Tissue-specific expression, heat inducibility, and biological roles of two *hsp16* genes in *Caenorhabditis elegans*

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Abstract In this report we have examined two new heat shock protein (HSP16) proteins in the nematode *Caenorhabditis elegans* encoded by the open reading frames F08H9.3 and F08H9.4. The F08H9.3 and F08H9.4 genes are oriented in the same direction next to each other on the chromosome, not sharing any promoter region, unlike other *hsp16* genes that share common promoters in pairs. The F08H9.3 and F08H9.4 proteins were expressed in a tissue-specific manner, unlike the other four HSP16 proteins. F08H9.3 was expressed in the pharynx, and F08H9.4 in the excretory canal and a few neuronal cells. While F08H9.3 was weakly induced by heat shock only in the same tissue as under the normal condition, F08H9.4 was newly induced in the intestine. RNA interference experiments showed that these two proteins are required for survival under the heat shock condition.

Key words: Heat shock protein 16; Heat shock; *Caenorhabditis elegans*; F08H9.3; F08H9.4; Tissue-specific expression

1. Introduction

Eukaryotic cells respond to heat shocks by the induction of a conserved set of proteins, the heat shock proteins (HSPs), many of which function as molecular chaperones [1,2]. HSPs are classified into several major families according to their size, structure and function. These are HSP100, HSP90, HSP70, HSP60, HSP40, HSP33 and the small heat shock proteins (smHSP). Large HSPs are highly conserved in species as diverse as bacteria, yeast and mammals. In contrast, for smHSPs the main region of homology is a hydrophobic stretch of only 80–100 amino acids showing sequence similarity to vertebrate α-crystallin [3]. The smHSPs form a diverse family of proteins that are produced in all organisms, and their size are about from 12 to 43 kDa. Although the major molecular chaperones are the large HSPs such as HSP60, HSP70 and HSP90, the smHSPs are also classified as molecular chaperones on the basis of their ability to prevent the aggregation and in some cases promote the renaturation of unfolded polypeptides in vitro [4–10]. The large HSPs are ATP-dependent, but smHSPs are ATP-independent and have low chaperone activity [5,7,11]. They mainly act as reservoirs of non-native refoldable proteins and need the help of large HSPs for refolding proteins [12].

In *Caenorhabditis elegans*, the smHSP16s are relatively well studied [13–16]. The major HSP16 genes are encoded by *hsp16-1/hsp16-48* and *hsp-2/hsp16-41* gene pairs. These four genes are very similar in their gene structures and amino acid sequence homology [15]. HSP16-2 is known to be increased by reactive oxygen species, β-amyloid peptide, heavy metals and electro-magnetic fields [16–19]. HSP16-1 and HSP16-48 are also increased by oxidative stress [20,21]. These genes are not expressed in a normal condition and only induced by stresses, indicating that these proteins may not have other roles than in stress responses. Two new HSP16 proteins in *C. elegans*, encoded by the open reading frames (ORFs) F08H9.3 and F08H9.4, as reported recently [22], have not been well characterized. In this paper we show that F08H9.3 and F08H9.4 are expressed in a tissue-specific manner, and that they are induced, although slowly and weakly, by heat shock in specific tissues. We also show by RNA interference (RNAi) experiments that they play an important role for survival under heat shock condition.

2. Materials and methods

2.1. Strains and cultures

The Bristol strain N2 was used as the standard wild-type strain. *C. elegans* culture was done as previously described by Brenner [23].

2.2. Sequence alignment

The sequences were aligned using the Macvector™ program (IBI). Dashes in the sequence alignment represent gaps introduced to optimize alignment. Gene bank accession numbers of genes and protein used in sequence alignments are: GenBank/EMBL: Z77657 and GenPep: CAB01146 for F08H9.3, GenBank/EMBL: Z77657 and GenPep: Z77657 for F08H9.4, GenBank/EMBL: AC006774 and GenPep: AAF60615 for Y46H3A.3, GenBank/EMBL: AC006774 and GenPep: AAF60616 for Y46H3A.2, GenBank/EMBL: U64837 and GenPep: AAB04839 for T27E4.2, and GenBank/EMBL: U64837 and GenPep: AAB04841 for T27E4.9.

2.3. Heat shock test and Northern blotting

We applied mild heat shock to animals. We kept 55 mm plates with well-fed worms in a 30°C incubator for heat shock. After 3 h, we transferred the worm plate to a 20°C incubator and observed the worms within 15 min. For Northern analysis, total RNA of the mixed stages of the worms was prepared by the general molecular method. 100 μg of RNA was loaded in each lane of the RNA gel and transferred to nitrocellulose membrane. The transcript level was detected by hybridization with ^{32}P-labeled cDNA probe of each gene.

2.4. Green fluorescence protein (GFP) fusion construct

The full-length F08H9.3::GFP fusion plasmid, pJS371, was constructed by genomic DNA PCR by using F08H9.3-1 and F08H9.3-2

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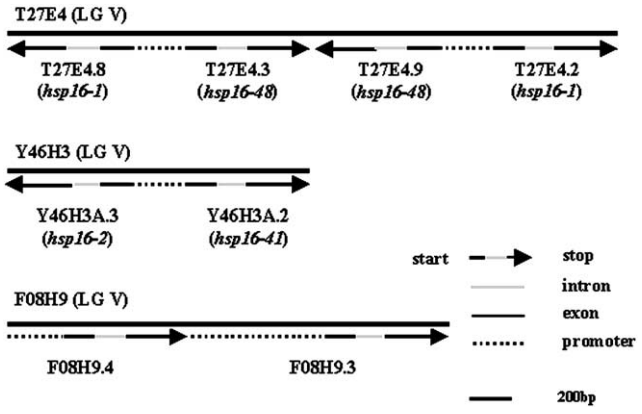

B

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F08H9.3   --MSVNPFFDPRTLGEMVFGDNGRLDREYVPISENND-DLSDCRNEIVDT
F08H9.4   --MSVSPFFNPKRDSQLGEMMRDMGGMQRRLMPISGTFNPMTDDSEIMNS
T27E4.2   --MSLYHYFRPAQRSVFGDLMRDMAQMERQFTPVCR--GSPSESSEIVNN
Y46H3A.3  --MSLYHYFRPAQRSVFGDLMRDMALMERQFAPVCR--ISPSESSEIVNN
T27E4.9   MLMLRSPFSDSNVLDHFLDEITGSVQFPYWRNADHNSFNFSDNIGEIVND
Y46H3A.2  MLMLRSPYSDSNALDHFLDELTGSVQFPYWRNADHNSFNFSDNIGEIVND

F08H9.3   HEKFSVNLNVPDVKPEELKINLEGRKLSIKAEHQEIENDNISTTQTYSKS
F08H9.4   NDKFAVNLNVSNFKPEELKVNLEGRQLSIQGE-HDVENEHGASRKSFSRM
T27E4.2   DQKFAINLNVSQFKPEDLKINLDGHTLSIQGE-QELKTEHGYSKKSFSRV
Y46H3A.3  DQKFAINLNVSQFKPEDLKINLDGRTLSIQGE-QELKTDHGYSKKSFSRV
T27E4.9   ESKFSVQLDVSHFKPEDLKIELDGRELKIEGI-QEKKSEHGYSKRSFSKM
Y46H3A.2  ESKFSVQLDVSHFKPENLKIKLDGRELKIEGI-QETKSEHGYLKRSFSKM

F08H9.3   IVLPEDVDVTHLSSNLSEDGKLLIEVPKVEAKKTNFFGFLSKFRCMPESQ
F08H9.4   ILLPEDVDITSVATNLSNDGKLCIEAPKLEGVCGRSVPVKEASMDHHHIE
T27E4.2   ILLPEDVDVGAVASNLSEDGKLSIEAPKKEAIQGRSIPIQQAPVEQKTSE
Y46H3A.3  ILLPEDVDVGAVASNLSEDGKLSIEAPKKEAVQGRSIPIQQAIVEEKSAE
T27E4.9   ILLPEDVDLTSVKSAISNEGKLQIEAPKKTN-SSRSIPINFVAKH-----
Y46H3A.2  ILLPEDADLPSVKSAISNEGKLQIEAPKKTN-SSRSIPINFVAKH-----
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C

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F08H9.3    1 ACATCATTTTCTTATCTTTCAAATTTGGAGTTCACGTACGCTAAACATGGTGAGCAGCGGAACCCGCAAAATGTCGGAAC  80
F08H9.4    1 ---CCAATTTGTCTCGCATCGCATTTTCGATT-ACCTA-ATTATATTACCGAAGCTACACCGGAAGTTTCTTTTTGGTAT  75

F08H9.3   81 ACACGAGCACACCACTCCCTCCACCCCCCGTCTCATAAACTTACCTGGCTGGGGGTTATTTCGCGATCAAGAAGGCGGAA 160
F08H9.4   76 TGACAAATAAAATGAATACTGTACTTT--GATATACACAATAAACGTATCTATAGAGACAATTCGATTTTGATTGAAGG- 152

F08H9.3  161 TCCCCATGGTGAGGGCCTACCCATTGCACTTTTGGGCGGGCTGACCTATGTGGCAGTCTCGAGTTGAGATTCGCCAACAGC 240
F08H9.4  153 ----TGGAGTACGGCCAGTTGCCAAGAATATAATATGAAATTATTTCTATGGCGCT---------AAAATGACCAGATAA 219

F08H9.3  241 TTAATTTTTGCGTATCGGGGCTGCGTGCGCGCGGCCCTGAAAAAAAGATATACATTAATGATTTTGAATCACTGCAACTA 320
F08H9.4  220 TAAATTTTTTCAAA--------AAATATTGGACAATTTTTTCAAACGGTATGAGATCTCAGTTTTCACCTAT-----CTA 286

F08H9.3  321 ATTTTTGGAAGTTCTAATTCATATAATGTGCTCTGTGACTACTATCTTCCATTTTGCTCGGCAACTGATGCTATTTCAAG 400
F08H9.4  287 ATTATTGAAAATTCTCTTTCAGACACTACGACCTGTATCTACTAGCTTCTATTTTGCTCGGCAACTGGTGTCATCT-ACT 365

F08H9.3  401 TTTACTTTTAACAGTTGGGAACATATTTTCAATTCAAGATAGCAAAGCTTTCGGATATTTCCCTTTTAACTGGCATTTTG 480
F08H9.4  366 TTTCGATTTATCTTTT-------TAATTTTGTTTCAGGAGAGTCTA-CTTTATCCTCTATTATGGATCACTGATGCCTTC 437

F08H9.3  481 AGAATATTATTATTTCCAGTACTGTTTCCATGTCTACATCATTTTGAACTACTGTCAAAGAGTTTATTACTTTGGAAAAC 560
F08H9.4  438 TAGAATTCTCCAGCAGACCTACAAGCGGAGACTTAGACGAAAAAATAACTGCGTCTCACCAAAATATTTACTCTCTAGAT 517

F08H9.3  561 ATTTTTTATCTAGAACTTTGATGCCTTGTCG-CATGGGCTCTCTGGTAAATAATCACGAGCATCATTTATACATACAGAA 639
F08H9.4  518 TTTTGATGCATCGAAGTTGGCTCTCTGGTCGACGACAACAACACAACAGAAAATAACTCGAGAAGTCTAGA---AAAGGA 594

F08H9.3  640 GGTTCTGAACAAATAACACTTATATAAATAGTCACACTTTTTGAGTTTTTGACAACAAAAA------------------- 700
F08H9.4  595 AAACAAAAACACAAAGT-CTCGTATAAAAAGGGCGAGATTTTCAGGTTTTCACAAACCAACCCCAAAATTTCTATATCGT 673

F08H9.3  701 --------------------------- 700
F08H9.4  674 CTACTCAACTTTTTCAACGAAAAAAAA 700
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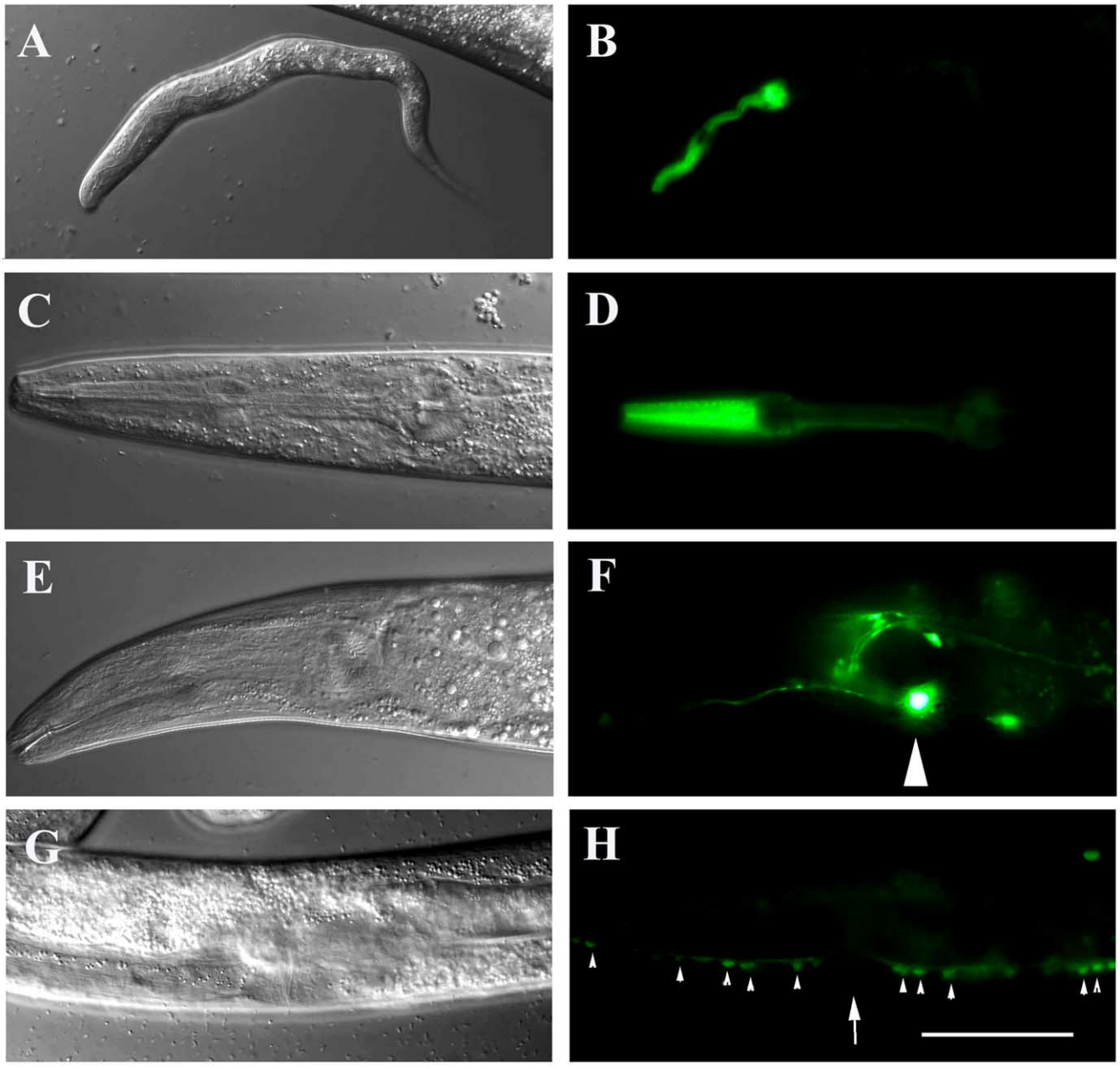

Fig. 2. The expression patterns of F08H9.3 and F08H9.4. The left panel shows the Nomarski images and the right panel the expression of GFP. A–D: Pharyngeal expression of F08H9.3. A,B: The L1 stage larva and (C,D) show the head region of an adult worm. E–H: The expression pattern of F08H9.4. E,F: The head region of adult worm, and (G,H) show the mid-body region of an adult worm. The large arrowhead in (F) indicates the cell body of the excretory cell, and the small arrowheads in (H) indicate the cell bodies of the ventral cord neurons. The arrow in (H) indicates the location of the vulva. The scale bar is 50 μm.

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Fig. 1. Genomic structures and sequence alignments of F08H9.3 and F08H9.4. A: The genomic structures of F08H9.3 and F08H9.4 compared with those of other HSP16 genes in *C. elegans*. F08H9.3 and F08H9.4 are located next to each other on a cosmid of chromosome V and are transcribed in the same direction. Both of the genes have one intron and two exons. The size of exons and intron is very similar. B: Amino acid sequence alignment of F08H9.3 and F08H9.4 against other HSP16 proteins. The overall amino acid sequences of the six HSP16 proteins show strong homology and the C-terminal α-crystallin domains are almost identical. The bold characters indicate amino acids that are identical among more than four proteins out of six proteins. The ORFs represent: T27E4.2 for HSP16-1, T27E4.9 for HSP16-48, Y46H3A.2 for HSP16-41, and Y46H3A.3 for HSP16-2. C: Sequence alignment of the promoters of F08H9.3 and F08H9.4 genes. The 5′-upstream regions of 700 nucleotides from the translation start codons are compared. The shadowed sequences 'nGAAnnTTCn' represent HSEs, and the underlined sequence 'TGGGGGT' is a putative STRE (see the text). There is a stretch of 34 nucleotides, underlined in the figure, exceptionally conserved between two promoters. The boldface nucleotides are the ones conserved at least in three consecutive positions.

primers, and subcloning the polymerase chain reaction (PCR) product into pPD95.77 vector (A. Fire, Carnegie Institute of Technology) using the *Hin*dIII and *Bam*HI restriction enzyme sites. The full-length F08H9.4::GFP fusion plasmid, pJS382, was constructed by genomic DNA PCR using F08H9.4-1 and F08H9.4-2 primer set, and subcloning the PCR product into pPD95.77 vector using the *Hin*dIII and *Bam*HI restriction enzyme sites. The sequences of primers used are: F08H9.3-1 (5′-CTA CTA TCT TCC ATT TTG CTC GGC AAC TGA TGC TAT TT-3′), F08H9.3-2 (5′-GGG ATC CTT GAG ATT CTG GCA TAC AC-3′), F08H9.4-1 (5′-TAA GCT TTC AGG TCA CGC TTC C-3′) and F08H9.4-2 (5′-GGG ATC CTT CAA TAT GAT GAT GGT CC-3′).

2.5. Microinjection, RNAi followed by heat shock, and microscopy

For the microinjection, DNA construct was prepared by a commercial mini prep kit (Qiagen, Cat. # 12145). Microinjection was carried out by injecting DNA into the gonads of young adult hermaphrodites with an Axiovert microscope and microinjection system (Carl Zeiss). The pRF4 plasmid carrying a dominant mutation in the *rol-6* gene was used as a selection marker. The concentration of total injected DNA including 75 μg/ml of marker DNA was always below 200 μg/ml.

For RNAi, templates for RNA synthesis were produced by PCR amplification of full-length cDNA using T3 and T7 primers. RNAs were synthesized using a commercially available in vitro transcription kit (Promega, Cat. # P2075, P2083) with T3 and T7 RNA polymerases. Unmodified RNA was resuspended for injection at 100 μg/ml concentration in DEPC-treated water. Following microinjection of double strand RNA, the injected animals (P0) were transferred to new plates every 12 h and F1 progeny were counted and analyzed. Between 6 and 9 h after RNAi, the worms are incubated at 30°C for 3 h for heat shock.

The Axioplan II microscope (Carl Zeiss) was used in all microscopy in this paper. All the photographs were taken using an Axiocam (Carl Zeiss). We treated with 2 mM levamisole (Sigma, Cat. # L-9756) to take pictures of living worms.

2.6. In vitro chaperone activity assay

We expressed recombinant F08H9.3, F08H9.4, and HSP16-1 with a 6× histidine tag in the *Escherichia coli* strain BL21, and purified them using Ni-NTA agarose beads (Qiagen, Cat. # RA9701) and glycerol gradient ultracentrifuge (Beckman LE80). For assaying the in vitro chaperone activity, we measured thermal aggregation of citrate synthase (Sigma, Cat. # C-3260).

3. Results and discussion

3.1. New HSP16 proteins in the nematode C. elegans with distinct genomic features

The smHSPs exist in all eukaryotes studied so far. *C. elegans* has diverse smHSPs including HSP12, HSP16, HSP20, HSP25 and HSP43, and stress-induced protein-1 (SIP-1) [24]. HSP16s of smHSPs in *C. elegans* belong to HSP20/α-crystallin families. This HSP20/α-crystallin family includes vertebrate HSP27, *Drosophila melanogaster* HSP22, -23, -26, -27, and *Saccharomyces cerevisiae* HSP26. Six *hsp16* genes encode four smHSP16 proteins in *C. elegans*. As reported in former studies, the *hsp16* genes of *C. elegans* are very similar. There are two pairs of *hsp16-1* (T27E4.2 or T27E4.8) and *hsp16-48* (T27E4.3 or T27E4.9) genes in *C. elegans* [15]. The characteristic of sharing a promoter region between the two ORFs also applies to the gene pair *hsp16-2* (Y46H3A.3) and *hsp16-41* (Y46H3A.2). Besides the previously reported *hsp16* genes, there are two new *hsp16*-like genes in *C. elegans*, encoded by F08H9.3 and F08H9.4, which are located on chromosome V like the other *hsp16* genes. The genomic structures of F08H9.3 and F08H9.4 are very similar to each other (Fig. 1A). They are transcribed in the same direction, unlike the other *hsp16* genes. Their amino acid sequences are similar, and C-terminal regions with α-crystallin domain sequences are almost identical (Fig. 1B). The overall identity between the two proteins and other HSP16 proteins is about 50%. However, the 5′-upstream regulatory regions of the two genes are very different, with limited sequence similarity, which may bear physiological importance. The HSP genes are reported to contain one or more repeat of heat shock responsive elements (HSEs), which are binding sites for the heat shock factor and allow rapid transcription of HSPs in response to heat shock [25,26]. Sequence analysis showed that the promoter of F08H9.3 had one HSE and F08H9.4 had two HSEs. The HSE of F08H9.3 is located close to the translational start

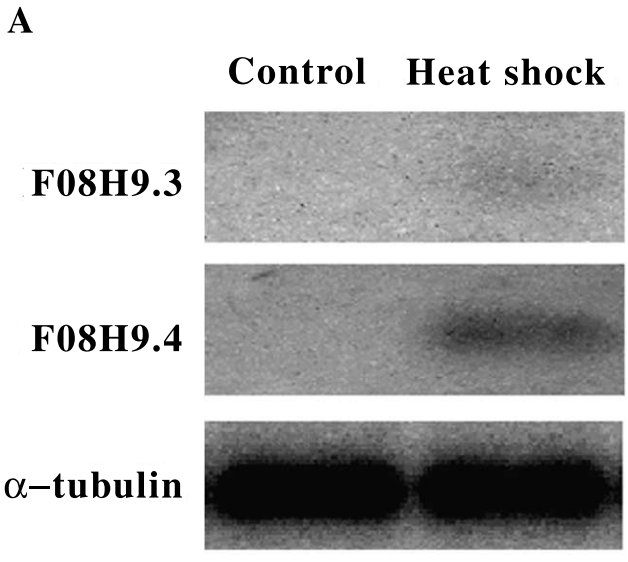

Fig. 3. The induced expression of F08H9.3 and F08H9.4 by heat shock. A: Northern blotting of F08H9.3 and F08H9.4 shows the induced transcript level by heat shock. α-Tubulin was used as the loading control. B: Induced expression of F08H9.3 and F08H9.4 at the tissue level. a,b,e,f are Nomarski images; c,d,g,h are GFP images. The left panel shows normal expression of F08H9.3 and F08H9.4 and the right panel shows their induced expression by heat shock. a–d: The expression of F08H9.3 in the pharynx and (e–h) show the expression of F08H9.4 in the neurons and the intestine. The pharyngeal expression of F08H9.3 by heat shock is stronger than normal expression and the expression of F08H9.4 is newly induced in the intestine by heat shock.

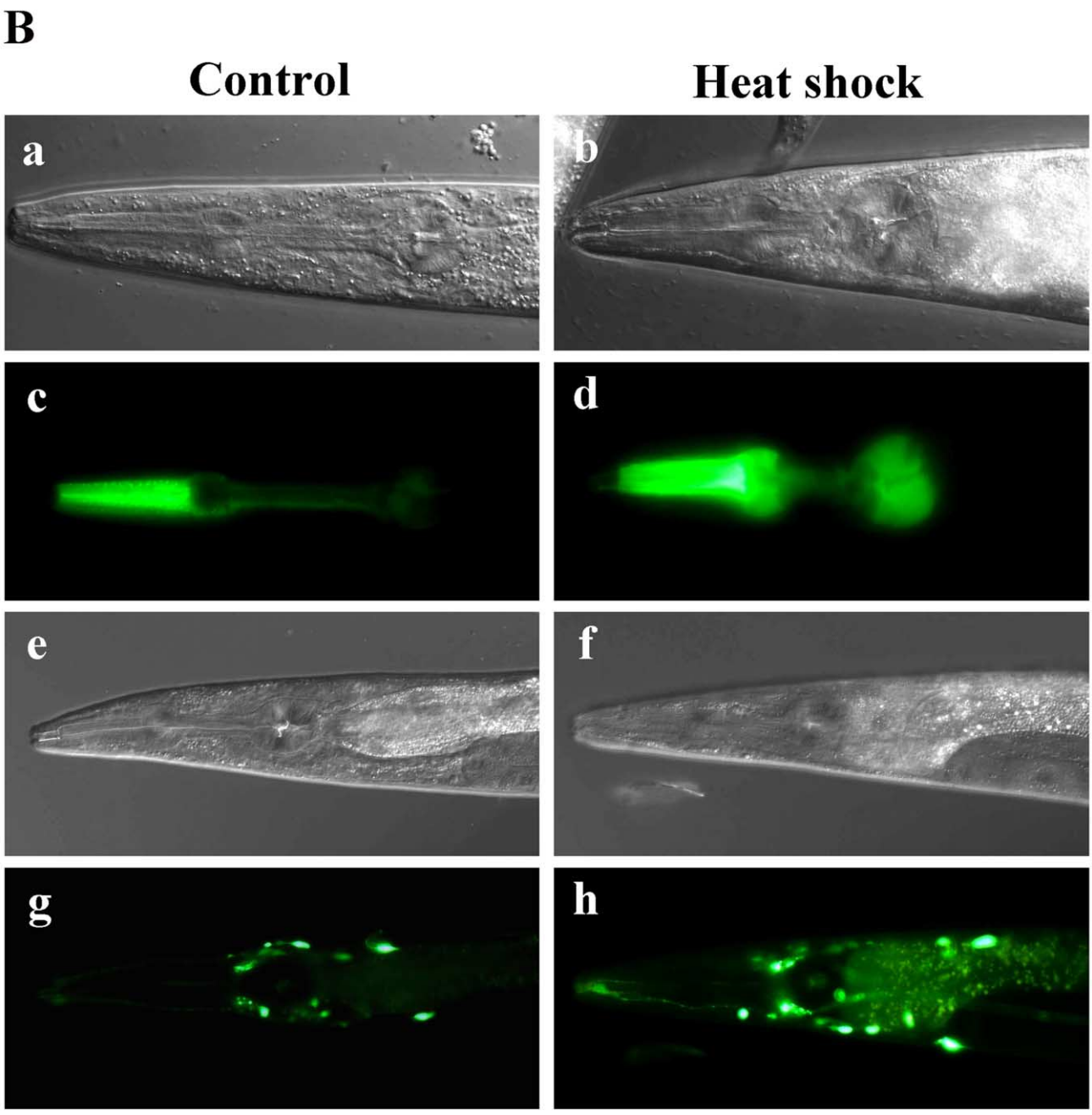

Fig. 3 (*Continued*).

site, but the HSEs of F08H9.4 are relatively far from the start codon (Fig. 1C). In *S. cerevisiae*, several *hsp* genes have UAS elements named general stress response elements (STREs), which mediate response to several stresses including nutrient starvation [27,28]. Although there is no evidence that the sequence corresponding to the yeast STRE is functional outside of this species, it is worth noting that the promoter of F08H9.3 had one putative STRE in the promoter region. In addition, there is a patch of almost identical nucleotides in the promoter region in the two genes. In this region, 31 out of 35 nucleotides were identical. This region may have important regulatory functions, since other regions are highly diverse. The two genes must have been duplicated early in evolution, and have undergone changes in the upstream regions with little changes in the ORFs.

3.2. Tissue-specific expression of F08H9.3 and F08H9.4

To determine the expression pattern of F08H9.3 and F08H9.4, we made GFP fusion constructs and prepared transgenic lines. They were expressed in a tissue-specific manner, but not a stage-specific manner, under normal conditions. F08H9.3 was expressed only in the pharynx, and F08H9.4 was expressed in the excretory canal and a few neuronal cells, including several head neurons and the ventral cord neurons, from the time the respective tissues emerged during development throughout adulthood (Fig. 2). Although we prefer the interpretation that F08H9.3 and F08H9.4 genes are constitutively expressed without any stress, we can not rule out the possibility that the reporter constructs showed constitutive expression as a result of the multicopy transgenes for the

examination of the expression patterns. The expression of reporter constructs contained in the extrachromosomal arrays could be leaky. Consistent with this possibility, search of the database revealed no apparent ESTs corresponding to either gene, which may indicate that the transcripts of these genes are of extremely low abundance.

3.3. Slow and mild induction of F08H9.3 and F08H9.4 by heat shock

The response of F08H9.3 and F08H9.4 to heat shock was slow and mild. These two proteins began to be induced after exposure to heat for 30 min whereas other HSP16 proteins were induced as early as 5 min (data not shown). The transcript level of F08H9.3 and F08H9.4 was very low in the normal condition. We tried several times to detect the transcripts of F08H9.3 and F08H9.4 under normal conditions, but failed. This low transcript level may result either from the restricted tissue-specific expression of F08H9.3 and F08H9.4, or from the extremely low abundance of the endogenous transcripts. In the heat shock condition, the transcript level of F08H9.3 and F08H9.4 was significantly increased (Fig. 3A). The extent of increase in the transcript level of F08H9.4 was much higher compared with that of F08H9.3. This different extents of increase in the transcript of F08H9.3 and F08H9.4 may result from the different expression pattern by heat shock. Examination of tissues in which these two genes are induced revealed that F08H9.3 was weakly induced only in the pharynx by heat shock, where the endogenous expression is detected. On the contrary, F08H9.4 was induced in new tissues that do not express F08H9.4 under the normal condition: the intestine (Fig. 3B).

3.4. F08H9.3 and F08H9.4 have an important role in heat shock condition

Slow but definite heat inducibility of F08H9.3 and F08H9.4 implies that they may function in the response to heat shock condition. We compared the lethality of worms after heat shock, following F08H9.3 or F08H9.4 RNAi (Fig. 4). Although their constitutive tissue-specific expression and inducible expression implicated their functions in these specific tissues not only in stress response but also in some essential biological processes, RNAi experiments did not show any significant difference compared with wild-type animals in normal conditions. Their mutational analysis followed by careful examination might reveal the biological functions of these genes. On the contrary, under heat shock condition, survival rates of the RNAi affected animals were lower. Embryogenesis was mostly affected, and embryo lethality was increased. After heat shock, F08H9.3 and F08H9.4 RNAi-affected animals exhibited much higher lethality than the mock N2 animals (Fig. 4). We then examined whether the F08H9.3 and F08H9.4 proteins purified after overexpression in bacteria contained chaperone activity by an in vitro chaperone assay using citrate synthease as substrate, but we found that they did not show any in vitro chaperone activity (data not shown). However, one still can not rule out the possibility that these proteins act as substrate-specific chaperone activity, and might play an important role in worm development after heat shock condition.

The smHSPs examined so far can be classified into two groups by their expression patterns. One group includes those whose expression is normally undetected, but induced by heat shock or other stresses. The four HSP16 proteins in *C. elegans*, HSP16-1 (T27E4.2 and T27E4.8), HSP16-48 (T27E4.3 and T27E4.9), HSP16-2 (Y46H3A.3) and HSP16-41 (Y46h3A.2) are examples of this group: they are induced

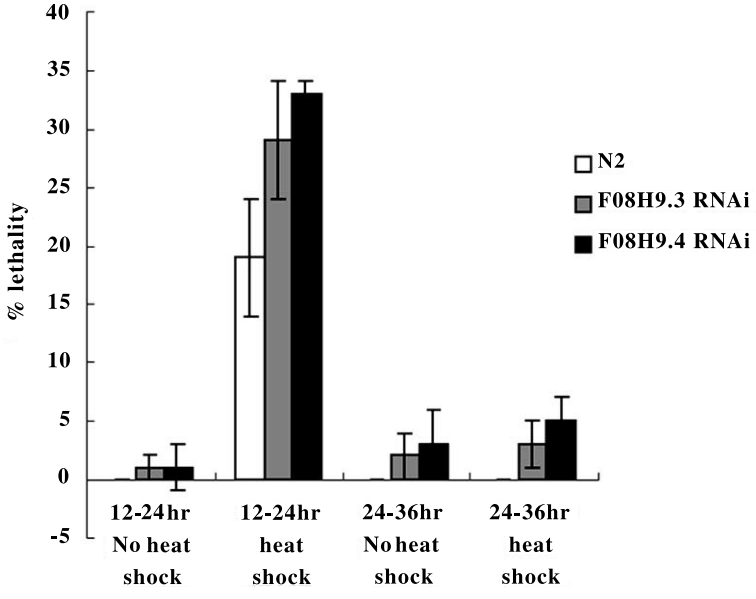

Fig. 4. Lethality promoted by F08H9.3 or F08H9.4 RNAi under heat shock conditions. The RNAi-affected animals or mock-treated animals were subjected to heat shock or no heat shock for 3 h at 6–9 h after RNAi; eggs were collected during the 12–24 h interval and 24–36 h interval, and examined for their survival rate. While the RNAi-affected animals showed virtually no lethality without heat shock, these animals showed much higher lethality than mock animals (N2) under heat condition. Note that the eggs collected at the interval of 24–36 h interval recovered from their survival defects, indicating that either RNAi effect or heat shock effect was diminished by that time. The data presented are the result of three independent experiments, involving observation of a total of 5428 animals. The increase in lethality attained significance for F08H9.4 ($P < 0.025$, Student's t-test) whereas for F08H9.3 the results were more variable.

quickly and strongly with ubiquitous expression [29,30]. A second group includes proteins whose expression is not induced by stress, but appears at specific developmental stage under normal conditions without any stress. One example is HSP12.6: it is expressed only in the L1 stage [31]. We propose that there is a third smHSP family: proteins that are expressed in specific tissues without stress and can be induced by stress, though slowly and weakly. According to our data, F08H9.3 and F08H9.4 proteins can be grouped into this third expression group. It would be interesting to identify such HSPs and determine their biological roles in other species.

In summary, we showed in this report that the *C. elegans* genome contains two genes encoding smHSPs that are expressed in a tissue-specific manner, induced by heat shock, and are important for the survival of the animals under the heat shock conditions.

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