che-2*

The *che-2(e1033)* mutation had been previously mapped (Avery, 1993; Jongeward et al., 1995) very close to *egl-17 X*, which has been cloned (Burdine et al., 1997). 15 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 restoration of dye-filling (Starich et al., 1995). The cosmid F38G1 and its 6.7-kb *Eco*T14I fragment rescued the defect of dye-filling in *che-2(e1033)*. The latter also rescued other *che-2* phenotypes, namely, osmotic avoidance (Fig. 1C), chemotaxis to NaCl and benzaldehyde (Fig. 1D) and male mating activity (Fig. 1E). The *C. elegans* DNA Sequence Consortium (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). We obtained the missing 5′ part by RT-PCR with a *C. elegans* trans-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 extrinsic 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). All 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.

Reporter constructs for studying *che-2* expression

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, J. Ahnn, G. Seydoux and S. Xu, personal communication). This construct had a 2.9-kb 5′ upstream sequence and 13 of the 14 exons of *che-2* gene.

pCHE-2::GFP2 was made by ligating 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 coding region of *che-2* gene) and the rest of the coding sequence from *che-2* cDNA, into the pPD95.69 GFP vector containing a nuclear localization signal (A. Fire, J. Ahnn, G. Seydoux and S. Xu, personal communication). The DNA sequence at the 3′ end of the insert had been modified mainly by PCR, so that the C-terminal amino acid Asp of CHE-2 was changed to Lys followed

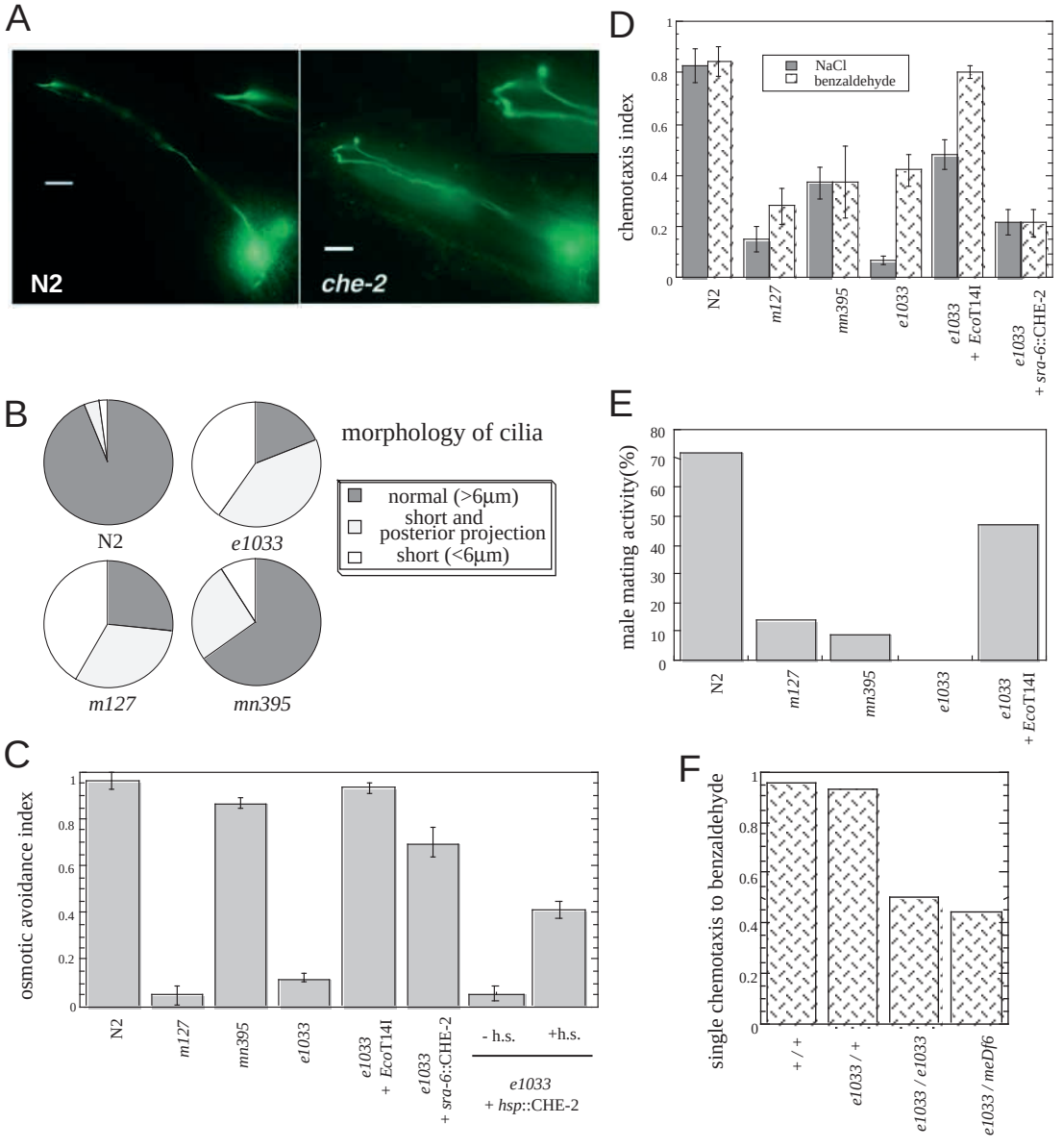


Fig. 1. Phenotypes of *che-2* mutants. (A) Cilium morphology of the wild-type (N2, left) and the *che-2 (e1033)* (right) adult. ASH and ASI neurons in the head are visualized by *sra-6*::GFP. Anterior is to the left. Bright spheres on the right are cell bodies, from which dendrites extend to the left, ending in cilia. Normal cilia are seen in the wild-type animal, while short cilia with (upper) and without (lower) a posterior projection are seen in the *che-2(e1033)* mutant. Bars, 10 μm. (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), a *che-2* gene under the control of the *sra-6* promoter (*sra-6*::CHE-2) and a *che-2* gene under the control of a heat shock promoter (*hsp*::CHE-2), with and without heat shock (h.s.). Each data point represents at least three independent assays. Error bars indicate ± s.e.m. (D) Chemotaxis to NaCl and to benzaldehyde of the same strains as C, but excluding *che-2(e1033)* carrying *hsp*::CHE-2. Each data point represents at least four independent assays. Error bars indicate ± s.e.m. (E) Male mating activity of the same strains as D, excluding *che-2(e1033)* carrying *sra-6*::CHE-2. The percentages of males that showed mating activity are shown. 15-20 independent assays were conducted for each strain. (F) Chemotaxis to benzaldehyde of N2, the *che-2(e1033)* heterozygote and homozygote, and *che-2(e1033)* over a deficiency deleting *che-2* gene. Single-animal assay was performed with more than 30 animals for each strain.

by a linker of six amino acids. This construct had a 650-bp 5′ upstream sequence and all the coding sequence.

pCHE-2::GFP3 was made by removing the *Kpn*I cassette containing the nuclear localization signal from pCHE-2::GFP2.

Germline transformation

We performed germline transformation by a microinjection method (Mello and Fire, 1995). For GFP expression, the DNA concentrations were 90 μg/ml for the GFP fusion constructs and 10 μg/ml for the semidominant *rol-6(su1006)* marker plasmid pRF4, except for the experiments with pCHE-2::GFP3 (5 μg/ml for the GFP fusion construct and 95 μg/ml for pRF4). For rescue experiments, the DNA concentrations were 5-20 μg/ml for the DNA to be tested and 10-95 μg/ml for the co-injection markers. The markers were *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 drive expression of GFP in body wall muscles, in a subset of neurons, and in almost all neurons, respectively.

Expression of *che-2* under the control of 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 eight 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 *sra-6*::GFP, *gcy-10*::GFP or *gpa-9*::GFP as the injection and cilium morphology markers, was used for the transformation of *che-2(e1033)* animals.

Rescue of *che-2* phenotypes by expression with a heat shock promoter

A *che-2* gene under the control of a heat shock promoter (*hsp*::CHE-2) was made by inserting *che-2* cDNA into the pPD49.78 vector (Mello and Fire, 1995). This vector contains a *C. elegans* heat shock promoter hsp16-2, which drives strong expression in neural and hypodermal cells. *che-2(e1033)* animals carrying an extrachromosomal array of *hsp*::CHE-2 (or pPD49.78 for a control experiment) and a marker were made by co-injection. The marker was *sra-6*::GFP for the observation of cilium morphology (injection and cilium morphology marker) and H20::GFP for the dye-filling assay (injection marker).

The animals were cultured at 20°C and heat-shocked at 30°C for 6 hours. Some of the animals were taken at various times after heat shock and checked for cilium morphology or the ability of dye-filling. As controls, we checked animals before or without heat shock and animals carrying the vector pPD49.78 after heat shock. In the dye-filling assay the animals were soaked in DiI for 2 hours before observation. Hence, time zero means that the animals were observed 2 hours after the end of the heat shock treatment.

The rescue of osmotic avoidance defects was assayed about 15 hours after heat shock, by the method mentioned above. In addition to the mass experiments, we checked the cilium morphology of the same young adult animals before and about 15 hours after heat shock.

RESULTS

Characterization of various *che-2* mutants

Mutants of the *che-2* gene show abnormalities in cilium morphology, dye-filling, osmotic avoidance, chemotaxis and male mating (Lewis and Hodgkin, 1977; Hodgkin, 1983; Perkins et al., 1986; Bargmann et al., 1993; Starich et al., 1995). However, most of the phenotypes were studied with only one allele, *e1033*. To characterize and compare various *che-2* alleles, we studied these phenotypes in three different *che-2* alleles (*e1033*, *m127*, and *mn395*) (see Materials and Methods for the reason we chose these alleles.)

As shown in Fig. 1B-E, *mn395* showed milder defects than *e1033* and *m127* in some phenotypes. Most sensory cilia of ASH and ASI neurons in *e1033* and *m127*, as visualized with *sra-6*::GFP (Troemel et al., 1995), were very short and often had an abnormal posterior projection, whereas 65% of ASH and ASI cilia in *mn395* looked apparently normal (Fig. 1A,B). While the fluorescent dye DiI did not penetrate into any neuron in *e1033* and *m127*, it often penetrated into some sensory neurons, especially ASK, ASH and ASJ, in *mn395*. In contrast, other fluorescent dyes, FITC and DiO, did not stain any neurons in all the *che-2* alleles. While *e1033* and *m127* showed a strong defect in osmotic avoidance, *mn395* showed only a weak defect: 86% of the animals could avoid high osmotic strength (Fig. 1C). This is consistent with the mild defect in the cilium morphology of ASH neurons, which mediate this behavior (Bargmann et al., 1990). Chemotaxis to the water-soluble attractant NaCl was strongly abnormal in *e1033* and *m127*, whereas *mn395* retained a weak response to NaCl (Fig. 1D), which is sensed mainly by ASE neurons. On the other hand, all the alleles showed a mild defect in chemotaxis to benzaldehyde, which is sensed by AWC neurons. Finally, males of all the alleles showed no or very weak mating activity (Fig. 1E).

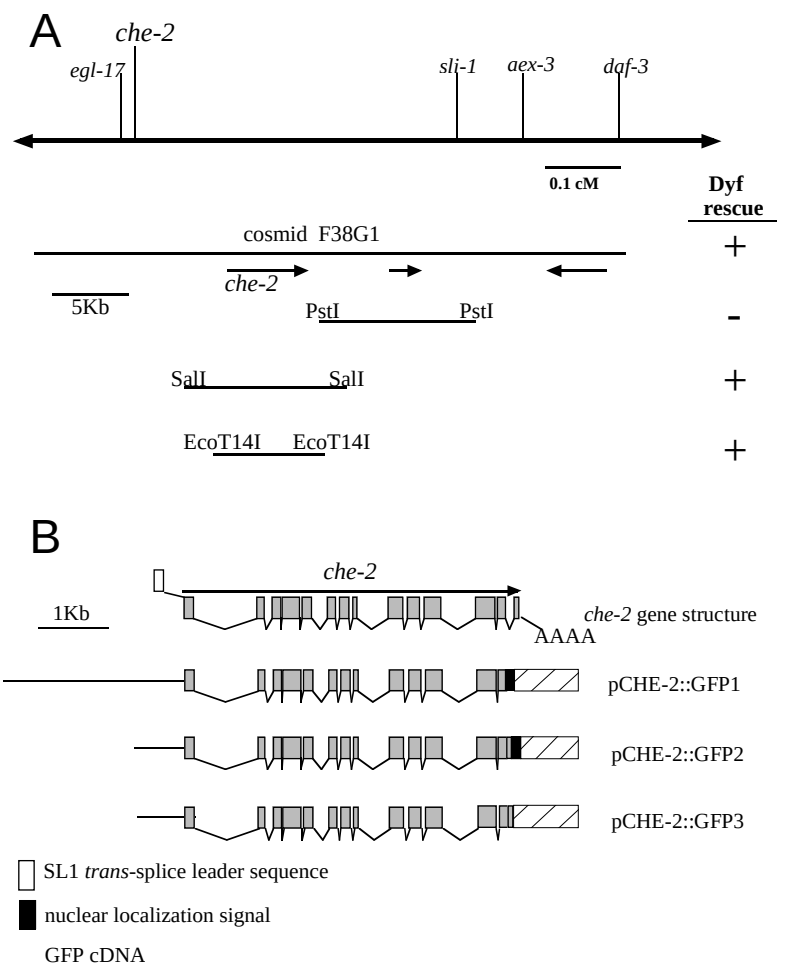
The heterozygote *e1033/meDf6* showed essentially the same phenotype as the *e1033* homozygote in growth, dye-filling (data not shown), and chemotaxis to benzaldehyde (Fig. 1F). Hence, *e1033* is a candidate for a null allele.

che-2 encodes a novel protein with WD40 repeats

The *che-2* gene was cloned by the rescue of the *che-2(e1033)* mutant phenotypes (Fig. 2A and Materials and Methods). The full-length cDNA consisted of 2375 bases excluding poly(A) and started with the trans-splice leader sequence SL1 (Krause and Hirsh, 1987). It had 14 exons that encoded a protein of 760 amino acids (Fig. 2B,C). 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 proteins of the WD40 family. CHE-2 satisfies the criteria for WD repeat proteins as defined by Neer et al. (1994). There are four WD40 motifs in the N-terminal region of CHE-2, from amino acid 100 to 297 (Fig. 2C). Although the WD40 protein family is divided into subfamilies (Voorn and Ploegh, 1992; Neer et al., 1994), CHE-2 does not belong to any of them.

The non-WD40 region of CHE-2 shows significant similarity to the conceptual translation product of the human EST clone zs23c07 (EMBL/GenBank/DDBJ accession no. AA262097). The similarity was found in all the sequenced regions of zs23c07: the third and the fourth WD40 repeats (54% identity; 46/85) and a non-WD40 72 amino acid stretch (amino acids 368-439) (39% identity; 28/72).

The coding regions of four known *che-2* mutant alleles (*e1033*, *mn395*, *m127*, *sa133*) were sequenced to identify the mutations responsible for their defects. The mutation *e1033* altered codon 601 from glutamine-encoding to a TAA stop codon, while the mutations *m127* and *sa133* were associated with the change of codon 599 from tryptophan-encoding to a TAG stop codon (Fig. 2C). These three mutants are predicted to produce truncated forms of CHE-2 protein lacking about 160 C-terminal amino acids. In the remaining allele, *mn395*, codon



C

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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 TTGTTATAATTTACTCAAAAAATTTCTAATTTCAAAAAAAAAAAAAA
```

Fig. 2. Cloning of *che-2* gene. (A) Genetic and physical map of the *che-2* region. The arrows below the cosmid F38G1 indicate genes predicted by the *C. elegans* genome project. (B) The structure of *che-2* gene and *che-2*::GFP fusion constructs in this study. *che-2* gene has 14 exons (heavily dotted box). (C) 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.

126 was changed from a glycine to a glutamate codon (Fig. 2C). This substitution was in the first WD40 repeat of CHE-2.

CHE-2 is localized at the cilia of most ciliated sensory neurons

To determine the localization of the *che-2* product, we used a GFP reporter gene pCHE-2::GFP3 (Fig. 2B), which contained a 650-bp 5′ upstream sequence and all the coding sequence. This construct rescued the dye-filling defect of *che-2 (e1033)*. Most of the GFP produced from this construct was localized at the cilia of most ciliated sensory neurons. This could be seen most clearly in amphid sensory neurons (Fig. 3A), whose 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 may act in sensory cilia.

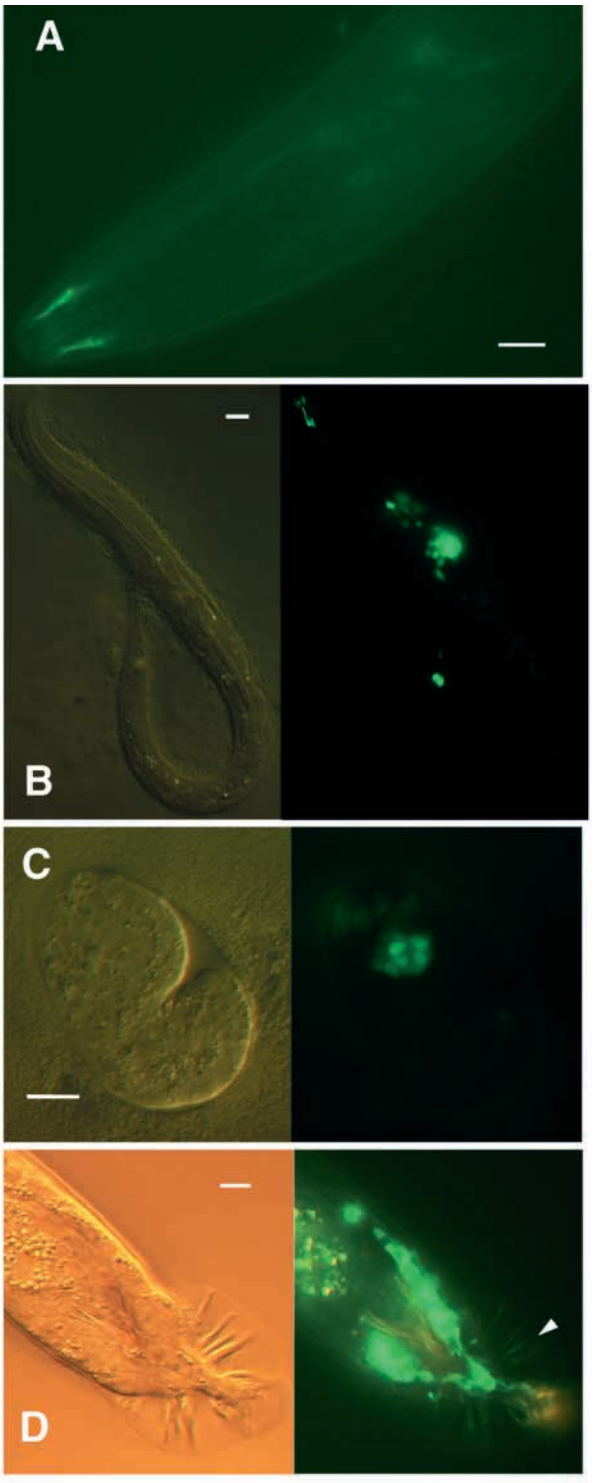

Fig. 3. Expression of *che-2*::GFP fusion genes. (A) Expression of pCHE-2::GFP3 in the amphid neurons of an L3 hermaphrodite. The cilia show strong fluorescence, while the processes and cell bodies show faint fluorescence. (B-D) Right panels show fluorescence of animals visualized by DIC microscopy in left panels. (B) Expression pCHE-2::GFP2 in the head of an L1 hermaphrodite. Almost all the ciliated sensory neurons show fluorescence. (C) Expression of pCHE-2::GFP2 in a mid-stage embryo. Some head neurons show fluorescence. (D) Expression of pCHE-2::GFP2 in the adult male tail. The arrow indicates rays. Bars, 10 μm.

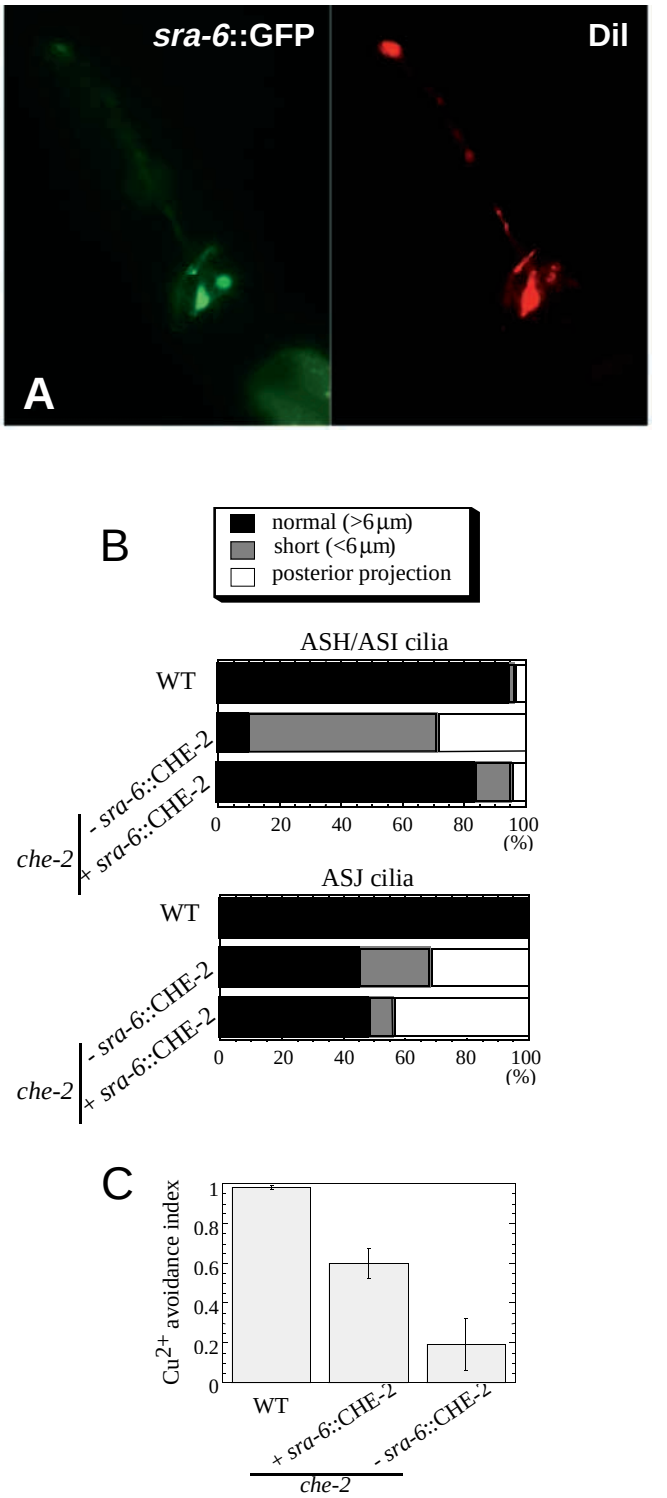

Fig. 4. (A) Dye-filling of *che-2(e1033)* animals carrying *sra-6*::CHE-2 and *sra-6*::GFP. The animal expresses *che-2* and GFP only in ASH and ASI neurons (left) and take up DiI in the same neurons (right). (B) Cilium morphology of *che-2(e1033)* carrying *sra-6*::CHE-2 as an extrachromosomal array, as compared with that of wild type (WT, N2) and *che-2(e1033)* without *sra-6*::CHE-2. Cilia of ASH and ASI neurons (top) and those of ASJ neurons (bottom) were visualized with *sra-6*::GFP, *gpa-9*::GFP, respectively. (C) Avoidance of Cu^{2+} ions by wild type (N2) and *che-2(e1033)* with and without *sra-6*::CHE-2.

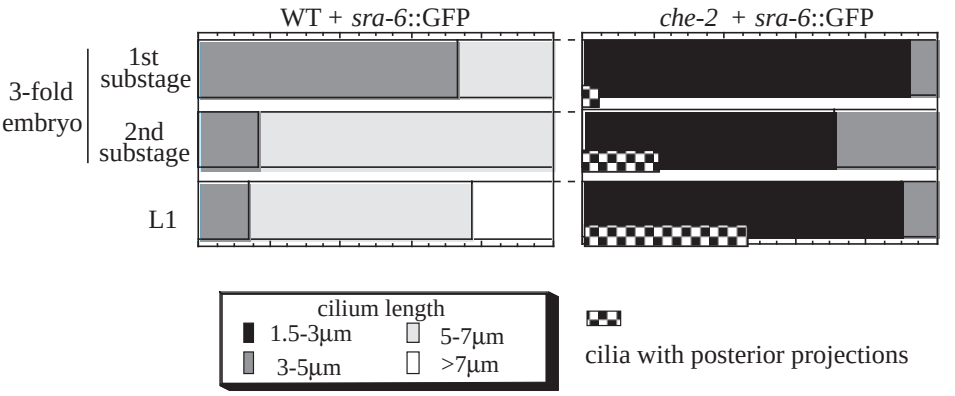

Fig. 5. Elongation of cilia and emergence of abnormal posterior projections during development. The cilium morphology of wild-type (WT, N2) and *che-2(e1033)* mutant animals was observed at the first and second substages of the latter half of the threefold stage and just after hatching. At each stage, 30-50 cilia of *che-2(e1033)* and over 15 cilia of N2 were observed. The first and second substages were defined by the mouth morphology (Sulston et al., 1983): in the first substage (650-770 minutes after the first cleavage) the embryo has a mouth plug, and in the second substage (770-800 minutes after the first cleavage) the mouth plug has already fallen away.

To identify the cells in which *che-2* is expressed, we used two GFP reporter genes containing a nuclear localization signal, pCHE-2::GFP1 and pCHE-2::GFP2 (Fig. 2B), which drove expression essentially in the same cells. The *che-2*::GFP transgenics first expressed GFP in some head neurons between the comma and 1.5-fold stage of embryogenesis (Fig. 3C). The number of cells expressing GFP increased, as the development proceeded to the adult stage. The expression at the adult stage (Fig. 3B) was detected in all the amphid sensory neurons except AFD, phasmid neurons PHA and PHB, all the inner and outer labial neurons (IL1, IL2, OLQ and OLL), CEP, PDE, FLP, PQR, and three unidentified neurons (perhaps AQR and ADEL/R). Thus, *che-2* seems to be expressed in all the ciliated sensory neurons (White et al., 1986) except BAG and AFD. The expression pattern is consistent with the report that most sensory cilia are morphologically abnormal (Lewis and Hodgkin, 1977; Perkins et al., 1986). The result also indicates that CHE-2 is a general component of sensory cilia.

Since *che-2(e1033)* males are defective in mating (Lewis and Hodgkin, 1977), we observed GFP expression in N2 male tails (Fig. 3D), where there are many male-specific sensory neurons required for mating behavior. Although we could not identify

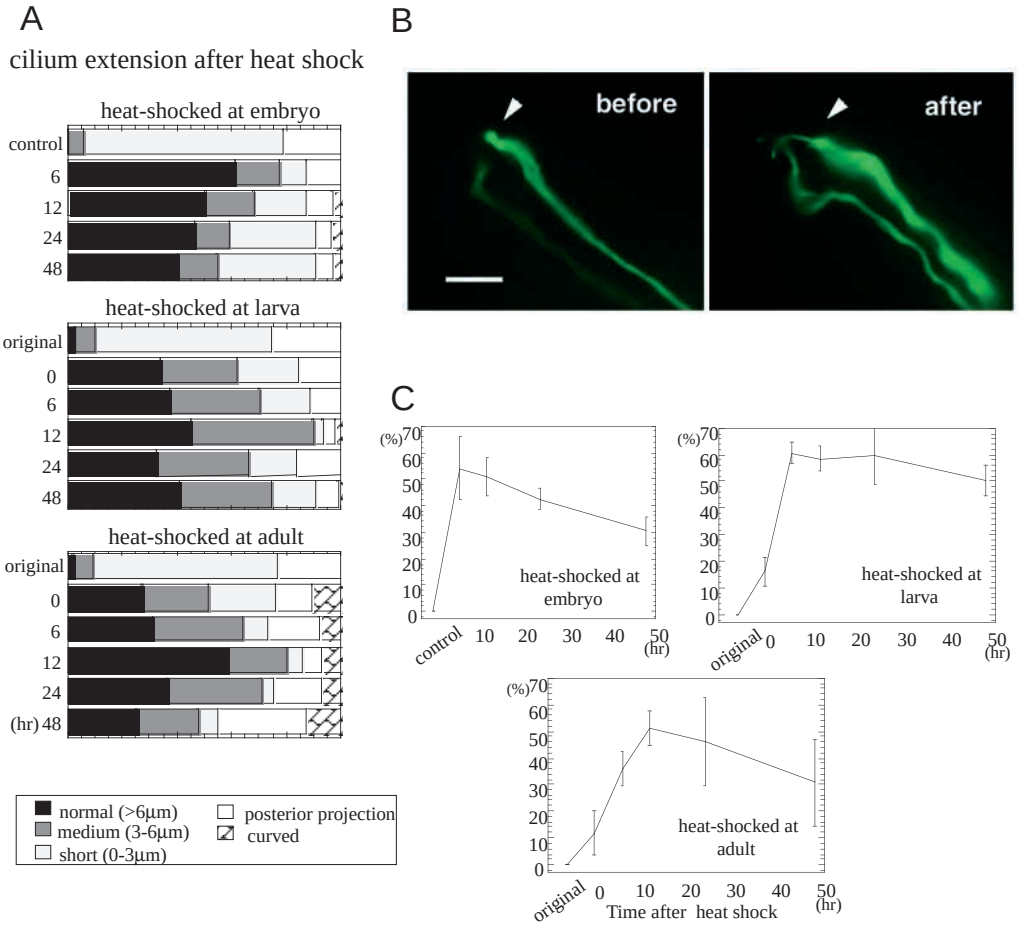

Fig. 6. Cilium morphology and dye-filling ability of *che-2(e1033)* animals carrying an *hsp::che-2* fusion gene (*hsp*::CHE-2) after heat shock induction of *che-2*. Heat shock was given to either embryos, L1/L2 larvae or young adults. (A) Time course analysis of cilium morphology of ASH and ASI neurons after heat shock. Each data point was obtained with over 30 cilia. Animals just before the heat shock ('original') were used as the controls in the heat shock experiments on larvae and adults. However, in the heat shock experiment on embryos, young adults grown without heat shock ('control') were used as the control, because even wild-type animals had short cilia just before the heat shock in this case. (B) Amphid cilia bundles of the same animal before and about 12 hours after heat shock at the adult stage. ASH and ASI neurons are visualized with GFP. The top right cilia elongated but in a curved form in this case. (C) Time course analysis of dye-filling, demonstrating the percentage of animals that showed dye-filling at least in one neuron. Each data point is the average of three independent assays, and over 30 animals were used for each assay. Error bars indicate ± s.e.m. The *che-2(e1033)* mutant carrying only the vector did not show dye-filling at any time point (data not shown).

all the GFP-expressing neurons, we 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, but we could not confirm the expression in spicules, due to strong autofluorescence.

che-2 acts cell-autonomously

To determine if *che-2* acts cell-autonomously, we expressed *che-2* cDNA only in a subset (ASH and ASI) of amphid neurons in the *che-2 (e1033)* mutant, by using *sra-6* promoter (Troemel et al., 1995) (*sra-6*::CHE-2). As mentioned above, the *che-2 (e1033)* mutant did not show dye-filling in any neuron. However, the *che-2 (e1033)* strain carrying the *sra-6*::CHE-2 took up DiI in ASH and ASI neurons (Fig. 4A). Moreover, the shape of the cilia of these neurons (as visualized by *sra-6*::GFP) became mostly normal (Fig. 4B). In contrast, the shape of ASJ cilia (as visualized by *gpa-9*::GFP) (Fig. 4B) and AWB cilia (as visualized by *gcy-10*::GFP) (data not shown) remained abnormal even in the presence of *sra-6*::CHE-2. Furthermore, this strain had an almost normal response to high osmotic strength (Fig. 1C), which is mediated by ASH neurons, but an abnormal response to NaCl and to benzaldehyde (Fig. 1D), which are mediated mainly by ASE and AWC neurons, respectively (Bargmann and Horvitz, 1991; Bargmann et al., 1993). In conclusion, the function and cilium morphology of only the cells that expressed *che-2* were restored, i.e. *che-2* acts cell-autonomously.

Avoidance from Cu^{2+} ion was restored partly with *sra-6*::CHE-2. This result confirms that *che-2* acts cell-autonomously, because ASH neurons seem to play an important role in Cu^{2+} avoidance. Sanbongi et al. (1999) showed that the ablation of ASH, ADL and ASE neurons results in almost complete loss of Cu^{2+} avoidance, while the ablation of ADL and ASE does not change it.

Developmental defects in *che-2(e1033)* cilium formation

Lewis and Hodgkin (1977) suggested that the abnormal posterior projection at the amphid sensory cilia in *che-2(e1033)* may be produced by the degeneration of cilia that once extended to the normal length. We therefore traced the cilium morphology during development, using *sra-6*::GFP. We could observe cilium morphology from the midpoint of the threefold stage of embryos (650 minutes after the first cleavage), when cilia were about 3 μm or longer in N2 animals (Fig. 5).

As shown in Fig. 5, the amphid sensory cilia of N2 extended rapidly during the latter half of the threefold stage. In *che-2(e1033)*, the cilia did not extend, but many posterior projections were formed during this period. We therefore conclude that the abnormal posterior projection is made due to a cilium formation defect and not by the degeneration of cilia once correctly formed.

Expression of *che-2* at the adult stage is sufficient for sensory cilium formation.

As shown above, *che-2* is expressed from embryos to adults. To determine the developmental stage of expression that is sufficient for cilium formation, we expressed *che-2* in *che-2(e1033)* animals at various stages by using a heat shock promoter to drive expression of *che-2* cDNA (*hsp*::CHE-2). The effect of heat shock was analyzed by cilium morphology as visualized with *sra-6*::GFP. Without heat shock, the animals showed no cilium extension. However, the extension of cilia was observed after heat shock treatment at embryos, at larvae (L1 to L2) or even at adults (Fig. 6A). The increase of cilium length was also confirmed by comparing the same cilia before and after the heat shock treatment (Fig. 6B). Abnormal posterior projections did not always disappear after cilia had extended. Some cilia formed by heat shock induction were unusually curved.

Dye-filling abnormality was also rescued, regardless of the stage of heat shock (Fig. 6C). There were no neurons that preferentially restored dye-filling ability. The function of ciliated sensory neurons was also restored at least after heat shock at adults, as judged by the ability of osmotic avoidance (Fig. 1C).

The effect of heat shock lasted at least for 2 days. However, the percentage of animals that had normal cilia and dye-filling ability showed a tendency to decrease. The *che-2* product might be required not only for the formation but also for the maintenance of sensory cilia.

DISCUSSION

che-2 encodes a new member of the WD40 family

che-2 encodes a new member of the WD40 protein family. Proteins of this family interact with other proteins through the WD40 repeat region and form multiprotein complexes (Neer et al., 1994; Sondek et al., 1996). Hence, the WD40 repeats of CHE-2 may be involved in the formation of a complex needed for sensory cilium formation.

The first WD40 motif probably plays an important role in CHE-2 function, because *che-2(mn395)* has a missense mutation in this repeat. Furthermore, the non-WD40 region in the C-terminal portion of the protein is also essential for CHE-2 function or stability, because three other alleles (*e1033, sa133, m127*) have nonsense mutations in this region but preserve the WD40 region. This argument is supported by the conservation of at least some of the non-WD40 sequence between *che-2* and zs23c07. The phenotypes of *e1033* are probably caused by complete loss of the CHE-2 activity, because *e1033/Df* shows essentially the same phenotype as the *e1033* homozygote.

The function of CHE-2 protein

This study showed that *che-2* is required for the formation of most sensory cilia and that it acts cell-autonomously. In the following we propose three possibilities for the function of CHE-2.

One possibility is that CHE-2 may be a structural component of sensory cilia, such as a microtubule-associated protein. CHE-2 may play a role in the assembly of microtubules or may 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* (Perkins et al., 1986).

A second possibility is that CHE-2 may act in the transport of cilium precursor proteins in cilia. In the *Chlamydomonas* flagella, a kinesin transports the precursor proteins 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 retrograde transport

(Pazour et al., 1998). Both the kinesin and the dynein are essential for normal flagellum formation. Since the *C. elegans* OSM-3 kinesin and CHE-3 dynein are also essential for normal cilium formation, they may play a role similar to the *Chlamydomonas* flagellar kinesin and dynein, respectively, and CHE-2 may help OSM-3 or CHE-3 in ciliary transport. Similarity of the transport system in *C. elegans* sensory cilia and in *Chlamydomonas* flagella is suggested by the presence of the homologs of *Chlamydomonas* IFT particle components in *C. elegans* cilium structure gene products (Cole et al., 1998).

A third possibility is that CHE-2 may be a component of a signal transduction system. Some G protein mutants (*gpa-3(gf)* and *odr-3(lf))* have defects in sensory cilium structure (Zwaal et al., 1997; Roayaie et al., 1998). These G proteins act in the signal transduction of pheromone-sensation (Zwaal et al., 1997) and that of olfaction, osmosensation and mechanosensation (Roayaie et al., 1998), respectively. Loss of activity in sensory neurons sometimes causes abnormalities in their structure (Kumar and Ready, 1995; Pecol et al., 1999). Furthermore, mutants of the *C. elegans osm-6* gene show phenotypes resembling those of *che-2* mutants, and *osm-6* encodes a novel protein with a motif that might interact with SH3 domains (Collet et al., 1998), which often play a role in signal transduction (Birge et al., 1996). CHE-2, using its WD40 repeats, may form a protein complex necessary for signal transduction, as *Drosophila* InaD does using its PDZ domains (Tsunoda et al., 1997; Montell, 1998).

Controlled expression of *che-2* can be used for determining the function of each type of ciliated sensory neurons

Since all the neurons in *C. elegans* can be identified in living animals, it is possible to determine their functions by killing them with a laser beam and by examining the abnormalities of the resulting animal (Chalfie et al., 1985; Avery and Horvitz, 1987). However, this method 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 we used the *sra-6* promoter to drive expression of *che-2* only in ASH and ASI neurons, investigated the cilium structure and behavior of the animals, and found that *che-2* acts cell-autonomously. This experiment provides the basis of an alternative method for determining the functions of each type of *che-2*-expressing neurons. Recent studies have revealed many promoters that drive expression in various subsets of sensory neurons (Troemel et al., 1995; Yu et al., 1997). Using these promoters, we can express *che-2* in various neurons in the *che-2* background, and determine if it is sufficient for a certain *che-2* function, for instance, dauer formation under the conditions of high population density and scarce food (Starich et al., 1995). This method does not have the drawbacks of the laser-killing method mentioned above. It is suited for determining which neurons are sufficient for a certain function, while the laser killing method is suited for determining which neurons are required.

Expression of *che-2* at the adult stage is sufficient for sensory cilium formation

Using a heat shock promoter, we showed that the *che-2* mutant can form functional cilia by transient expression of *che-2* at the adult stage, although cilium formation takes place at the embryonic stage in normal development. It remains to be examined how *C. elegans* retains such ability. One possibility is that cilium formation continues even in wild-type larvae and adults, because cilia are in dynamic equilibrium between formation and degradation. If this is true, cilia should degenerate when cilia formation is blocked. This seems to be the case with *Chlamydomonas* flagella. Temperature-sensitive mutants in the *fla10* (kinesin-II) gene lose their flagella when the temperature is raised from permissive to non-permissive range (Lux and Dutcher, 1991). Also in our heat shock experiments, cilia tended to become shorter after expression of *che-2* was terminated.

A general mechanism for the formation of cilia and flagella may exist

Two pieces of evidence suggest that there may be a general mechanism for the formation of sensory cilia and motile cilia/flagella in most eucaryotes. First, the cDNA represented by the EST clone zs23c07 may encode a human ortholog of CHE-2. 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 structural genes and on the *Chlamydomonas* IFT particle components, will benefit each other in elucidating the general mechanisms involved in the formation of sensory cilia and motile cilia/flagella.

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