Extended longevity of *Caenorhabditis elegans* by knocking in extra copies of hsp70F, a homolog of mot-2 (mortalin)/mthsp70/Grp75

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Abstract The *Caenorhabditis elegans* homolog of mortalin/mthsp70/Grp75 (called mot-2 hereafter) was isolated by screening of a nematode cDNA library with mouse mot-2 cDNA. The isolated clone matched to hsp70F of *C. elegans*. Analysis with two of the antibodies raised against hsp70F revealed that unlike mammalian mot-2, it is heat inducible. Transient induction of hsp70F by heat shock led to a slight ($<13\%$) extension in the *C. elegans* life span. The transgenic worms that constitutively over-expressed hsp70F predominantly in muscle showed life span extension ($\sim 43\%$ for mean and $\sim 45\%$ for maximum life span) as compared to the wild-type and green fluorescent protein-transgenic worms. Life span extension of human cells was obtained by over-expression of mot-2 [Kaul et al. (2000) FEBS Lett. 474, 159–164]. Our results show, for the first time, that this member of the hsp70 family governs the longevity of worms and thus there are common pathways that determine mammalian and worm longevity.

Key words: Heat shock protein; Mortalin; Life span; Over-expression; Transgenic nematode; *Caenorhabditis elegans*

1. Introduction

Aging is an intricate phenomenon which remains a major challenge to modern biology. It is a universal attribute determined by the interaction of the environment with the genetic predisposition of the individuals [1–3], and is most invariably defined by functional deterioration and declined adaptability. Nevertheless, the mechanisms that limit life span remain largely unknown. In a mammalian system, tumor suppressor genes such as p53 and pRB play a significant role in safeguarding against unlimited proliferation and, therefore, aging has been coined as a tumor suppressor mechanism. The role of DNA damage, particularly at chromosome ends, telomeres [4], has also emerged as a significant mechanism for determination of life span. Other genes implicated in the aging process include repair genes and stress-response genes [5–8]. Many of these studies suffer the limitation of their universality which is partially because of the lack of experimental data in whole organisms. Single gene mutations have been a powerful technique in defining the genes controlling the aging process in *Caenorhabditis elegans*, the nematode worm that undergoes characteristic morphological as well as physiological signs of senescence. These age mutations not only prolong life, but also confer a complex array of other phenotypes that provide clues to the evolutionary origins of these genes. Stress proteins, including the heat shock proteins (hsps), function in combating the stress-induced damage and most of these do so by their chaperone [9] and intracellular trafficking activity [10].

The hsp70 family protein, mortalin/Grp75/mthsp70/PBP74 has been shown to be differentially distributed in normal and immortal cells from mouse and human. There are two alleles [11] coding for two minutely different proteins (mot-1 and mot-2) with contrasting biological activities in mouse [12,13]. Human cells were shown to have only one kind of protein that achieves its differential distribution [14] in normal and immortal cells by mechanism(s) that remain largely unknown to date. Human mortalin cDNA was shown to possess activity similar to the mouse mot-2 cDNA and causes malignant transformation of mouse immortal cells and life span extension of normal human cells [7,12]. This effect was ascribed partly to the p53-inactivating function of mot-2 [7,15,16]. To follow these studies in an animal model, we aimed to isolate the *C. elegans* homolog of mortalin/Grp75/mthsp70/PBP74 (called mot-2 hereafter) and elucidate its effect on life span.

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Abbreviations: GFP, green fluorescent protein; hsp, heat shock protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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2. Materials and methods

2.1. Strain and culture of nematodes

All *C. elegans* strains were derived from the wild-type Bristol strain N2, and were maintained using the standard technique [17]. The animals were harvested by washing off the plates with ice-cold 0.1 M NaCl. The dead animals were separated by centrifugation in 30% (w/w) sucrose at $600 \times g$ for 5 min. The live animals were suspended in 0.1 M NaCl at 22°C for 30 min to allow complete digestion of the bacteria remaining in the worm guts and then washed three times with 0.1 M NaCl. Generally, 60 mg of worms was recovered from a 60-mm plate. The collected worms were used to carry out Western blotting and reverse transcription-polymerase chain reaction (RT-PCR).

2.2. Life span determination

The life span was determined by the standard method. Animals were allowed to lay eggs overnight and were then removed from the day of hatching ($t = 0$) for life span measurement of their progeny. At the L4 or young adult stage, five animals were placed on each plate. They were then transferred to new plates day by day. Once reproduction ceased, the nematodes were transferred to new plates approximately once a week. The worms were judged dead when they did not move after repeated prodding with a pick, or after being tugged gently on the tail by a pick covered with bacteria. Worms that crawled off the plates were not included in the analysis.

2.3. Cloning of hsp70F and its expression construct

To obtain the mot-2 homolog of *C. elegans*, the cDNA library of N2 mixed stages was screened with mouse mot-2 cDNA [18]. A clone containing the full-length *hsp70F* gene was isolated. It showed 73.2% homology to mot-2. The Xba*I* and Sac*I* sites were introduced into *hsp70F* by PCR amplification using *Ex*Taq polymerase (TaKaRa). The sequences of the amplified DNA fragments were confirmed using an ABI-373A sequencer (PE Applied Biosystems, USA). The Xba*I* site was ligated into the green fluorescent protein (GFP) expression plasmid pPD91.14 [19] containing *unc*-54, a promoter for muscles, by site-directed mutagenesis (SDM) using a quick-change SDM kit (Stratagene, USA). The constructed plasmid contained the *hsp70F* gene in the Xba*I* and Sac*I* site of pPD91.14 and was named pPDhsp70f.

2.4. Preparation of transgenic lines

DNA injection into the *C. elegans* germ line was carried out as described by [20]. The plasmid pRF-4 (*rol-6*), whose successful transformation exhibit *Rol* phenotype, was co-injected as a transformation marker. The total DNA concentration of the injected mixture was 3 μg/μl. The resultant transformants were X-ray-irradiated to insert the transgene into worm chromosomes and crossed with wild-type cells to stabilize them as described [19].

2.5. Preparation of antibodies

Two peptides, ^{31}NDQGNRTTPSTV**C** (close to its N-terminal sequence plus cysteine) and **C**^{472}QEAKTAEEPKKEQN (cysteine plus its C-terminal sequence), were designed as epitopes of the antigen and were conjugated to keyhole lympet hemocyanin with maleimidobenzoyloxy-succinimide and injected subcutaneously in the back into two rabbits, respectively. Peptides were purchased from Biologica (Nagoya, Japan). The resultant anti-sera from rabbits injected with NDQGNRTTPSTV**C** were named anti-serum 70fn, and the one from rabbits injected with **C**QEAKTAEEPKKEQN were named anti-serum 70fc, respectively.

2.6. Western blot analysis

Proteins were separated by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then electrophoretically transferred to the polyvinylidene difluoride membrane. The blots were then incubated with the anti-serum (1:500). For pre-treatment with antigen each anti-serum was incubated with a 10 times higher amount of the corresponding antigen at 4°C for 12 h, the insoluble materials generated were removed by centrifugation, and the resultant supernatant was used for Western blotting analysis. The Western blots were developed by an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Uppsala, Sweden). ECL was recorded by a LAS-1000 luminescence analyzer (Fuji Film, Tokyo, Japan) and the images were analyzed using the software 'Image Gauge V3.1' (Fuji Film).

3. Results

3.1. Nematode homolog of mortalin (mot-2)/mthsp70/Grp75

We used mouse mot-2 cDNA to screen its nematode homolog from *C. elegans* cDNA library as described in Section 2. The isolated cDNA clone encoded a protein of 657 amino acids that matched to hsp70F [21], 73.2% homology to mouse mot-2/mthsp70 (Fig. 1). Similar to mot-2 it shares a higher degree of identity with the *Escherichia coli* dnaK protein than with eukaryotic hsp70 proteins. The predicted amino-terminal half of the hsp70F polypeptide also contains a long, amphiphilic leader sequence similar to mitochondrial import leader sequences. We selected two peptides (one each from its unique amino- and carboxy-terminus) from the predicted amino acid sequence of hsp70F and raised anti-serum against these. The anti-sera were tested for their reactivity to *C. elegans* proteins by Western blotting of whole worm lysates. Both anti-sera detected a protein of predicted molecular weight (70 kDa). Furthermore, pre-treatment of the anti-serum with a 10-fold excess of the antigen abolished the detection of the 70-kDa protein suggesting the specificity of anti-serum against the 70-kDa protein (hsp70F, *C. elegans* homolog of mot-2/mthsp70/Grp75) (Fig. 2).

Unlike other members of the hsp70 family of proteins, mot-2 remains uninduced by heat shock [18,22]. We first tested if this characteristic also persisted in *C. elegans*. Four day-old adults of *C. elegans* were exposed to 30°C for 2–6 h. As shown in Fig. 3a,b, the expression level of hsp70F of heat-treated worms as detected by Western blotting with anti-hsp70F increased 2–5-fold to that of untreated worms suggesting that

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Mot-2 :   MISA-SRAAA ARLVGTAASR SPAAARPQDG WNGLSHEAFR FVSRRDYASE AIKGAVVGID
          * ** *     **   * *   *   **                        *    ** * ***
HSP70F :  MLSARSFLSS AR---TIARS SLMSAR---- ---------- ------SLSD KPKGHVIGID

    60 :  LGTTNSCVAV MEGKQAKVLE NAEGARTTPS VVAFTADGER LVGMPAKRQA VTNPNNTFYA
          ********   ****  ** * **** *****  ********** *** ****** ***  **  *
    38 :  LGTTNSCVSI MEGKTPKVIE NAEGVRTTPS TVAFTADGER LVGAPAKRQA VTNSANTLFA

   120 :  TKRLIGRRYD DPEVQKDTKN VPFKIVRASN GDAWVEAHGK LYSPSQIGAF VLMKMKETAE
          *********  ******* *  ** *** *** ******* **  ***** *** **********
    98 :  TKRLIGRRYE DPEVQKDLKV VPYKIVKASN GDAWVEAQGK VYSPSQVGAF VLMKMKETAE

   180 :  NYLGHTAKNA VITVPAYFND SQRQATKDAG QISGLNVLRV INEPTAAALA YGLDK-SEDK
           *** *  ** * ******** ********** ********** ********** *****   **
   158 :  SYLGTTVNNA VVTVPAYFND SQRQATKDAG QISGLNVLRV INEPTAAALA YGLDKDAGDK

   239 :  VIAVYDLGGG TFDISILEIQ KGVFEVKSTN GDTFLGGEDF DQALLRHIVK EFKRETGVDL
           ********* *** ****** ********** ********** * **   * *  *** * ****
   218 :  IIAVYDLGGG TFDVSILEIQ KGVFEVKSTN GDTFLGGEDF DHALVHHLVG EFKKEQGVDL

   299 :  TKDNMALQRV REAAEKAKCE LSSSVQTDIN LPYLTMDASG PKHLNMKLTR AQFEGIVTDL
          ***  * **  ********** ***  ***** *** *** ** ***** **** * ** ** **
   278 :  TKDPQAMQRL REAAEKAKCE LSSTTQTDIN LPYITMDQSG PKHLNLKLTR AKFEQIVGDL

   359 :  IKRTIAPCQK AMQDAEVSKS DIGEVILVGG MTRMPKVQQT VQDLFGRAPS KAVNPDEAVA
          ***** ** * *  ****  *  *  * **** * ****** * **  **  ** **********
   338 :  IKRTIEPCRK ALHDAEVKSS QIADVLLVGG MSRMPKVQAT VQEIFGKVPS KAVNPDEAVA

   419 :  IGAAIQGGVL AGDVTDVLLL DVTPLSLGIE TLGGVFTKLI NRNTTIPTKK SQVFSTAADG
           ****** ** ********** ********** ****  ****  ********** **********
   398 :  MGAAIQGAVL AGDVTDVLLL DVTPLSLGIE TLGGIMTKLI TRNTTIPTKK SQVFSTAADG

   479 :  QTQVEIKVCQ GEREMAGDNK LLGQFTLIGI PPAPRGVPQI EVTFDIDANG IVHVSAKDKG
          **** *** * ******  ** ***** * ** **********  ********** ** *** * *
   458 :  QTQVQIKVFQ GEREMATSNK LLGQFSLVGI PPAPRGVPQV EVTFDIDANG IVNVSARDRG

   539 :  TGREQQIVIQ SSGGLSKDDI ENMVKNAEKY AEEDRRKKER VEAVNMAEGI IHDTETKMEE
          ** ******* ******** * *** * ***  * **   **  **  * **** ***** ** *
   518 :  TGKEQQIVIQ SSGGLSKDQI ENMIKEAEKN AAEDAKRKEL VEVINQAEGI IHDTEAKMTE

   599 :  FKDQLPADEC NKLKEEISKV RALLARKDSE TGENIRQAAS SLQQASLKLF EMAYKKMASE
          * **** ***   *   *       *  ** * * * *  *    *** ***** * *** **
   578 :  FADQLPKDEC EALRTKIADT KKILDNKDNE TPEAIKEACN TLQQQSLKLF EAAYKNMAAK

   659 :  REGSGSSGTG EQKEDQKEEK Q
            * *         *  * *
   638 :  NSG-GDAQEA KTAEEPKKEQ N
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Fig. 1. Amino acid sequence comparison of mot-2/mthsp70/Grp75 and its nematode homolog hsp70F.

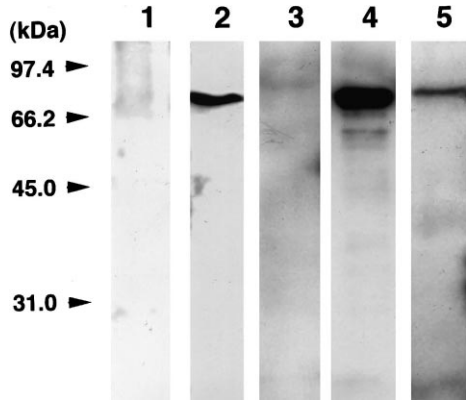

Fig. 2. Characterization of anti-peptide anti-sera against hsp70F. Proteins (50 μg) from the whole cell lysate of nematode were separated in 12.5% SDS–PAGE and electrophoretically transferred to polyvinylidene difluoride membrane. Western blotting was performed with antibodies (1:500, pretreated or untreated). For pretreatment with antigen, each anti-serum was incubated with a 10-fold amount of the corresponding antigen at 4°C for 12 h, the insoluble materials generated were removed by centrifugation, and the resultant supernatant was used for Western blotting analysis. Lane 1, pre-immune; lane 2, anti-serum 70fn; lane 3, anti-serum 70fn pre-treated with the antigen; lane 4, anti-serum 70fc and lane 5, anti-serum 70fc pre-treated with the antigen.

unlike its homolog in mammals, hsp70F is induced by mild heat shock.

3.2. Heat shock induces a slight life span extension of C. elegans

We next treated worms at 30°C for 2, 4 or 6 h followed by a shift to their normal culture temperature of 25°C. Fig. 3c shows typical survival curves of untreated and heat-treated (2, 4 and 6 h) worms. Heat treatment for 6 h increased the life span slightly ($P < 0.005$, Table 1), however, 2 or 4 h heat treatment did not affect the life span of the *C. elegans*. Western blotting analysis of the heat-treated worms and those cultured at 25°C revealed that the expression level of hsp70F decayed to the original level in 3 days (Fig. 4). Similar results were obtained from three independent experiments and suggested that heat shock treatment transiently increases the expression level of hsp70F and caused a slight extension in the life span of *C. elegans*.

3.3. Transgenic expression of hsp70F in C. elegans

Transgenic worms expressing extra copies of hsp70F were obtained as described in Section 2. The obtained hsp70-transgenic lines showed almost the same properties as that of GFP-transgenic lines, for example, body size, mean lengthening of

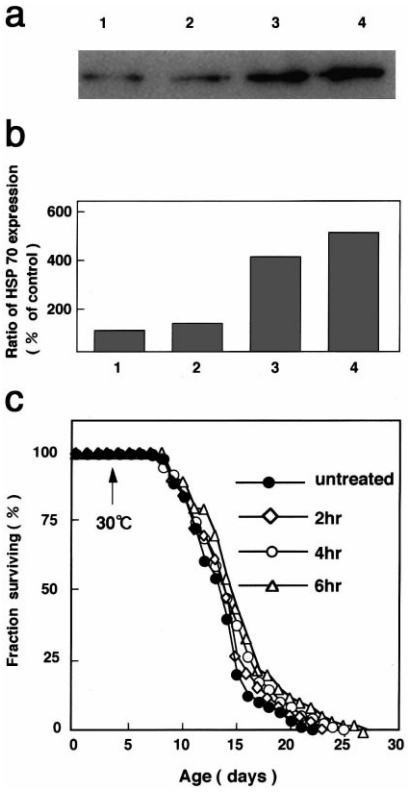

Fig. 3. Effect of mild heat stress on the expression of hsp70F and the life span of *C. elegans*. Young adult hermaphrodite animals were exposed to 30°C for 2, 4 or 6 h, and then collected for Western blotting analysis, or returned to 25°C for survival assay. The other procedures were as described in Section 2. a: Western blotting. b: Quantification of the Western blotting data showing the amount of hsp70F relative to untreated control animals. c: Survival curves. 1, untreated control animals; 2–4, four day-old adults (shown by arrow) were exposed to 30°C for 2, 4 or 6 h, respectively.

embryonic development, and the cycles of swimming and pumping (data not shown). The whole cell lysate of the transgenic lines carrying pPDhsp70f were analyzed for the expression of the transgene of hsp70F by Western blotting using anti-hsp70F anti-serum. As shown in Fig. 5a,b, the amount of hsp70F in the transgenic line carrying pPD70f increased two- to three-fold relative to the wild-type animals. The expression of hsp70F as detected by immunohistochemical staining with the anti-hsp70F anti-serum (Fig. 5c) shows tissue specific expression of hsp70F in the muscle cells of the *C. elegans* including body walls and anus muscle. The expression pattern of hsp70F in transgenic lines carrying pPD70f

Table 1
Summary of survival data of heat-treated and transgenic worms

	Mild heat shock treatment (h)				Transgenic line[a]	
	0	2	4	6	GFP	hsp70F
Number of animals	200	200	200	200	200	200
Maximum life span (days)	22	23	25	26	22	32
50% survival age (days)	12.5	13.8	13.8	14.4	13.7	19.2
Mean life span (days)	13.6	14.1	14.4	15.3	14.5	19.3
t-test	control	1.15	1.71	3.37	control	8.39
Significance level	–	< 0.5	< 0.1	< 0.005	–	$\ll 0.001$

'*t*-test' indicates the probability that the mean life span is the same as the control (Student's *t*-test).
[a]GFP, transgenic line carrying pPD91.14; hsp70F, transgenic line carrying pPDhsp70f.

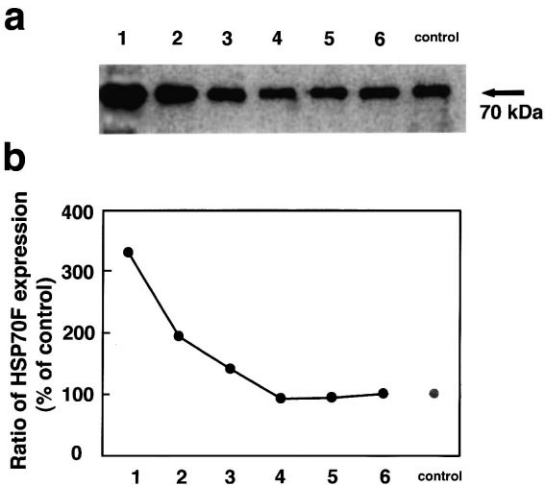

Fig. 4. Stability of mild heat shock-induced hsp70F. The *C. elegans* were exposed to 30°C for 6 h, and then maintained at 25°C for 5 days. The worms collected were analyzed for hsp70F by Western blotting. Other procedures were as described in Section 2. a: Western blot analysis. b: The amount of hsp70F relative to that in control untreated worms. After pre-exposure to 30°C for 6 h (1), animals were maintained at 25°C for 1–5 days (2–6). Control: untreated animals.

were almost the same as that of the expression of GFP in transgenic line carrying pPD91.14 (Fig. 5c, panel 4).

3.4. *hsp70F-transgenic worms show extended life span*

The life span of wild-type and transgenic worms was studied by typical survival curves (Fig. 6). Hsp70F-transgenic worms lived significantly longer than the control wild-type animals and the transgenic lines carrying pPD91.14. The mean and the maximum life spans of the transgenic lines carrying pPD70f was extended by 6.2 days (43%) and 10 days (45%), respectively, as compared to the control animals (Table 1). In several independent experiments, we obtained the similar results of extended longevity of worms by overexpression of hsp70F.

4. Discussion

C. elegans is a well accepted model for aging studies. Many studies, especially the single gene mutations, have contributed to our present understanding of aging [23–25]. The hsp70 family shows tight conservation in evolution. In *C. elegans*, three kinds of hsp70 member, hsp70F, hsp70A, hsp70C, were reported [20,26]. Hsp70F is moderately heat inducible [21], and shares a high degree of identity with mot-2/mthsp70/Grp75 and has a mitochondria leader sequence. Hsp70A has a high degree of identity with the eukaryotic hsc70 family found in the cytosol and the nucleus. Hsp70C shows similarity to eukaryotic GRP78/BiP mainly found in the endoplasmic reticulum. Although the major functions of hsp70 family proteins are described in relation to stress response and chaperone activity, their specific functions are largely undefined. Mot-2 was shown to have differential cellular distribution in normal and immortal cells and was shown to bind to and inactivate p53 [14,15] elucidating, at least in part, its role in control of cell proliferation. We report here that the hsp70F, homolog of mot-2 in *C. elegans* is inducible by mild heat shock. Transient induction of hsp70F led to slight but notice-

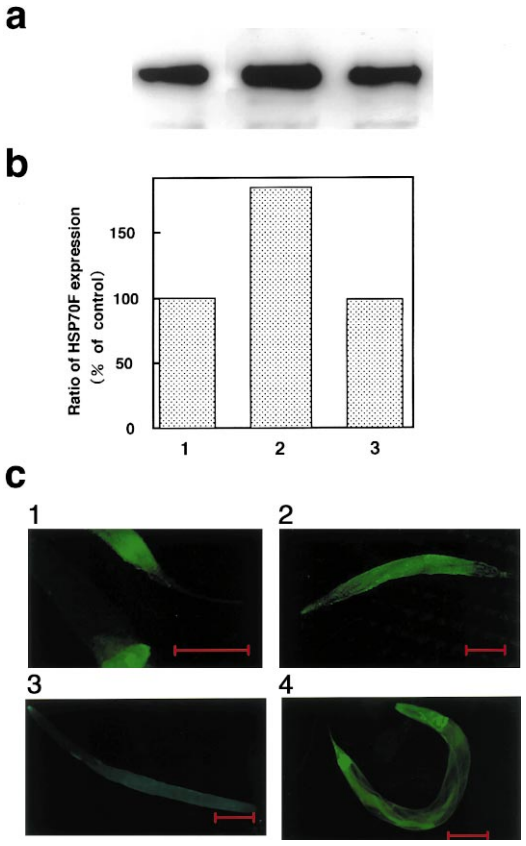

Fig. 5. Expression of hsp70F in transgenic animals. a: Western blot analysis. b: Amount of hsp70F relative to control untreated animals. 1, wild-type animals (N2); 2, transgenic lines carrying pDP70f; 3, transgenic line carrying pPD91.14. c: Immunohistochemical staining of transgenic lines. The transgenic nematodes carrying pPD70f (1, 2) or pPD91.14 (4) were stained immunohistochemically. 1 shows staining in anus muscles and 2 shows body wall muscles. The fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (COSMO BIO) was used as a secondary antibody. The localization of GFP expressed in muscle cells in transgenic lines carrying pPD91.14 was detected by fluorescence microscopy. Other procedures were as described in Section 2. Bars represent 100 μm. Both Western blot analysis and immunohistochemical staining were carried out with anti-serum 70fc.

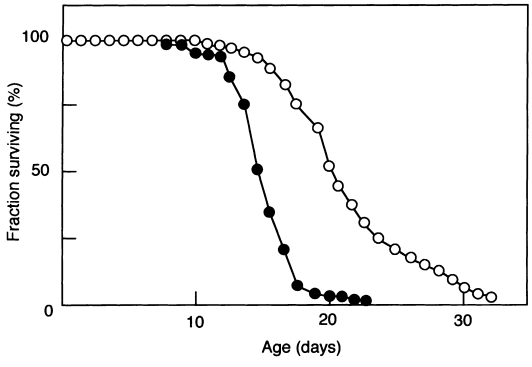

Fig. 6. Survival curves of the control and hsp70F-transgenic *C. elegans*. Survival curves of control (filled circles) and hsp70F-transgenic (open circles) worms.

able increase in the longevity of worms. Transgenic *C. elegans* over-expressing hsp70F possess a significantly increased life span. Spe-10 mutant nematodes were shown to have an extended life span and increased resistance to both UV light and to heat [27]. *Drosophila melanogaster* with a higher amount of hsp70 induced by mild heat shock also showed an increased life span [28,29]. Furthermore, transgenic *C. elegans* over-expressing hsp70A also possessed a significantly increased life span (data not shown). Taken together, these results suggest the importance of stress genes in determining longevity. An over-expression of mot-2 was shown to extend the life span of normal human cells in culture suggesting that there are common pathways to life span determination in nematodes and human and these involve hsp mot-2/mthsp70/Grp75. It was shown to inactivate p53 in human cells [15,16]. Since the homolog of p53 is not known in nematode, it is likely that some of the other predicted functions of mot-2/hsp70F contribute to the life span extension in worms. These include chaperone [8], mitochondrial importer [10], intracellular trafficking [30] and other yet to be defined functions predicted from its multiple residing sites.

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