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Direct observation of stress response in *Caenorhabditis elegans* using a reporter transgene

Christopher D. Link, James R. Cypser, Carolyn J. Johnson and Thomas E. Johnson

Institute for Behavioral Genetics, Campus Box 447, University of Colorado Boulder, CO 80309–0447, USA

Abstract Transgenic *Caenorhabditis elegans* expressing jellyfish Green Fluorescent Protein under the control of the promoter for the inducible small heat shock protein gene *hsp-16–2* have been constructed. Transgene expression parallels that of the endogenous *hsp-16* gene, and, therefore, allows direct visualization, localization, and quantitation of *hsp-16* expression in living animals. In addition to the expected upregulation by heat shock, we show that a variety of stresses, including exposure to superoxide-generating redox-cycling quinones and the expression of the human β amyloid peptide, specifically induce the reporter transgene. The quinone induction is suppressed by coincubation with L-ascorbate. The ability to directly observe the stress response in living animals significantly simplifies the identification of both exogenous treatments and genetic alterations that modulate stress response, and possibly life span, in *C. elegans*. © Harcourt Publishers Ltd 1999

INTRODUCTION

The small heat shock proteins (HSPs) are a collection of low molecular weight polypeptides (15–30 kD), identified in all eukaryotes examined, that show sequence similarities to the α B crystallins (Arrigo and Landry 1994). Strong transcriptional upregulation of small HSPs is a common component of the cellular response to thermal stress (Lee et al. 1996), and has been observed in *Drosophila* cells exposed to H_2O_2 (Courgeon et al. 1990), plant cells exposed to ozone (Eckey-Kaltenbach et al. 1997) and yeast cells exposed to low pH (Carmelo and Sa-Correia 1997). In the nematode *Caenorhabditis elegans*, the major small HSPs are a collection of 16 kD species encoded by six closely related genes (Candido et al. 1989). Candido and colleagues have extensively characterized the temporal and spatial expression of these *hsp-16* genes (Stringham et al. 1992), and have used transgenic animals containing *hsp-16::lacZ* reporter transgenes to demonstrate induction of *hsp-16* in response to heavy metals, paraquat, and the fungicide captan (Stringham and Candido 1994, Jones et al. 1996).

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Correspondence to: Christopher D. Link, Institute for Behavioral Genetics, Campus Box 447, University of Colorado Boulder, CO 80309–0447, USA. Tel.: 303 735–5112; Fax: 303 492–8063; E-mail: linkc@colorado.edu

Interest in the stress response of *C. elegans* has been stimulated by the striking correlation between increased longevity and enhanced resistance to stress in single-gene mutants that display increased lifespans (Age mutants: Larsen 1993, Vanfleteren 1993, Lithgow et al. 1994, Lithgow et al. 1995, Murakami and Johnson 1996, Martin et al. 1996). The physiological mechanisms underlying enhanced stress resistance and increased longevity have not been determined, although Age mutants have been shown to have increased levels of superoxide dismutase and catalase (Larsen 1993, Vanfleteren 1993), metabolic throughput (Vanfleteren and De Vreese 1995) and protein kinase activity (Vanfleteren and De Vreese 1997). Also unknown is whether the stress response of Age mutants is altered in onset, tissue distribution, or kinetics. The ability to continually monitor the stress response in living animals would, therefore, be a very useful tool in characterizing both wild-type and Age animals under a variety of conditions. Direct visualization of the stress response could also have clear applications in *C. elegans* models of cellular pathology, including transgenic models of amyloid diseases (Link 1995), and genetic neurodegeneration models (Driscoll and Chalfie 1991). We have, therefore, employed the inherently fluorescent Green Fluorescent Protein (GFP, Chalfie et al. 1994), in conjunction with an *hsp-16–2* promoter, to generate a stress reporter transgene that allows visualization of the stress response in living animals.

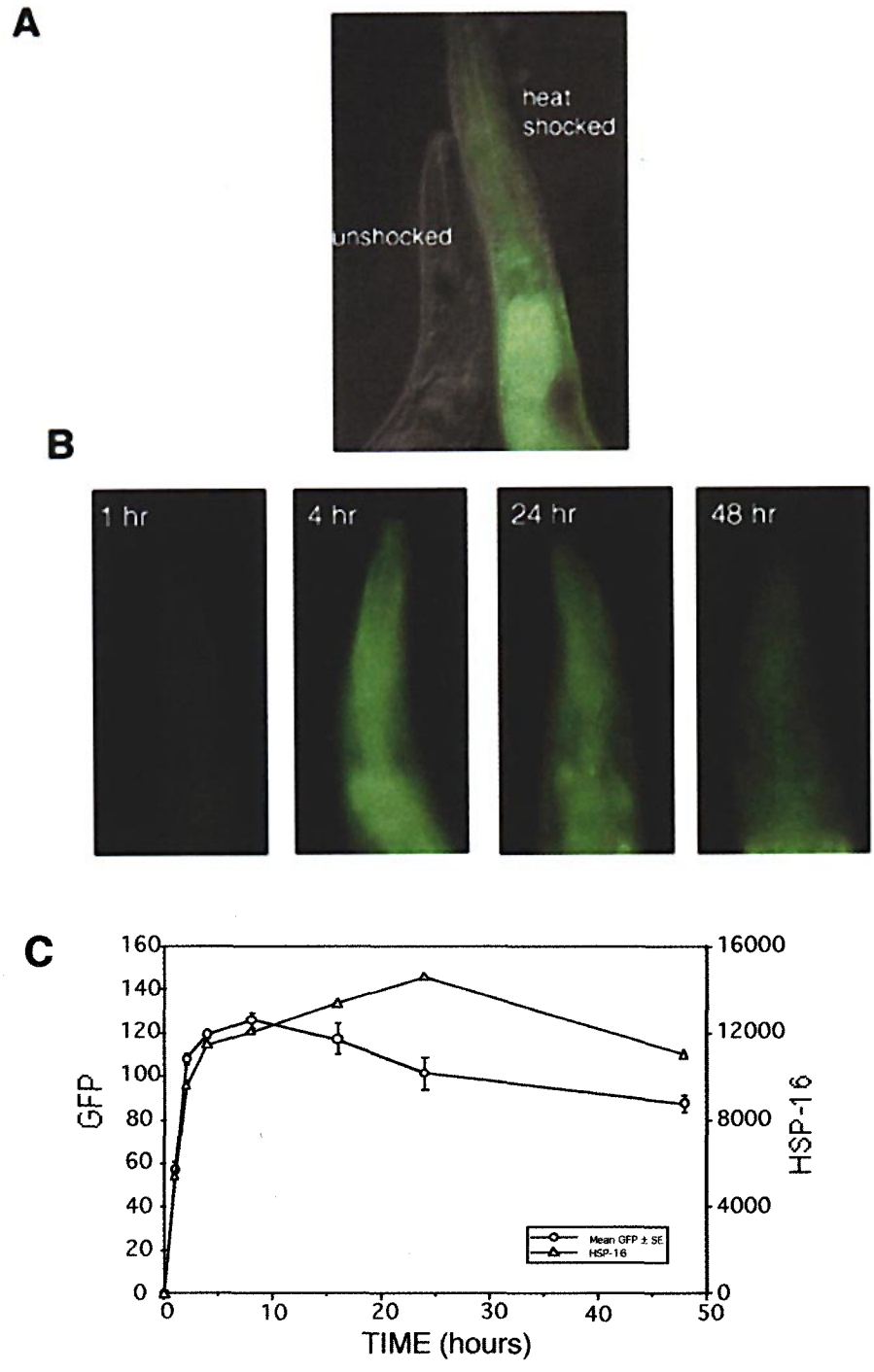

Fig. 1 Heat shock induction of the *hsp-16-2::GFP* reporter transgene. Four-day-old (young adult) CL2070 animals were heat shocked for 2 h at 35°C, then returned to 20°C. (All animals in **A** and **B** are oriented with anterior end at top) **A.** Anterior region of two CL2070 animals (left animal unshocked, right animal shocked), photographed 8 h after the end of heat shock. This image was generated by digitally fusing differential interference contrast (DIC) and epifluorescence (GFP) images of these animals. Note strong GFP expression in all tissues of the shocked animal, and absence of detectable GFP in the unshocked control. **B.** Time series of anterior GFP expression in single heat shocked animal. This animal was mounted for photomicroscopy at the times shown (measured from the end of the heat shock), then returned to the culture plate between imaging. **C.** Comparison of GFP and endogenous HSP16 expression in a reporter strain. A synchronous population of CL2070 animals was heat shocked, and sibling cohorts were measured for GFP and endogenous HSP16 expression (see Materials and Methods). Note similar induction kinetics of reporter transgene and endogenous *hsp-16* genes.

Here we demonstrate that tissue-specific induction of *hsp-16* is observed in dual transgenic animals expressing human β amyloid peptide. We have also employed the transgenic reporter strains to specifically examine the response of *C. elegans* to redox quinones shown to generate intracellular superoxide. Exposure to one such quinone, juglone, effectively induces the *hsp-16::GFP* reporter transgene. This juglone-induced expression of the reporter transgene is suppressed by coincubation with anti-oxidants, suggesting an oxidative mechanism for this induction.

MATERIALS AND METHODS

Strain maintenance

Strains were routinely propagated at 20°C on Nematode Growth Media (NGM) plates with *Escherichia coli* strain OP50 as a food source, as previously described (Wood 1988). Liquid transfers of animals were performed in S Basal media (Wood 1988).

Construction of transgenic nematodes

The 341 bp *hsp-16–2* promoter region was recovered as a *Hin*D III/*Bam* HI fragment from expression vector pPD49.78 (gift of A. Fire), and ligated between the unique *Hin*D III/*Bam* HI sites of pGFP-TT (gift of Y. Jin and H.R. Horvitz). The pGFP-TT vector was derived from pTU61 (Chalfie et al. 1994) by site-directed mutagenesis, and contains Ser65Thr and Ile167Thr mutations that increase the fluorescence of GFP. The resulting plasmid (pCL25) was microinjected, along with the *rol-6* morphological marker plasmid pRF4 (Mello et al. 1991), into wild-type *C. elegans*, and chromosomally integrated, stable transgenic lines were recovered as previously described (Link 1995). The transgenic strains used for the studies described in this work, CL2070 (*dvls70*) and CL2071 (*dvls71*), were outcrossed 9X before use. Animals transgenic for both the β amyloid peptide (βAP) transgene (*dvls1*) and the *hsp-16::GFP* reporter transgene (*dvls70*) were constructed by mating *dvls70*/+ male animals to *dvls1* hermaphrodites (strain CL2005), and recovering *dvls1*/+; *dvls70*/+ cross-progeny. (Homozygous *dvls1*; *dvls70* animals could not be constructed, possibly due to excessive expression of the *rol-6* marker gene.)

Photomicroscopy and quantitation of GFP levels

Microscope images were acquired with a Zeiss Axioskop using 35 mm color slide film, then scanned and adjusted for brightness and contrast using Adobe Photoshop. For quantitative measurements of GFP expression, images were acquired using 10X and 40X objectives and a Cohu High Performance charge-coupled diode camera, allowing direct archiving of digital images.

For measuring the response of a population of reporter animals to heat shock (Fig. 1C), synchronized cohorts of 10 animals were examined for each set of conditions. (Corresponding groups of 50 animals were frozen simultaneously for later analysis by immunoblot.) Animals were mounted on 2% agarose pads and observed under a coverslip using a 40X objective. (To avoid repeated remounting of animals, a given cohort was assayed at only one time point.) Each 40X image extended anteriorly from the back of the pharynx, thus including 90–100% of the most anterior part of the animal and excluding the intestine, which displays an endogenous fluorescence that cannot be distinguished from GFP with the monochromatic camera used in these studies.

Images were analyzed using NIH Scion Image software (version 1.60). In each image the animal was outlined, black-to-white inverted, and the mean pixel density measured. A background region in the image was then selected, inverted, and measured in like manner. The difference between the two values was treated as the brightness of the animal in that particular image. Statistical analyses were carried out using Statistica version 5.1 and SPSS version 6.1.4.

Immunoblotting and immunohistochemistry

Protein samples were obtained for each time point after heat shock by collecting 50 animals in 1X S Basal, freezing immediately in liquid nitrogen, and maintaining samples at –80°C until electrophoresis. Worm samples were boiled for 5 min in sample buffer (1.2 mM Tris HCl, pH 6.8, 3.5% sodium dodecyl sulfate, 3.6% glycerol, 20 μM dithiothreitol, 0.004% bromphenol blue, final concentrations) fractionated on SDS-polyacrylamide gels (10%), and electroblotted onto a 0.45 micron nitrocellulose membrane using a BioRad Trans-blot cell for 45 min at 150 v. Protein gels and membranes were stained with Coomassie Blue and Ponceau S, respectively, to ensure even transfer. Blots were incubated for 1.5 h in 3% milk powder in TBS (Tris-Buffered Saline, 100 mM Tris-HCl, pH 7.5, 0.9% [w/v] NaCl) with a polyclonal anti-HSP16 primary antibody (1:8000) courtesy of D. Jones and E. P. M. Candido. After TBS washes, blots were probed with alkaline phosphatase-conjugated anti-rabbit IgG antibody (1:33 000) for 1.5 h and visualized upon incubation with BioRad AP Conjugate Substrate Kit. Blots were scanned and bands were quantified using NIH Image software. Expression of β amyloid peptide (βAP) in dual transgenic animals was assayed by immunohistochemistry as described (Link 1995), using anti-βAP monoclonal antibody 4G8 (Senetek) as the primary antibody.

Exogenous stress treatments

Heat shock was carried out on NGM plates spotted with *E. coli* strain OP50. Worms were observed at 20°C prior to

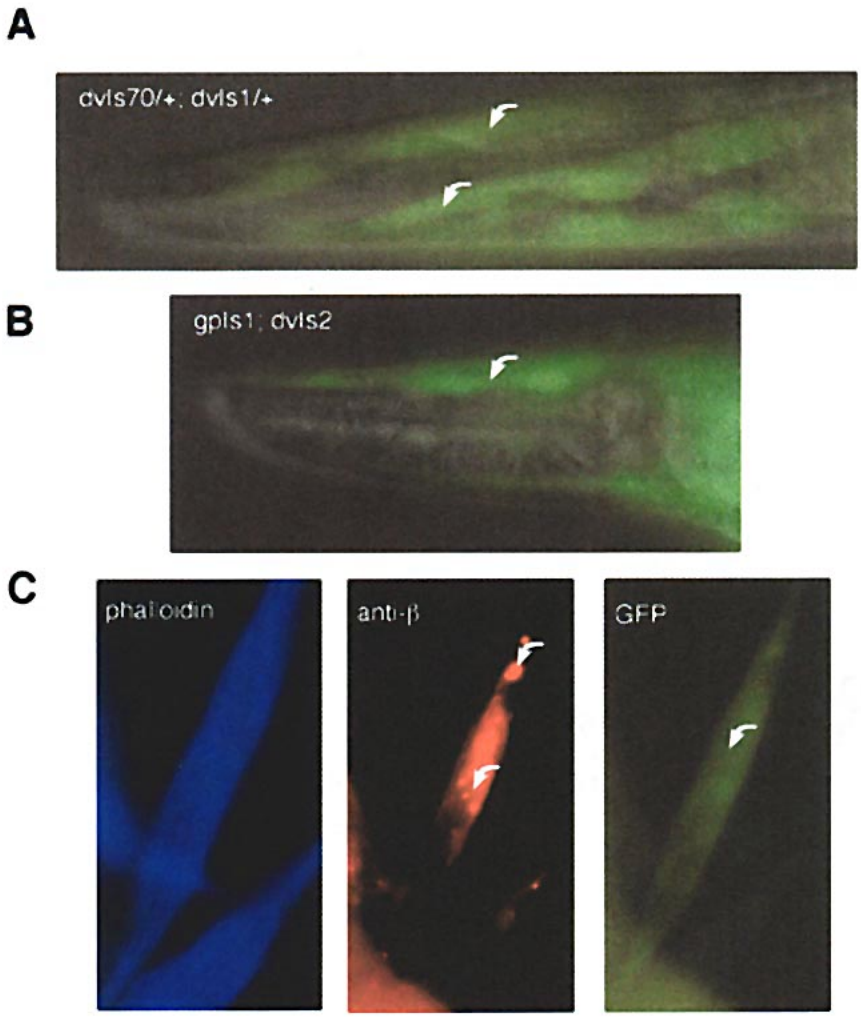

Fig. 2 Expression of GFP in dual transgenic animals containing *hsp-16::GFP* reporter and human β amyloid peptide transgenes. **A.** Anterior of living dual transgenic animal (*dvIs70*/+; *dvIs1*/+), digitally fused DIC and GFP epifluorescence images. (See Materials and Methods for strain construction). Note strong induction of GFP expression in body wall muscle quadrants (arrows). **B.** Similar fused DIC/GFP epifluorescence image of dual transgenic animals homozygous for independent reporter (*gpIs1*) and amyloid (*dvIs2*). Note similar induction of GFP in body wall muscle (arrow). **C.** Isolated muscle cells from dual transgenic animal fixed with paraformaldehyde and probed with anti-β peptide monoclonal antibody 4G8. Left panel: muscle fibers visualized with coumarin-phalloidin. Central panel: staining with MAb 4G8 and Texas Red-conjugated secondary antibody. Note deposits in central muscle cell (arrows). Right panel: induction of GFP in muscle cells expressing β amyloid peptide. Note higher expression of GFP in the central muscle cell (arrow), which appears to have a higher amyloid load.

heat shock to establish a zero point, then exposed to 35°C for 2 h. Worms were then transferred to 20°C for recovery and further observation. For exposure to redox quinones, animals were propagated on NGM plates, then transferred as young adults to liquid Survival Media (Johnson and Wood 1982) containing 40 μM of the appropriate quinone. Treated animals were assayed for GFP expression at 24 h (using 40X objective, as described above) after transfer to liquid media.

RESULTS

Reporter transgene response to heat shock

Larval or young adult worms from transgenic GFP reporter strain CL2070 (*dvIs70*) do not express detectable GFP when propagated under standard conditions, but display intense GFP expression after heat shock (Fig. 1A). The kinetics of this heat shock induction can be followed in single worms by repeated observations (Fig. 1B). To determine if the reporter transgene expression was reflective of endogenous HSP16 levels, the time course of both GFP and HSP16 expression after heat shock was followed by digital quantitation of GFP and quantitative immunoblotting, respectively. As shown in Figure 1C, GFP expression roughly parallels HSP16 expression over the 48 h time period examined. (The differences in time of peak expression between HSP16 and GFP may result from shorter perdurance of GFP than the native HSP16 protein.) Similar results have been obtained with two

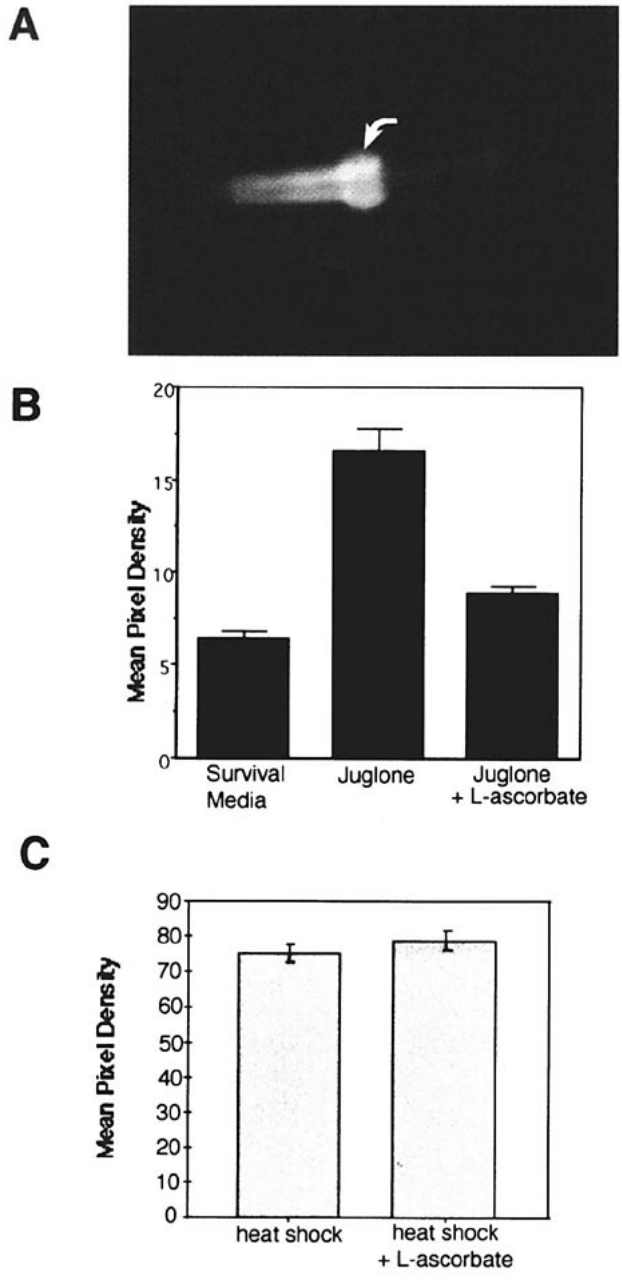

Fig. 3 Induction of the *hsp-16::GFP* reporter transgene in response to the redox quinone juglone. **A.** Anterior of CL2070 animal exposed for 24 h to 40 μM juglone, 20°C. This animal is representative of the typical response observed under these conditions, with intense GFP observed in the anterior bulb of the pharynx (arrow), less intense expression in the posterior bulb, and no intestinal GFP. **B.** Quantitation of GFP response to 40 μM juglone or 40 μM juglone in the presence of the antioxidant L-ascorbate (1 mM). **C.** Effect of L-ascorbate on heat shock induction of reporter gene. CL2070 was exposed to a standard heat shock in liquid survival media with or without 1 mM L-ascorbate. Note that L-ascorbate does not significantly suppress the heat shock induction of the reporter transgene. All graphs are mean ± SEM.

other reporter transgenic strains (CL2071 and CL2074) that contain independent chromosomal insertions of the *hsp-16::GFP* reporter construct (data not shown).

Reporter transgene induction by human β amyloid peptide

To investigate the response of the *hsp-16::GFP* reporter transgene in models of cell pathology, dual transgenic animals were constructed that contained both the reporter transgene (*dvls70*) and a previously described transgene (*dvls1*) that produces high levels of the human β amyloid peptide (βAP) (Link 1995). This amyloid transgene, which uses the body-wall-muscle-specific promoter *unc-54* to drive the expression of a β peptide minigene, is toxic in *C. elegans*, leading to progressive paralysis. Dual transgenic animals were constructed by mating *dvls70*/+ male animals to CL2005 (*dvls1*) hermaphrodites and recovering cross-progeny. Animals containing both transgenes show body-wall-muscle-specific expression of GFP independent of heat shock (Fig. 2A). An identical result is observed when dual transgenic animals are generated using independent *hsp-16::GFP* reporter (*gpls1*) and *unc-54*::β amyloid (*dvls2*) transgenes (Fig 2B). GFP expression in dual transgenic animals closely parallels β peptide deposition (Fig. 2C). No reporter GFP expression was observed when equivalent dual transgenic animals were constructed by replacing CL2005 with control strains CL2122 (*dvls15*), which contains only the *unc-54* expression vector, or CL2008 (*dvls3*) which expresses human transthyretin under control of the *unc-54* promoter (Link 1995). Because there is no measurable GFP signal in these control constructs, we cannot directly calculate the degree of reporter induction resulting from β peptide expression. The level of amyloid-induced muscle GFP does appear to be of a magnitude similar to that observed in heat-shocked animals. Muscle-restricted expression of GFP has not been observed in CL2070 animals at any age or under any other heat shock conditions. Thus, we conclude that expression of human β amyloid in *C. elegans* induces HSP16 expression, likely in a cell-autonomous manner.

Reporter transgene response to oxidative stress

To investigate the possibility that HSP expression can result from oxidative stress (implicated in both aging and β amyloid toxicity), the transgenic reporter strain (CL2070) was exposed to a panel of redox quinones (juglone, menadione, paraquat, phenazine and plumbagin) that can cause intracellular production of superoxide (Hassan and Fridovich 1979). *hsp-16* induction was observed after exposure to juglone, paraquat, and plumbagin; 40 μM juglone was particularly effective at reproducibly inducing HSP16 after a 24 h exposure. In contrast to heat and β amyloid induction, HSP16 expression in juglone-exposed animals was restricted predominantly to the pharynx (Fig. 3A). To demonstrate that juglone induction of *hsp-16* involves an oxidative

mechanism, worms from the reporter strain were coincubated with 40 μM juglone and the anti-oxidant L-ascorbate (1 mM), chosen for its aqueous solubility and effectiveness in cell culture models of βAP toxicity (Behl et al. 1994). L-ascorbate significantly reduces induction of the reporter transgene by juglone (Fig. 3B). L-ascorbate does not reduce reporter transgene induction by standard heat shock (Fig. 3C), suggesting that L-ascorbate does not non-specifically inhibit reporter transgene induction.

DISCUSSION

We have used a novel *hsp-16::GFP* reporter transgene to monitor HSP16 expression in response to heat shock and several novel stressors. These results suggest that this reporter transgene provides a direct and general measure of *hsp-16* induction and thus provides one measure of stress response in *C. elegans*. Unlike previous *lacZ*-based constructs (Stringham et al. 1992, Stringham and Candido 1994), this reporter can be monitored in living animals. Moreover, there are no complications resulting from variable fixation, permeabilization, or substrate availability, allowing accurate reporter quantitation. We have used this novel reporter to both examine *hsp-16* response to novel stressors, and to follow the kinetics of *hsp-16* induction in living individual animals.

Increased expression of small heat shock proteins and the related α B crystallins has been observed in the brains of Alzheimer's disease patients (Shinohara et al. 1993, Renkawek et al. 1994), as has increased HSP70 expression (Perez et al. 1991, Hamos et al. 1991). We show here that muscle-specific expression of human β amyloid peptide induces muscle-specific expression of the *hsp-16::GFP* reporter gene. Endogenous HSP16 is also induced in transgenic animals expressing human β amyloid (our unpublished data). At present, it is unclear whether this induction represents a true cellular response to β amyloid toxicity, or a more general response to the expression of an aggregating protein. Expression of actin missense mutations in *Drosophila* has also been found to induce small HSP expression (Hiromi et al. 1986, Drummond et al. 1991). As one control for such artifacts, we monitored induction of the HSP16 reporter in dual transgenic animals expressing an *unc-54*::human transthyretin construct. No induction was observed. This result suggests that high level expression of a foreign protein is not in itself sufficient to induce *hsp-16*. However, we cannot exclude the possibility that it is the aggregation of the β peptide (likely occurring, in part, in the secretory pathway), and not its intrinsic toxicity, that is inducing *hsp-16*. As small HSPs have been shown to have chaperone activity (Leroux et al. 1997, Lee et al. 1997), it would not be surprising if the reporter transgene was induced by high levels of misfolded peptides. It would be interesting to determine if induction of small HSP genes occurs in cell culture models of β peptide toxicity (reviewed in Iverson et al. 1995) using exogenously added β peptide, as this would address whether expression of small HSPs is a direct result of β peptide toxicity.

There is extensive evidence that oxidative damage increases with age (Sohal and Weindruch 1996), and production of reactive oxygen species (ROS) has also been observed in cell culture models of β amyloid toxicity (Behl et al. 1994, Mattson and Goodman 1995). Given that small HSPs can protect cells in culture from oxidative stress (Mehlen et al. 1995), we sought evidence that ROS could directly induce the reporter transgene. As candidate inducers, we used a panel of redox-cycling quinones that have previously been shown to have activity in *C. elegans* (Blum and Fridovich 1983). These compounds can generate superoxide by the reduction of O_2 following their own reduction by NAD(P)H (Hassan and Fridovich 1979). Our standard conditions of 40 μM juglone exposure for 24 h produces a strong, reproducible induction of *hsp-16*. This induction can be suppressed by coincubation with the anti-oxidant L-ascorbate (observed in 4/4 experiments). L-ascorbate does not reduce heat shock induction of the reporter transgene, indicating that its inhibition of juglone-induced reporter expression does not result from a general suppression of transgene expression. Animals incubated in juglone plus anti-oxidant media were noticeably healthier than animals incubated in juglone alone, indicating that the suppression of *hsp-16* induction is not trivially due to the coincubated animals being 'sicker' and physiologically unable to produce GFP. We have also observed suppression of juglone-induced reporter expression by incubation in 100 μM N-tert-Butyl-α-phenyl-nitrone (BPN, 2/3 experiments). Suppression of the heat shock response as monitored by the *hsp-16::GFP* reporter is, therefore, likely to be via the classic anti-oxidant activity of these compounds, as it is unlikely that these chemically dissimilar anti-oxidants both react with and inactivate juglone directly. We have also observed induction of the reporter transgene after direct exposure to H_2O_2 and T-butyl hydroperoxide, although these effects have been variable. We, therefore, conclude that *hsp-16* induction can result from an intracellular increase in ROS.

The experiments described do not address the actual biochemical mechanism of induction (i.e. whether *hsp-16* transcriptional up-regulation is directly triggered by sensing of oxidative stress, or by some resultant byproducts, such as denatured proteins), nor do they demonstrate that the reporter induction resulting from β amyloid is necessarily moderated by oxidative stress.

These experiments do establish the *hsp-16::GFP* reporter transgene as an important tool for assaying stress response in *C. elegans*. We anticipate that this tool will be productively employed to investigate the relationship between stress response, aging, and the cellular response to pathological conditions.

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