RESEARCH COMMUNICATION

Association of several small heat-shock proteins with reproductive tissues in the nematode *Caenorhabditis elegans*

Lily DING and E. Peter M. CANDIDO[1]

Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada V6T 1Z3

Immunohistochemical data on 10 of the 14 small heat-shock (smHSPs) proteins in fourth larval stage and adult *Caenorhabditis elegans* show that the tissues expressing the greatest number of smHSPs are vulva (HSP12s, HSP43 and, under stress, HSP16s) and spermatheca (HSP12s, HSP25, HSP43 and, under stress, HSP16s). HSP43 is also expressed in male tail structures, and following heat-shock HSP16s are expressed in spermatids and spermatozoa.

Key words: α-crystallin, immunocytochemistry, tissue specificity.

INTRODUCTION

Small heat-shock proteins (smHSPs) share a similar core sequence with α-crystallins. A BLAST [1] search of the *Caenorhabditis elegans* genome, using the sequence of the stress-inducible HSP16-48 protein as query, yields 16 genes encoding 14 distinct smHSPs [2]. According to their molecular masses and sequence similarity, these smHSPs can be roughly divided into HSP12s (four members), HSP16s (six members), HSP25, HSP43, HSP17.5 and stress-induced protein-1 (SIP-1).

C. elegans HSP12s are constitutively expressed and not significantly up-regulated by a wide range of stressors. Their native structure seems to consist of small oligomers (monomer-tetramer), and the recombinant proteins lack chaperone activity in the standard *in vitro* assays [3], suggesting that they may have relatively specialized functions *in vivo*.

The HSP16s include six members, of which four (HSP16-2, HSP16-41, HSP16-1 and HSP16-48) have been extensively studied in our laboratory. These smHSPs are encoded by paired genes (*hsp16-2/16-41*, *hsp16-1/48*), and controlled by closely spaced heat-shock promoter elements [4,5]. HSP16-1, HSP16-2, HSP16-41 and HSP16-48 are made only in response to heat-shock and other stresses [6–8], and are active as molecular chaperones *in vitro* [9].

HSP25 is constitutively associated with dense bodies (analogues of the Z-line in vertebrates) and M-lines in body-wall muscle and also with cell junctions of the spermathecal wall [2], whereas HSP43 is localized to specific cells (utse and uv1) of the vulva and to spermathecal desmosomes [10]. SIP-1, originally called small embryonic chaperone-1 (SEC-1) [11], was detected only in oocytes and developing embryos, and antisense experiments showed that it is required for embryonic development; SIP-1 presumably has chaperone activity since its expression in *Escherichia coli* confers thermotolerance [11]. HSP17.5 has not been studied to date.

HSP12s and HSP16s have been studied biochemically *in vitro*, and message levels have been measured throughout *C. elegans* development [3,6]. In addition, the control of HSP16 synthesis has been studied using *hsp16–lacZ* fusions in transgenic animals [7,8]. However, the normal tissue distributions of endogenous HSP12s and HSP16s have not been examined. Here we employ immunohistochemical techniques to determine the tissue specificity of these smHSPs in fourth larval stage (L4) and adult nematodes, and of HSP43 in males. The results indicate that many of the smHSPs are prominently expressed in reproductive tissues and structures in mature animals.

MATERIALS AND METHODS

Nematode culture and immunohistochemistry

Mixed populations of N2 nematodes and a *him-8* (high incidence of males) mutant (*him-8 e1489*) were cultured at 20 °C [12] or heat-shocked at 33 °C, as previously described [7].

Immunofluorescence staining was a modification of the method of Loer and Kenyon [13], as previously described [2], or according to the method of Finney and Ruvkun [14]. After permeabilization and blocking in BSA at room temperature for 1 h, nematodes were incubated with rabbit anti-HSP12.6 [3], anti-HSP16-41 or anti-HSP16-2 [8,15] at 1:250 dilution (pre-treated with 1% *E. coli* acetone-dried powder), and/or mouse monoclonal antibody DM5.6 [16] at 1:250 dilution at room temperature for 2 h. Samples were washed and incubated with fluorescent-conjugated secondary antibodies at 1:250 dilution [Alexa™ 488 anti-rabbit conjugate (Molecular Probes, Eugene, OR, U.S.A.) and/or Texas Red dye-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.)] at room temperature for 2 h. In addition, some nematodes were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and Texas Red-X phalloidin (Molecular Probes). In control experiments, anti-smHSP antibodies were pre-incubated with 1 mg/ml of the corresponding recombinant smHSP. After staining, nematodes were viewed by fluorescence microscopy (Axioplan 2 microscope; Zeiss).

RESULTS AND DISCUSSION

The HSP12s are the smallest known members of the smHSP family known from any organism [3]. The tissue specificity of HSP12s was studied by immunohistochemistry using a polyclonal anti-HSP12.6 antibody (Figure 1, A1–A14), which cross-reacts

Abbreviations used: SIP-1, stress-induced protein-1; (sm)HSP, (small) heat-shock protein; DAPI, 4,6-diamidino-2-phenylindole; L1 and L4, first and fourth larval stages respectively.

[1] To whom correspondence should be addressed (e-mail epmc@interchange.ubc.ca).

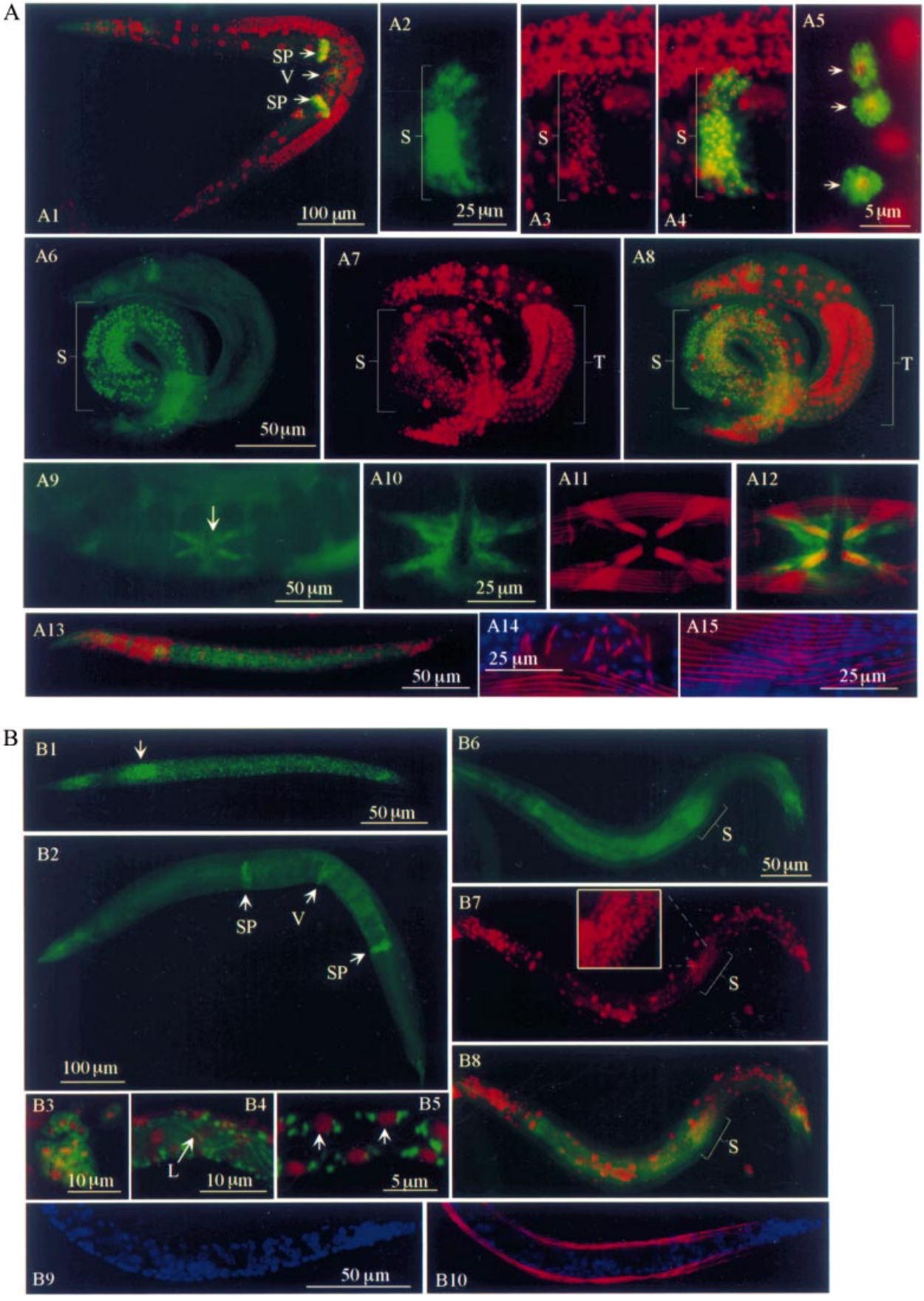

Figure 1 For legend see opposite page

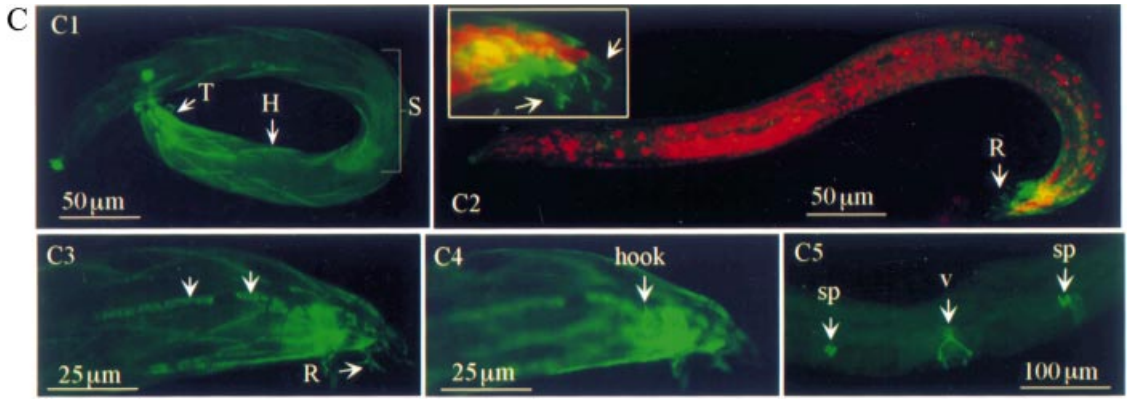

Figure 1 Tissue distribution of small heat-shock proteins

In all panels anterior is on the left and dorsal towards the top. Small heat-shock proteins were labelled with rabbit polyclonal antibodies as previously described [2], and the images are shown in green. (**A**) HSP12s (HSP12.6 antibody). A1, overview of HSP12 expression in L4 hermaphrodite. Spermathecae are strongly labelled and vulva is lightly labelled (arrows), with nuclei (red, DAPI) showing the positions of the tissues. A2–A5, staining in spermatheca (A2) is confirmed by co-staining of sperm nuclei (A3; red, DAPI). The images in A2 and A3 are merged in A4. A5, HSP12s localized in cytoplasm of sperm cells (green), with nuclei shown in red (DAPI). A6–A8, adult male showing HSP12s in sperm (S). The positions of sperm cells are indicated by nuclear counterstaining with DAPI (A7), and the distal loop of the testis (T) is indicated. Images A6 and A7 are merged in A8. A9–A12, anti-HSP12 labels vulva in adult hermaphrodites. A9 and A10, staining pattern of HSP12s in vulval region. A11, vulval muscles are revealed by counterstaining with a monoclonal antibody to myosin heavy chain, DM5.6 [16]. Images A10 and A11 are merged in A12. A13, HSP12s are ubiquitously expressed in L1 (green) with nuclei shown in red (DAPI). A14 and A15, controls: HSP12.6 antibody was pre-incubated with an excess of recombinant HSP12.3. The nematode was counterstained with DAPI to show nuclei, and with DM5.6 to label myosin. (**B**) HSP16 localization with polyclonal anti-HSP16-2 antibody [15]. B1, in late L1, HSP16s are ubiquitously expressed, although particularly prominent in nerve ring (arrow). B2, adult hermaphrodite: in addition to general tissue staining, anti-HSP16 strongly labels spermathecae (SP), and, more lightly, the vulval region (V). B3, HSP16s (green) are localized in sperm cytoplasm, with nuclei shown in red (DAPI). B4 and B5, HSP16s (green) are concentrated in discrete cytoplasmic regions in intestinal cells and along the border of the lumen (L); large intestinal nuclei (arrows, B5) are shown in red (DAPI). B6, HSP16s are expressed throughout the body in males, slightly concentrated over sperm (S). Sperm nuclei were stained with DAPI (B7), and the images are merged in B8. B9, unstressed L2–L3 animal, showing lack of HSP16 staining (green). The animal was counterstained with DAPI, and nuclei are shown in blue. B10, as a control for permeabilization, the animal in B9 (DAPI nuclei in blue) was also counterstained with monoclonal antibody DM5.6 [15] to show body-wall muscle (red). (**C**) HSP43. C1, overview of HSP43 staining pattern in male. HSP43 staining is conspicuous in the tail (T) and at hypodermal/muscle cell contacts (H), and less prominent over the location of spermatids in the testis (S). C2, anti-HSP43 labels tail rays in males (R and arrows, inset). Nuclei (red, DAPI) show the position of the tissues. C3, tail region of male, with anti-HSP43 labelling of vas deferens or, possibly, copulatory spicules (arrows). C4, HSP43 labels the hook and other structures in the male tail (arrow). C5, hermaphrodite, showing anti-HSP43 labelling of spermathecae and vulva.

with the other nematode HSP12s [3,17]. In previous work, examination of HSP12 levels throughout development revealed that first larval stage (L1) larvae have the highest levels of this smHSP [3], and immunohistochemistry shows that L1 larvae have high HSP12 levels throughout the body (Figure 1, A13). Although overall levels of HSP12s are much lower in L4 larvae (the stage at which sexual maturation occurs) and in adult hermaphrodites, these smHSPs are locally abundant in the spermatheca and in specific vulval cells under unstressed conditions (Figure 1, A1–A12).

In the spermatheca of hermaphrodites, HSP12s are localized in sperm (Figure 1, A2–A5). At higher magnification, the distribution of HSP12 cytoplasmic staining in sperm cells is seen to be spherically symmetrical (Figure 1, A5), indicating that the pseudopod of the cell [18] is not labelled by anti-HSP12. In males, spermatids are stored in the seminal vesicle, and most sperm cells mature following ejaculation [18]; the extensive labelling of HSP12 in male germ cells (Figure 1, A6–A8) therefore indicates localization of this smHSP to spermatids and perhaps spermatocytes. Cells in the mitotic region of the male gonad, however, are not labelled (Figure 1, A8). In hermaphrodites, HSP12s are also expressed in a subset of vulval muscle cells (A9–A12). The specificity of the antibody for HSP12s is demonstrated by the loss of signal when the antibody is pre-incubated with excess recombinant HSP12.3 (Figure 1, A14 and A15).

The expression and chaperone activity of HSP16s in *C. elegans* have been extensively analysed in our laboratory. These small heat-shock proteins (HSP16-2/HSP16-41, HSP16-1/HSP16-48) are strictly stress-inducible and act as molecular chaperones. It is believed that HSP16s play important roles in enhancing survival of the animal under conditions of chemical and physical stress.

The tissue specificity of HSP16s was studied by immunohistochemistry with a polyclonal anti-HSP16-2 antibody [15] before and after heat-shock. HSP16-2 is ubiquitously expressed throughout most somatic tissues of larvae after heat-shock (Figure 1, B1), and the intensity gradually decreased with age (results not shown). HSP16 labelling is prominent in spermathecae and in the vulval region in L4 and adult hermaphrodites (Figure 1, B2). The spermathecal labelling seems to consist of cytoplasmic localization of HSP16-2 in sperm (Figure 1, B3), and perhaps also in the spermathecal cage cells (results not shown). Counterstaining with DAPI revealed HSP16 localized in discrete regions within the cytoplasm of intestinal cells, characterized by their large nuclei (Figure 1, B4 and B5). Concentrations of HSP16-2 occur along the luminal border of these cells (Figure 1, B4). The prominent localization of HSP16 to intestinal cells was previously noted in transgenic animals expressing a *lacZ–HSP16* gene fusion [7]. Vulval expression of HSP16 was also seen in transgenic reporter experiments [7]. On the other hand, the expression of transgenes in meiotic cells tends to be repressed in *C. elegans* [19], so the lack of sperm-cell labelling in transgenic reporter strains is not surprising. In males, HSP16-2 was distributed more generally, though it was slightly concentrated in the lower testis in the region corresponding to spermatids, and in tail structures (Figure 1, B6–B8). The latter expression pattern was also seen with transgenic reporter constructs [7].

Similar patterns of HSP16 distribution were observed with two other polyclonal antibodies, one with greater specificity for

HSP16-41 [15] and another which cross-reacts with all four stress-inducible HSP16s [8] (results not shown). Thus the staining pattern described here reflects the distribution of the HSP16 family in general. Non-stressed animals showed little or no detectable signal (Figure 1, B9 and B10).

The tissue distribution of HSP43 in N2 hermaphrodites has recently been determined [10]. It is localized to specific cells of the vulva, to the spermathecal valve and junctions between cells of the spermathecal cage. It is also concentrated in regions of contact between muscle cells or between muscle cells and the overlying hypodermis. The latter pattern was also seen in males in the present study (Figure 1, C1). In males, HSP43 was also found to be concentrated in specialized structures of the tail, including rays (Figure 1, C2), copulatory spicules (C3), hook (C4) and other structures too poorly resolved to permit reliable identification. This signal was HSP43-specific, and not due to autofluorescence of tail structures, since it was abolished when the antibody was pre-incubated with excess HSP43 protein (results not shown).

The results reported here, together with recent studies on the tissue distribution of HSP25 [2] and HSP43 [10], provide immunohistochemical data on the localization of 10 of the 14 distinct smHSPs in *C. elegans*. The remaining smHSPs include HSP17.5 and two proteins which are similar to HSP16-2, none of which have been studied to date, and SIP-1/SEC-1, which is expressed in embryos [11].

In hermaphrodites, the spermatheca expresses HSP12s, HSP25 and HSP43 under normal growth conditions, and under stress conditions HSP16s are added to this repertoire. The vulva expresses HSP12s and HSP43 normally and HSP16s additionally under stress conditions. In males, HSP12s are expressed in sperm and HSP43 in tail structures. Besides the reproductive tissue expression, HSP12s and HSP16s are also expressed ubiquitously, whereas HSP25 is found in body-wall muscle and HSP43 in hypodermis. Following a heat-shock, HSP16 staining was strongest in L1, and the response gradually decreased in successive stages. This is in good agreement with HSP16 mRNA levels determined in earlier work [6], in which it was found that following heat induction, HSP16 mRNA levels were highest in L1 and became progressively lower in later larval stages and adults.

In mature *C. elegans*, the tissues which express the greatest number of smHSPs are vulva (HSP12s, HSP43 and, under stress conditions, HSP16s) and spermatheca (HSP12s, HSP25, HSP43 and, under stress conditions, HSP16s). Interestingly, prominent expression of smHSPs in reproductive tissues has been noted in other species. Hsp25 is localized specifically to spermatocytes, but not spermatids or spermatogonia in mouse seminiferous tubules [20]. Mouse HSP27 is localized in testis under both unstressed and heat-shock conditions [21]. *Drosophila* Hsp23 and Hsp27 are expressed in unstressed and stressed male gonad [22], and Hsp27 is associated with germline nurse cells throughout *Drosophila* egg development in unstressed flies [23].

Our results demonstrate that a given *C. elegans* smHSP may be present in several different tissues at a given developmental stage, and that the tissue specificity may vary at different stages. At this time it is not clear whether these distributions indicate that smHSPs perform different functions in different tissues, or whether they interact with a common or related set of target proteins. Small HSPs have been associated with a bewildering variety of functions, most notably protection from oxidative stress [24] and the control of actin filaments [25] and intermediate filaments [26]. With regard to the latter, particularly intriguing is the association of HSP12 with sperm cells, and HSP25 and 43 with muscle cell junctions and male tail structures. Interestingly, *C. elegans* sperm cells contain little or no actin, but have large amounts of specialized major sperm proteins (MSPs) believed to be involved in amoeboid movement [18]. It would thus be of interest to determine whether HSP12s interact with MSPs.

We thank Don Jones for critical reading of the manuscript. This research was supported by the Medical Research Council of Canada and the Natural Sciences and Engineering Research Council.

REFERENCES

1 Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**, 3389–3402
2 Ding, L and Candido, E. P. M. (2000) HSP25, a small heat shock protein associated with dense bodies and M-lines of body wall muscle in *Caenorhabditis elegans*. J. Biol. Chem. **275**, 9510–9517
3 Leroux, M. R., Ma, B. J., Batelier, G., Melki, R. and Candido, E. P. M. (1997) Unique structural features of a novel class of small heat shock proteins. J. Biol. Chem. **272**, 12847–12853
4 Russnak, R. H. and Candido, E. P. M. (1985) Locus encoding a family of small heat shock genes in *Caenorhabditis elegans*: two genes duplicated to form a 3.8-kilobase inverted repeat. Mol. Cell. Biol. **5**, 1268–1278
5 Jones, D., Russnak, R. H., Kay, R. J. and Candido, E. P. M. (1986) Structure, expression, and evolution of a heat shock gene locus in *Caenorhabditis elegans* that is flanked by repetitive elements. J. Biol. Chem. **261**, 12006–12015
6 Jones, D., Dixon, D. K., Graham, R. W. and Candido, E. P. M. (1989) Differential regulation of closely related members of the hsp16 gene family in *Caenorhabditis elegans*. DNA **8**, 481–490
7 Stringham, E. G., Dixon, D. K., Jones, D. and Candido, E. P. M. (1992) Temporal and spatial expression patterns of the small heat shock (hsp16) genes in transgenic *Caenorhabditis elegans*. Mol. Biol. Cell **3**, 221–233
8 Jones, D., Stringham, E. G., Babich, S. L. and Candido, E. P. M. (1996) Transgenic strains of the nematode *C. elegans* in biomonitoring and toxicology: effects of captan and related compounds on the stress response. Toxicology **109**, 119–127
9 Leroux, M. R., Melki, R., Gordon, B., Batelier, G. and Candido, E. P. M. (1997) Structure-function studies on small heat shock protein oligomeric assembly and interaction with unfolded polypeptides. J. Biol. Chem. **272**, 24646–24656
10 Ding, L. and Candido, E. P. M. (2000) HSP43, a small heat-shock protein localized to specific cells of the vulva and spermatheca in the nematode *Caenorhabditis elegans*. Biochem. J. **349**, 409–412
11 Linder, B., Jin, Z., Freedman, J. H. and Rubin, C. S. (1996) Molecular characterization of a novel, developmentally regulated small embryonic chaperone from *Caenorhabditis elegans*. J. Biol. Chem. **271**, 30158–30166
12 Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. Genetics **77**, 71–94
13 Loer, C. M. and Kenyon, C. J. (1993) Serotonin-deficient mutants and male mating behavior in the nematode *Caenorhabditis elegans*. J. Neurosci. **13**, 5407–5417
14 Finney, M. and Ruvkun, G. B. (1990) The unc-86 gene product couples cell lineage and cell identity in *C. elegans*. Cell **63**, 895–905
15 Hockertz, M. K., Clark-Lewis, I and Candido, E. P. M. (1991) Studies of the small heat shock proteins of *Caenorhabditis elegans* using anti-peptide antibodies. FEBS Lett. **280**, 375–378
16 Miller III, D. M., Ortiz, I., Berliner, G. C. and Epstein, H. F. (1983) Differential localization of two myosins within nematode thick filaments. Cell **34**, 477–490
17 Kokke, B. P. A., Leroux, M. R., Candido, E. P. M., Boelens, W. C. and de Jong, W. W. (1998) *Caenorhabditis elegans* small heat-shock proteins Hsp12.2 and Hsp12.3 form tetramers and have no chaperone-like activity. FEBS Lett. **433**, 228–232
18 L'Hernault, S. W. (1997) Spermatogenesis. In *C. elegans II* (Riddle, D., Blumenthal, T., Meyer, B. J. and Priess, J. R., eds.), pp. 271–294, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
19 Seydoux, G., Mello, C. C., Pettitt, J., Wood, W. B., Priess, J. R. and Fire, A. (1996) Repression of gene expression in the embryonic germ lineage of *C. elegans*. Nature (London) **382**, 713–716
20 Wakayama, T. and Iseki, S. (1999) Specific expression of the mRNA for 25 kDA heat-shock protein in the spermatocytes of mouse seminiferous tubules. Anat. Embryol. **199**, 419–425
21 Biggiogera, M., Tanguay, R. M., Marin, R., Wu, Y., Martin, T. E. and Fakan, S. (1996) Localization of heat shock proteins in mouse male germ cells: an immunoelectron microscopical study. Exp. Cell Res. **25**, 77–85
22 Michaud, S., Marin, R., Westwood, J. T. and Tanguay, R. M. (1997) Cell-specific expression and heat-shock induction of Hsps during spermatogenesis in Drosophila melanogaster. J. Cell Sci. **110**, 1989–1997

23 Marin, R. and Tanguay, R. M. (1996) Stage-specific localization of the small heat shock protein Hsp27 during oogenesis in Drosophila melanogaster. Chromosoma **105**, 142–149

24 Preville, X., Salvemini, F., Giraud, S., Chaufour, S., Paul, C., Stepien, G., Ursini, M. V. and Arrigo, A.-P. (1999) Mammalian small stress proteins protect against oxidative stress through their ability to increase glucose-6-phosphate dehydrogenase activity and by maintaining optimal cellular detoxifying machinery. Exp. Cell Res. **247**, 61–78

Received 17 July 2000/10 August 2000; accepted 11 August 2000

25 Lavoie, J. N., Hickey, E., Weber, L. A. and Landry, J. (1994) Modulation of actin microfilament dynamics and fluid phase pinocytosis by phosphorylation of heat shock protein 27. J. Biol. Chem. **268**, 24210–24214

26 Perng, M. D., Cairns, L., van den IJssel, P., Prescott, A., Hutcheson, A. M. and Quinlan, R. A. (1999) Intermediate filament interactions can be altered by HSP27 and alphaB-crystallin. J. Cell Sci. **112**, 2099–2112