Expression of a Single-Copy hsp-16.2 Reporter Predicts Life Span

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The level of green fluorescent protein expression from an hsp-16.2–based transcriptional reporter predicts life span and thermotolerance in Caenorhabditis elegans. The initial report used a high-copy number reporter integrated into chromosome IV. There was concern that the life-span prediction power of this reporter was not attributable solely to hsp-16.2 output. Specifically, prediction power could stem from disruption of some critical piece of chromatin on chromosome IV by the gpIs1 insertion, a linked mutation from the process used to create the reporter, or from an artifact of transgene regulation (multicopy transgenes are subject to regulation by C elegans chromatin surveillance machinery). Here we determine if the ability to predict life span and thermotolerance is specific to the gpIs1 insertion or a general property of hsp-16.2–based reporters. New single-copy hsp-16.2–based reporters predict life span and thermotolerance. We conclude that prediction power of hsp-16.2–based transcriptional reporters is not an artifact of any specific transgene configuration or chromatin surveillance mechanism.

Key Words: Biomarker of life span—Transgene—Heat shock—Aging—Longevity.

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The ability to predict individual longevity has been a standing goal of aging research for decades (and for millennia in the popular literature). Researchers imagined some measurable parameter with better predictive value than chronological age—a biomarker of life span. The biomarker concept has been postulated by many individuals (1). A formal definition of a biomarker was clearly articulated in 1988 by Baker and Sprott (2):

... a biological parameter of an organism that either alone or in some multivariate composite will, in the absence of disease, better predict functional capability at some late age than will chronological age. ... the most useful biomarkers would be those which could be assessed very early in the life span and have predictive values later in the life span.

Thus, biomarkers are of interest for many reasons. They can be used to identify at-risk individuals for intervention and to study efficacy of treatments in a variety of different levels of frailty. Biomarkers may indicate distinct physiological states (1). Here we use the simplest possible concept of a biomarker: a parameter that, at a young age, predicts viability at some later age: a biomarker of life span.

There are few empirically tested biomarkers of life span in model organisms, as defined by Baker and Sprott (2). Specifically, many parameters have been shown to change with age and/or be correlated with different age-related outcomes, but few parameters have been successfully used to predict subsequent differences in longevity. Here, we will provide a brief overview of reported biomarkers of life span (as described previously) in the model nematode Caenorhabditis elegans.

Biomarkers of Life span in C elegans

In C elegans, movement capability has been repeatedly shown to predict subsequent longevity (3–8). Both quantitative and qualitative changes in body movement correlate with differences in subsequent life span; animals could be classified by differences in number of body bends per second in liquid or by qualitative response to touch. Pharyngeal pumping also predicts life span better than chronological age (3,5,8). Pharynx pumping is a visually detectable contraction of the pharynx and is the means by which C elegans ingest their food (Escherichia coli in the laboratory); this phenotype is measured fairly easily under a stereoscope, like motility. Animals that spend a greater portion of their early life actively pumping or pump at higher rates have a higher chance for a longer life span. Finally, expression levels of several different green fluorescent protein (GFP)-based transgenes have been shown to predict subsequent life span (9,10).

In 2005, we identified a GFP-based biomarker of life span in young adult hermaphrodites of C elegans (9). We demonstrated that the level of GFP expressed from a multicopy transgene (gpIs1[Phsp-16.2::gfp::unc-54] IV) under
control of the promoter of the small heat-shock protein, hsp-16.2, predicted both thermotolerance and subsequent life span. Brighter green animals lived longer and survived lethal heat longer than dimmer animals. However, there were concerns with this result because only one allele of the hsp-16.2–based transcriptional reporter was tested for predictive capability. Other transgene-based results have been found to be artifacts of transgene technology. For example, life-span extension based on sirtuin overexpression has been found to be an artifact of the transgene technology used to create the original strains (11). Thus, we sought to determine if the results were an artifact of transgene technology in *C. elegans* by generating new alleles of the hsp-16.2–based transcriptional reporter with relatively new, more precise transgene technology.

Transgene Technology in *C. elegans*

In 2008, the Jorgensen Lab developed another technology to construct transgenic strains of *C. elegans*; the transgenes can be inserted at defined loci in a single copy with a defined orientation (12). This technology was called MosSCI (Mos Single-Copy Insertion); insertion is dependent on creation of a double-stranded break in the genome at a defined locus via excision of a single Mos transposon. Transposon excision is achieved through the injection of a plasmid containing the promoter region of the hsp-16.2 gene fused to the coding sequence for GFP and the unc-54 3′untranslated region (UTR) from *unc-54*, which was empirically used to drive expression of transgenes (14). This construct was manually injected into hermaphrodite gonads, and the progenies were screened for GFP expression. GFP-transmitting lines were subsequently exposed to gamma irradiation, causing chromosomal integration of the extrachromosomal array, and this was followed by 10 rounds of outcrossing to remove unlinked mutations generated by the gamma irradiation. The result from the earlier process was the TJ375 strain carrying the *gplIs1* insertion [Phsp-16.2::gfp::unc-54] IV (9).

Prior to MosSCI, there were three methods for constructing transgenic worms: (i) construction of extrachromosomal arrays through injection of DNA and selection for animals that could transmit the injected DNA at high frequency but had not integrated the injected DNA into their genome, (ii) radiation-induced integration of injected DNA, and (iii) construction of low-copy number arrays through biolistic transformation using gold particles (13). Integration of extrachromosomal arrays leads to the formation of chromosome-integrated arrays that are variably arranged (subject to apparently random rearrangement and integration at high copy numbers as is the case with *gpIs1*) and are variably silenced in the soma and usually silenced in the germ line. Biolistic transformation generates low-copy number transgenes that are less frequently germ line silenced but are still sometimes subject to germ line silencing.

The original *gpIs1* transgene (pCL25, Figure 1) was created using a plasmid that contained the promoter region of the *hsp-16.2* gene fused to the coding sequence of GFP and the 3′untranslated region (UTR) from *unc-54*, which was empirically used to drive expression of transgenes (14).

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This insertion on chromosome IV is composed of an array of about 530 copies (Table 1). Differences in GFP expression could be attributable to differences in somatic silencing or activation of the transgene, and this phenomenon could somehow also specify differences in life span. Specifically, expression of multicopy arrays is a concern.
because there are several cases where the chromatin modification machinery can either increase or decrease somatic expression levels (not just germ line silencing). Activation of multicopy transgenes is known to occur via TAM-1 (speculated to be an artifact gene from some transposon), and silencing can occur from a retinoblastoma-like protein, LIN-35, acting in the LET-60 Ras pathway (15).

The technology used to construct gpls1 involves several possible confounding factors: (i) mutations arising during the construction of the injected transgene, (ii) transgene-linked mutations generated by irradiation, (iii) uncontrolled multicopy array formation, (iv) random sites of integration, and (v) altered expression of the multicopy arrays due to chromatin modification machinery (15). However, MosSCI technology (due to the defined site and single-copy configuration of the insertion), combined with appropriate sequencing to confirm lack of mutations, alleviates these concerns (12).

To determine if the prediction capabilities are unique properties of the gpls1 insertion, we constructed additional GFP-based, hsp-16.2 reporter strains, carrying single-copy insertions at a defined locus and integrated without the use of radiation. This permits us to address three potential artifacts that could have been responsible for the prediction power of the gpls1 insertional allele: (i) the site of integration, (ii) the possibility of a linked mutation unrelated to hsp-16.2, and/or (iii) the regulation of transgenes in multicopy arrays. We show later that GFP expression levels from new single-copy Phsp-16.2::gfp transgene insertions are also predictive of subsequent longevity.

**MATERIALS AND METHODS**

**Strains, Growth, and Assay Conditions**

Strains (Table 1) were grown at 20°C for all experiments, except where noted. Measurement and sorting of isogenic populations of *C. elegans* using the Copas Biosort proceeded as in Rea et al. (9) using the 1-hour, liquid pulse heat-shock method. Expression measures occurred approximately 24 hours after heat shock using the brightest and dimmest 5%–10% of the population. Life-span and thermotolerance assays were conducted as in Rea et al. (9). Briefly, animals were sorted into bright, dim, and unselected classes or picked from un–heat shocked animals from the source population. Subsequent life-span or thermotolerance assays were performed on 60 mm, OP50-seeded, small nematode growth medium Petri dishes. All life-span assays were performed at 20°C except life spans displayed in Figure 5, which were conducted at 16°C.

**Polymerase Chain Reaction Conditions for Strain Validation, Mapping, and Copy Number Measurements**

Single-copy chromosome II locus, rescuing insertion and lack of a Mos transposon were determined via polymerase chain reaction (PCR) as described in Frokjaer-Jensen et al. (12). Specifically, the locus was confirmed via PCR that extended from the insert into the flanking genomic region. The lack of Mos transposon was determined by comparing a lack of a Mos-sequence–generated PCR band to positive and negative control strains (wild type and EG4322). No Mos transposons were detected in any MosSCI strains used in this study. Single-copy insertions were size and sequence validated. PCR products were consistent with a single-copy insertion; deletions or duplications would have been detected as differences in size. 5′UTR, 3′UTR, and coding sequence for GFP were all intact and identical to the injected plasmids; the GFP coding region was not entirely covered by sequencing, but the protein product is functional in all strains. Copy number of multicopy transgenes was determined using sybr-green–based quantitative PCR with *ama-1* as a single-copy control; measures were made across a threefold range of DNA concentrations, in duplicate within each of the three independent trials. Final copy numbers were the average of values obtained in three independent trials. All strains were outcrossed with N2CGCb at least five times.

**Microscopy**

Animals were mounted on a 1% agarose pad (to minimize distortion of the worm from mounting) and anesthetized with a tricaine/tetramisole solution as in McCarter et al. (16). A Zeiss LSM 510 confocal microscope was used for imaging, utilizing a ×20 objective; laser power to the sample was the same between groups, but gain was increased for visualization of the 10-fold dimmer TJ3001.
plasmid used to create the original transgenic array: gpIs1. By injecting pAM102, the appropriate transposase-containing plasmid, and co-injection markers, we generated two new, MosSCI-based, single-copy GFP-based transcriptional reporters of hsp-16.2: zSi3000 and zSi3001 in strains TJ3000 and TJ3001, respectively. Both insertions were sequenced and found to be molecularly identical to the original pCL25 and pAM102 vectors (Figure 1).

Expression of GFP in MosSCI Strains
TJ375 and TJ3001 differ in the level of GFP expression about 10-fold, even though the absolute difference in copy number is several 100-fold (Table 1 and Figure 2; N > 300 per strain). The distribution of GFP expression among individual worms of each strain appears dramatically different from each other (Figure 2A), but when the data sets are normalized by dividing each data point by the mean expression level, the distributions are quite similar (Figure 2B). The similarity in distribution is mathematically reflected by the similar coefficients of variation: 19.4% for TJ3001 population versus 20.9% for the TJ375 population (17). In all strains, the majority of the observed expression came from the intestine (Figure 3), as previously reported (18). Cellular expression patterns in the two independently derived stains, TJ3000 and TJ3001, were not visually distinguishable. Mean expression levels were also not significantly different between populations of TJ3000 and TJ3001 (Table 1).

Single-Copy Reporters Predict Thermotolerance and Life span
Expression level of the gpIs1 insertion is a good predictor of life span, so we asked whether levels of expression of these single-copy reporters could also predict subsequent longevity. Animals were shocked for 1 hour at 35°C, in a liquid pulse fashion, allowed to recover at 20°C until the next day, and then sorted into bright and dim classes (9). These classes were then tested for life span or thermostolerance as in Rea et al. (9). All life-span and thermostolerance data were analyzed with the log-rank survival test and are tabulated in Table 2.

We found that sorting out the brightest and dimmest 5%–10% resulted in populations that had significantly different life spans. Both single-copy strains showed differential longevity and stress resistance (Figures 4–6; Table 2). Bright worms from TJ3001 had life spans that were several days longer than dim worms, and life-span prediction ability was very similar to the multicopy strain (TJ375; Figure 4A). Differential thermostolerance of the bright and dim classes of TJ3001 animals were also comparable to the original TJ375 (Figure 4B). Differential life-span prediction also works at 16°C (Figure 5). Similar effects were seen with the TJ3000 strain, which was constructed completely independently of TJ3001 and was also predictive of subsequent life span (Figure 6).
Figure 3. Scanning confocal micrographs of worms carrying Phsp-16.2::gfp reporters. (A) A merged Nomarski differential interference contrast/GFP cross-sectional image of an animal (TJ375) showing typical expression of gpIs1 multicopy array. (B) A merged Nomarski differential interference contrast/GFP cross-sectional image of a typical zSi3001 (TJ3001) animal. GFP intensity of animals is artificially similar because different gains had to be used. TJ375 animals are about 10 times brighter than TJ3001 animals (Table 1 and Figure 2).

Bright TJ3001 animals lived an average of 14.7 ± 4.3 days, and dim TJ3001 animals lived an average of 11.5 ± 2.6 days (Figure 4A; \( p = .002, N > 25 \) for both groups). The bright TJ375 animals lived an average of 15.1 ± 4.9 days, whereas dim TJ375 animals lived an average of 11.8 ± 3.6 days (Figure 4A; \( p = .005, N > 25 \) for all groups). In thermotolerance assays, bright TJ3001 animals survived an average of 17.2 ± 2.8 hours, whereas dim TJ3001 animals survived for an average of 15.4 ± 3.5 hours (Figure 4B; \( p = .01, N > 34 \) for both groups). Bright TJ375 animals survived an average of 16.7 ± 2.6 hours as compared with the dim TJ375 animals’ average survival of 14.6 ± 4.5 hours (Figure 4B; \( p = .01, N > 32 \) for both groups).

The life-span prediction power of the zSi3001 single-copy insertion at 16°C is shown in Figure 5, where expression levels are again predictive of life span. Note that life-span prediction still works when animals are assayed at lower temperature. The brightest animals lived an average of 18.7 ± 5.9 days after heat shock, which was significantly longer than all other groups; dim animals lived for 7.6 ± 7.1 days. Worms, which were not selected (but were measured by being put through the stress of the flow sorter), lived for 10.1 ± 7.6 days, and unheated animals lived for 11.4 ± 8.4 days (\( p < .01 \) for all comparisons vs bright; \( N > 25 \) for all groups, except \( N = 17 \) for dims). At 20°C, GFP expression levels in the TJ3000 strain, carrying zSi3000, are able to predict differences in life span as well. Bright animals lived for an average of 21.1 ± 6.9 days, whereas dim animals lived about 18.8 ± 6.4 days (Figure 6; \( p = .029, N > 38 \) for all groups).

**DISCUSSION**

Replicating earlier studies using multicopy transgenic reporters, we find that expression levels of single-copy Phsp-16.2::gfp transcriptional reporters predict subsequent life span. This observation significantly strengthens earlier observations and alleviates concerns that the ability of the original gpIs1 insertion to predict life span may be a special case and/or could result from unusual properties of the multicopy array, its site of insertion, or some linked mutation. Furthermore, we found that expression was still predictive of life span even while using a less extreme criterion for selecting bright and dim worms (5%–10% of the extremes vs 2%)

<table>
<thead>
<tr>
<th>Experiment Type</th>
<th>Strain/Transgene Insertion</th>
<th>Figure</th>
<th>Bright Group (mean ± SD, ( N ))</th>
<th>Dim Group (mean ± SD, ( N ))</th>
<th>( p ) Value</th>
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</thead>
<tbody>
<tr>
<td>Life span</td>
<td>TJ375/gpIs1</td>
<td>4</td>
<td>15.1 ± 4.9, 32</td>
<td>11.8 ± 3.6, 26</td>
<td>.005</td>
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<tr>
<td>Life span</td>
<td>TJ375/gpIs1</td>
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<td>24.2 ± 5.9, 41</td>
<td>21.0 ± 7.1, 40</td>
<td>.03</td>
</tr>
<tr>
<td>Life span</td>
<td>TJ3000/zSi3000</td>
<td>6</td>
<td>21.1 ± 6.9, 40</td>
<td>18.8 ± 6.4, 39</td>
<td>.03</td>
</tr>
<tr>
<td>Life span*</td>
<td>TJ3001/zSi3001</td>
<td>5</td>
<td>18.7 ± 5.9, 27</td>
<td>7.6 ± 7.1, 17</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Life span</td>
<td>TJ3001/zSi3001</td>
<td>4</td>
<td>14.7 ± 4.3, 30</td>
<td>11.5 ± 2.6, 26</td>
<td>.002</td>
</tr>
<tr>
<td>Thermotolerance</td>
<td>TJ375/gpIs1</td>
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<td>16.7 ± 2.6, 41</td>
<td>14.6 ± 4.5, 34</td>
<td>.01</td>
</tr>
<tr>
<td>Thermotolerance</td>
<td>TJ3001/zSi3001</td>
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<td>17.2 ± 2.8, 44</td>
<td>15.4 ± 3.5, 42</td>
<td>.01</td>
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<td>Thermotolerance</td>
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<td>No figure</td>
<td>16.8 ± 2.5, 35</td>
<td>15.3 ± 2.3, 44</td>
<td>.008</td>
</tr>
</tbody>
</table>

*This life-span assay was conducted at 16°C. All other life-span assays were conducted at 20°C.

**Notes:** Life-span measurements are reported in days; thermotolerance measurements are reported in hours.
This extends the range of stress over which spans of dim groups of animals that experienced the 1-hour regimen used in this report did not reduce life span. The life spans of dim animals containing \( z\text{Si3001} \) are displayed. Assays were conducted at 20°C.

Another new observation is that the 1-hour heat-shock regimen used in this report did not reduce life span. The life spans of dim groups of animals that experienced the 1-hour heat-shock regimen ranged from 11 to 21 days, which is within the range of reported wild-type median life spans at 20°C (4,19–21). This extends the range of stress over which this reporter predicts life span to milder heat-shock conditions where dim animals are not very short lived: 11–21 days for dims in this report versus 5–8 days for dims from a 2-hour heat shock in the 2005 report (7).

The \( C\text{ elegans} \) genome contains few tandem arrays; known exceptions are genes encoding ribosomal RNAs (22), but they are arranged head to tail and not randomly like the high-copy number transgenes described within. The native \( hsp-16.2 \) gene exists as a single copy (though there are at least six distinct paralogs encoded by eight loci), not in an operon or tandem array, and is most likely not subject to the same type of regulation of multicopy transgene arrays that may affect \( gpIs1 \) (15). Expression patterns and levels of the single-copy reporters are probably more reflective of natural transcription.

Expression Biomarkers in Model Systems

The advent of GFP-based reporter gene technology has allowed the easy use of individual gene expression levels as biomarkers (23). GFP-based reporter genes have subsequently been determined to be fruitful biomarkers of life span in both \( C\text{ elegans} \) and \( D\text{ melanogaster} \) (9,10,23).

In a search for other predictors of longevity, GFP levels from eight different transcriptional reporters were recently shown to be correlated with remaining life span, when measured in middle-aged \( C\text{ elegans} \) (10); these reporters included transgenes reporting on transcription of \( sod-3 \) and \( daf-16 \), two other stress-responsive genes. In fruit flies, levels of HSP-70 and HSP-22 reporter expression predict subsequent life span (24). Clearly, transgenes reporting on stress-related genes are proving to be useful biomarkers of life span. Also clear from recent reports is that transgenic biomarkers predict life span in distinct tissues at distinct ages. Therefore, the use of multiple transgene-based biomarkers to classify and reclassify individuals may also prove to be more predictive than the use of single biomarkers alone (2,25). Though not a transgene, lipofuscin is
a fluorescent pigment that accumulates with age in many species, and it may be another biomarker of life span that could be easily measured with a microscope or the Copas Biosort. Lipofuscin is significantly different between motility classes, which predict life span better than chronological age. Thus, lipofuscin levels can be considered a biomarker of life span by proxy, but strictly speaking, lipofuscin levels have not been directly tested (26). By directly tested, we mean that, to our knowledge, there are no reports wherein animals are first classified by lipofuscin levels and then shown to have different life spans predicted by the initially measured lipofuscin level.

**Phsp-16.2::gfp** expression is a good biomarker because it predicts life span early in life and better than chronological age. Phsp-16.2 expression predicts life span at an earlier age than do other reported biomarkers. The early prediction ability of this biomarker is significant because there are no major visual discrepancies (no signs of aging) between individuals in young (2-day-old adults) populations of nematodes, similar to the lack of measurable physiological decline in younger humans (27). Other biomarkers that are based on transgene expression, lipofuscin, rate of pharyngeal pumping, or motility are well correlated with life span, but distinguishable differences in those parameters do not exist until the population is older (day 2 of adult life for the hsp-16.2 biomarker vs days 5–10 for other markers). Again, using these biomarkers together through the course of an animal’s life may provide even greater prediction ability.

**Life-span Prediction Signals and Potential Mechanisms of Life-span Prediction**

In multicopy reporters, the majority of the GFP signal is due to expression in the intestine (18); this same expression pattern also holds for single-copy reporters. Thus, transcription of hsp-16.2 in the intestine may be important for life span. Supporting a strong role of intestinal hsp-16.2 expression in life span are three critical observations: (i) hsp-16.2 is regulated in an insulin-like signaling–dependent manner (28); (ii) hsp-16.2 overexpression is sufficient to increase life span and is dependent upon insulin-like signaling (29); and (iii) intestinal expression alone of DAF-16/FOXO is sufficient for life-span extension in insulin-signaling mutants (30).

We believe that hsp-16–based reporters predict life span because they report on pathway outputs reflective of hsp-16.2 transcription. We have previously shown that many other GFP reporters were not predictive of life span when measured in populations of young adults; specifically, GFP-based transcriptional reporters of myo-2, gst-4, mtl-2, and pes-10 did not predict subsequent life span (9). Therefore, prediction power was not simply a function of GFP folding capacity or turnover. These data could be used to form a model to explain how hsp-16.2 transcription could predict subsequent life span: (i) Different life spans result from differences in individual interpretation of environmental and genetic information, stemming from both intrinsic and stochastic differences in thermosensory and insulin-like signaling and perhaps other pathways, still to be studied. (ii) The differences in interpretation of environmental and genetic information result in differences in hsp-16.2 expression (and probably in expression of many other co-regulated genes). (iii) The sum of this differential expression results in differences in life span, perhaps resulting from differences in stress response and proteostatic capabilities. The precise nature of the mechanisms governing the differences in environmental and genetic interpretations and generating worm-to-worm differences in life span and Phsp-16.2::gfp expression remain to be experimentally determined.

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**Conflict of Interest**

All authors declare that they have no competing financial interests.

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LIFE-SPAN PREDICTION BY HSP-16.2 REPORTER