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Shared Transcriptional Signature in *Caenorhabditis elegans* Dauer Larvae and Long-lived *daf-2* Mutants Implicates Detoxification System in Longevity Assurance*[S]

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In the nematode *Caenorhabditis elegans*, formation of the long-lived dauer larva and adult aging are both controlled by insulin/insulin-like growth factor-1 signaling. Potentially, increased adult life span in *daf-2* insulin/insulin-like growth factor-1 receptor mutants results from mis-expression in the adult of a dauer larva longevity program. By using oligonucleotide microarray analysis, we identified a dauer transcriptional signature in *daf-2* mutant adults. By means of a nonbiased statistical approach, we identified gene classes whose expression is altered similarly in dauers and *daf-2* mutants, which represent potential determinants of life span. These include known determinants of longevity; the small heat shock protein/α-crystallins are up-regulated in both milieus. The cytochrome P450, short-chain dehydrogenase/reductase, UDP-glucuronosyltransferase, and glutathione *S*-transferase (in *daf-2* mutants) gene classes were also up-regulated. These four gene classes act together in metabolism and excretion of toxic endobiotic and xenobiotic metabolites. This suggests that diverse toxic lipophilic and electrophilic metabolites, disposed of by phase 1 and phase 2 drug metabolism, may be the major determinants of the molecular damage that causes aging. In addition, we observed down-regulation of genes linked to nutrient uptake, including *nhx-2* and *pep-2*. These work together in the uptake of dipeptides in the intestine, implying dietary restriction in *daf-2* mutants. Some gene groups up-regulated in dauers and/or *daf-2* were enriched for certain promoter elements as follows: the *daf-16*-binding element, the heat shock-response element, the heat shock-associated sequence, or the *hif-1*-response element. By contrast, the *daf-16*-associated element was enriched in genes down-regulated in dauers and *daf-2* mutants. Thus, particular promoter elements appear longevity-associated or aging associated.

The biological processes that determine adult life span remain largely unknown. Over the last decade, analysis of mutants with altered rates of aging has led to the discovery of many genes and molecular pathways that act as determinants of life span. For example, the insulin/insulin-like growth factor 1 (IGF-1)[1] signaling system is a powerful regulator of aging in *Caenorhabditis elegans*: lowered insulin/IGF-1 signaling (IIS) can lead to more than a doubling of life span (the Age phenotype) (1–3). Similar effects have been observed in the fruit fly *D. melanogaster* (4, 5). Recent findings suggest that life span in the mouse is influenced by both insulin and IGF-1 signaling (6, 7). Thus, the role of IIS in the control of life span shows evolutionary conservation. However, the life span-determining processes that IIS regulates remain to be identified and are the topic of this report.

The IIS system is one of several molecular signal transduction pathways that regulate larval diapause in *C. elegans* (8). Under adverse environmental conditions (*e.g.* high temperature, low food, and high population density) developing larvae can form a long-lived, nonfeeding, and stress-resistant form: the dauer larva (9). This diapausal form can survive for more than 3 months, during which time it can resume development if dauer-inducing conditions are reversed; by contrast, the adult *C. elegans* die of old age after only 2–3 weeks (10). Many mutations that reduce IIS result in constitutive dauer larva formation (the Daf-c phenotype), even under nondauer inducing conditions (8). For example, mutations affecting the genes *daf-2* (insulin/IGF-1 receptor) (3) and *age-1* (phosphatidylinositol 3-kinase) (11) have this effect.

Potentially, the large increase in life span seen in many IIS mutant adults reflects expression in the adult of dauer-associated longevity assurance processes (2). A number of observations support this view; for example, dauer larvae and long-lived *daf-2* and *age-1* mutant adults have increased activity levels of the antioxidant enzyme superoxide dismutase (SOD) (12–14), and the mitochondrial Mn-SOD gene *sod-3* shows increased mRNA levels in both dauer larvae and *daf-2* mutants (15–17). Additionally, severe *daf-2* mutant adults exhibit a number of dauer-like behavioral characteristics, including cessation of feeding and adoption of an immobile, dauer-like posture (18).

Extensive information about gene expression patterns in dauer larvae and IIS mutant adults has been generated re-

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[S] The on-line version of this article (available at http://www.jbc.org) contains Fig. 1, Tables I–III, additional text, and additional Refs. 1–7.

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[1] The abbreviations used are: IGF-1, insulin-like growth factor 1; CYP, cytochrome P450; DBE, *daf-16*-binding element; DAE, *daf-16*-associated element; GST, glutathione *S*-transferase; HSE, heat shock element; HRE, *hif-1*-response element; HSAS, heat shock-associated site; IIS, insulin/IGF-1 signaling; L4, fourth stage larva; SDR, short-chain dehydrogenase/reductase; smHSP, small heat shock protein; TRP, transthyretin-related protein; UGT, UDP-glucuronosyltransferase; SOD, superoxide dismutase; PSSM, position specific score matrix; RBH, reciprocal best BLAST hit; RNAi, RNA interference; SAGE, serial analysis of gene expression; GO, gene ontology; Mt., topomountain.

cently by transcriptional profile studies. Differences in mRNA abundance between dauer larvae and mixed stage growing populations have been measured by using serial analysis of gene expression (SAGE) (19). More recently, microarray analysis was used to examine gene expression changes occurring during dauer exit and identified 1,984 genes showing significant expression changes (20).

The increase in adult life span resulting from reduced IIS is suppressed by loss of function of the gene *daf-16* (2, 21, 22). This gene encodes a FOXO class forkhead transcription factor (23, 24), and it is therefore likely that the action of genes differentially regulated by DAF-16 is a major determinant of the effects of IIS on aging. Previously, several gene classes were identified that are regulated by IIS in a *daf-16*-dependent manner. For example, increases have been reported in *age-1* and *daf-2* mutants of enzyme activity or gene expression levels of antioxidant proteins such as catalase, Cu/Zn-SOD (cytosolic), and Mn-SOD (13, 15, 25). Also up-regulated are a number of heat shock proteins (26), and the heat shock transcription factor (*hsf-1*) is required for the Age phenotype (27).

Genome-wide surveys of transcriptional changes resulting from reduced IIS have been performed by using spotted DNA microarrays (16, 17). These have led to broad observations of correlations between genes regulated by IIS and particular functional processes or gene groups. An earlier comparison of data from over 500 microarray experiments identified a number of clusters of genes with correlated expression (28). These clusters were represented in the form of a three-dimensional topomap, in which co-expressed genes form "topomountains." Reduced IIS increases expression of many genes associated with topomountain (Mt.) 15, and down-regulation of genes associated with Mts. 8, 19, 27, and 22 (16). Moreover, reduced IIS leads to up-regulation of several functional gene classes (*e.g.* antibacterial peptides) and down-regulation of others (*e.g.* vitellogenins) (17).

Although insights have emerged from these studies, interpreting such transcriptional profiles remains difficult. Microarray experiments often generate lists of genes that are difficult to interpret in an unbiased fashion. Given a long enough list of differentially expressed genes, proof may be found for whatever theory is cherished by the investigator or fashionable in the research community. To circumvent this problem, we have taken a novel approach. We make the following assumptions. (*a*) Some common mechanisms increase life span in dauer larvae and *daf-2* mutant adults. (*b*) These shared mechanisms are reflected by common changes in gene transcription. We have compared previously reported expression profiles of genes altered during dauer exit (20) with profiles for genes regulated by *daf-2* in a *daf-16*-dependent manner, newly generated using whole genome oligonucleotide microarray analysis. We find that transcriptional changes occurring in dauer larvae are partially recapitulated in long-lived *daf-2* adults. By using a nonbiased statistical analysis, we have identified a number of gene categories that are significantly enriched for members with altered expression in both dauer and *daf-2* adult animals. This provides information about dauer-specific processes, which are mis-expressed in long-lived *daf-2* mutant adults and potentially control longevity and aging. We also identify several promoter elements that are enriched among dauer- and *daf-2*-regulated genes.

EXPERIMENTAL PROCEDURES

Strains and Media—The following strains were used for strain constructions: SS104 *glp-4*(*bn2ts*) *I* (29); DR1563 *daf-2*(*e1370*) *III*; DR1567 *daf-2*(*m577*) *III* (18); and GR1307 *daf-16*(*mgDf50*) *I* (23). From these, the following strains were constructed by standard means: *glp-4 I*; *daf-2*(*m577*) *III*, *glp-4 daf-16*; *daf-2*(*m577*), *glp-4*; *daf-2*(*e1370*), and *glp-4 daf-16*; *daf-2*(*e1370*). *glp-4* is included in all strains to prevent egg and progeny production, thereby limiting mRNA analysis to that of somatic adult tissues.

For routine strain maintenance, strain constructions, and life span analysis, nematodes were maintained on NGM agar plates, and *Escherichia coli* strain OP50 was used as a food source for all aspects of this study (30). For preparation of large numbers of nematodes for RNA isolation, 9-cm diameter enriched peptone NGM plates were used to maximize growth (31).

Analysis of Life Span—For life span analysis, animals were raised at 15 °C and transferred to 25 °C at L4 stage. Life span of populations was measured on NGM plates as described previously (18). Kaplan Meier analysis was conducted on survival data, and survival of populations of different genotypes were compared by using the nonparametric log rank test, using the statistical analysis package JMP 3.2.2 (SAS Institute Inc. Cary, NC).

Isolation of RNA for Microarray Analysis—Nematodes were prepared for RNA extraction as follows. Eggs from each strain were first isolated from three large (9 cm) plates of just-starved, mixed-stage populations by alkaline hypochlorite treatment (30). These were allowed to hatch overnight in 50 ml of S media in 250-ml Erlenmeyer flasks at 20 °C with rotary shaking (200 rpm). The resulting synchronized L1 larvae were concentrated by centrifugation, pipetted onto eight large plates (subsequently pooled to give one biological replicate), and incubated at 15 °C. When most animals had reached L4 stage, plates were shifted up to 25 °C. This temperature shift at L4 served a dual purpose; it prevented dauer formation in the *daf-16*(+) strains (which are past the developmental point at which they can form dauers), and it ensured sterility in all strains (via *glp-4*). Worms were harvested for RNA extraction the following day (1-day-old sterile adults), by washing worms off plates with M9, followed by three additional washes with ice-cold M9 to remove residual bacteria. At the time of harvesting, cultures were sampled to check for the presence of dauer larvae, males, and carcasses, which might still remain from the hypochlorite treatment. Details of this and other aspects of this work are available at AgeBase (haldane.biol.ucl.ac.uk/publications.html). Whereas no dauer larvae were seen in *daf-16*(*0*) strains, a very small number were seen in one of the *daf-16*(+) samples. Small numbers of males and non-dauer larvae were observed in some samples to varying degrees; however, we did not observe any systematic contamination of particular sample genotypes. We did not observe any carcasses remaining from hypochlorite treatment in any samples. Finally, total RNA was isolated using TRIzol reagent (Invitrogen), followed by purification and concentration using RNEasy columns according to the manufacturer's instructions (Qiagen, www1.qiagen.com). The quality of RNA samples was confirmed on an Agilent Bioanalyzer 2100 (Agilent Technologies, www.chem.agilent.com).

Oligonucleotide Microarray Analysis—Changes in transcript abundance was measured using *C. elegans* whole genome oligonucleotide microarrays (Affymetrix). We performed five biological replicates of each genotype. All Affymetrix protocols were performed at the University College London Institute of Child Health Gene Microarray Centre. The cRNA probe was generated by using standard Affymetrix protocols (www.affymetrix.com). Fragmented biotinylated probe was then hybridized to *C. elegans* whole genome arrays. Washing, labeling (streptavidin-phycoerythrin), and scanning followed standard procedures at the Institute of Child Health Gene Microarray Centre.

Statistical Analysis—To calculate gene expression measures, the data sets were normalized as follows. Raw image files were converted to probe set data (.cel files) in Microarray Suite (MAS 5.0) (32) (www.stat.berkeley.edu/users/terry/zarray/Affy/GL_Workshop/genelogic2001.html). The 20 probe set data files were normalized together, and expression values were determined, using the Robust Multichip Average method (33), implemented in the Affymetrix package (version 1.4.14) of the free statistical programming language R (www.r-project.org). Full array gene expression measures are available on the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo), accession numbers GSM28389–GSM28408. To identify differential gene expression between genotypes, we used significance analysis of microarrays, implemented in Microsoft Excel. A Δ value of 0.819 was chosen, which called 2,274 significant probe sets (1,348 up-regulated, 926 down-regulated), with a median false discovery rate of 5.06% (median number of falsely called probe sets = 116).

To create the list of genes differentially regulated during dauer recovery, we re-analyzed the raw data from a previous study (20). Expression differences between time 0 and 12 h after dauer recovery were used to identify probes that were differentially expressed. Additionally, we calculated a new *t* test value for all gene changes, using a more stringent two-tailed Student's *t* test and assuming unequal vari-

ance. We then selected probes that showed at least a 2-fold (up or down) difference in expression and had a p value of <0.005. This resulted in a list of 2,535 differentially regulated genes (1,160 up-regulated, 1,375 down-regulated).

To ensure the reliability of our data, we also performed several quality control procedures for both microarray formats. Briefly, we verified the accuracy of the microarray probes, and we identified probes that are predicted to hybridize to more than one gene (promiscuous probes) or to no gene (orphan probes). (For full analysis, see haldane-.biol.ucl.ac.uk). Probes that could unambiguously be assigned to a single gene were used for EASE analysis (see below), whereas promiscuous probes were analyzed by hand for any potential functional significance.

EASE Analysis—For our EASE analysis (see "Results" for explanation of the EASE application), we annotated every probe set on the *C. elegans* Affymetrix microarray and the Stanford cDNA microarray using information from a number of different data bases and data sets. We first associated each oligonucleotide probe set (or spotted array DNA probe) with its corresponding current curated gene entry. Next, the gene entry of each probe set was used to annotate the probe set or cDNA probe with functional information from Gene Ontology (GO) (34) (www.geneontology.org) and Interpro (35) (www.ebi.ac.uk/interpro). We also added functional information from several previous microarray and SAGE studies (16, 17, 19, 20), as well as the yeast two-hybrid map of *C. elegans* (36). For a full description of source information and the files used in our complete EASE analysis, see haldane.biol.ucl.ac.uk.

Analysis of Promoter Sequences—Genes with a given gene promoter motif in a defined region were identified using the program DNA Motif Searcher. This program uses a set of user-supplied DNA sequence motifs to search for additional motif instances in the genome of *C. elegans*. The set of motifs supplied by the user are interpreted into a position-specific score matrix (PSSM) that is used in the search step. By using this PSSM, the program can then identify the closest matching occurrences of the motif based on either a score cut-off, or it can identify the top X number of occurrences of the motif. For download or for a detailed explanation of how DNA Motif Searcher works, please see calliope.gs.washington.edu/papers/mcelwee2004a/software.html.

For the analyses presented here, we identified the top 10,000 occurrences of each motif using local background nucleotide frequencies and then filtered the hits to identify genes that contained motif occurrences within 1,000 bp upstream of the translational start site and/or within intron 1. The PSSM for each search were derived from DBE (37), DAE (17), HSE, HSAS (38), and HRE.[2]

Analysis of Frequency of Drosophila Orthologues in Topomountains—Fly gene orthologues were identified by using an all-*versus*-all BLAST search for both worm and fly using a cut-off value of 1×10^{-8}. These results from these searches were then used to identify reciprocal best BLAST hits using RBH Assigner (calliope.gs.washington.edu/papers/mcelwee2004a/software.html). The resulting list of RBHs were used for this analysis, using a score cut-off of 100.0 or greater (2,623 of 3,609 RBHs). By using this list of RBHs, the fly orthologues were placed into orthologous mountains. The total number of *C. elegans* genes in each mountain was calculated, and the percent representation was determined as the fraction of fly genes found in the orthologous mountain.

RESULTS

Genes that are transcriptionally regulated by the DAF-16 transcription factor must include some that are direct determinants of life span. DAF-16-regulated genes have been identified by using DNA microarray analysis (spotted arrays) (16, 17). Here we have taken the approach of comparing lists of genes whose expression is altered in *daf-2* mutants and in dauer larvae, performing a nonbiased search for classes of genes that are enriched in both cases. To this end we prepared a new list of *daf-16*-regulated genes, using whole genome oligonucleotide (Affymetrix) arrays. The dauer-regulated gene list was derived from a previous study (20).

daf-2 mutants are all long-lived but show variable degrees of pleiotropy (18). To reduce the probability of identifying allele-specific targets unlinked to aging, we used two *daf-2* alleles for the microarray analysis: *daf-2*(*m577*) (class 1) and *daf-2*(*e1370*) (class 2, more pleiotropic). We studied age-synchronized 1-day-old adults, in which reproduction was blocked using the *glp-4*(*bn2ts*) mutation (29). This temperature-sensitive mutation blocks mitotic proliferation of the germ line at 25 °C but is fertile at 15 °C. Nematodes were shifted from 15 to 25 °C at the L4 stage. In *C. elegans*, an increase in life span results from removal of the germ line, whether by microsurgery (39) or by mutation (40). We therefore checked the effect of *glp-4*(*bn2ts*) on life span in animals raised as above in two trials. Whereas *glp-4* alone did not increase life span, it causes a slight but significant enhancement of the *daf-2* Age phenotype ($p < 0.05$ in all cases, log rank test) (supplemental Fig. 1).

To identify differentially expressed genes, we pooled data for the two *daf-2* alleles and compared gene expression between *daf-2* and *daf-16* (*0*); *daf-2* (10 replicates each). We identified probe sets where transcript abundance is significantly different between *daf-2* and *daf-16* (*0*); *daf-2* with a 5% false discovery rate. This identified 1,348 probe sets up-regulated and 926 probe sets down-regulated in *daf-2* compared with *daf-16*; *daf-2*. Of these probe sets, 2,052 could be unambiguously assigned to a single gene. For the dauer data set, we identified differentially expressed genes as those that showed at least a 2-fold (up or down) difference in expression during dauer recovery, with a p value of <0.005. This resulted in a list of 2,535 genes differentially regulated probes (1,160 up-regulated and 1,375 down-regulated). Of these probes, 2,348 could be unambiguously assigned to a single gene. Both gene sets are listed at haldane.biol.ucl.ac.uk/publications.html.

Comparison with Spotted Array Results—We first compared our lists of *daf-16*-regulated genes with lists from two previous analyses of the effects of reduced IIS on gene transcription in *C. elegans* (16, 17). In the two earlier studies DNA microarrays spotted with PCR-amplified DNA were employed. In principal, the use of oligonucleotide microarrays is more sensitive, because they employ multiple oligonucleotides designed from each gene to be unique in the genome, thus greatly reducing the problem of cross-hybridization between closely related genes. Moreover, there appears to be significantly lower variance among replicates when using oligonucleotide arrays (Affymetrix) compared with spotted arrays.[3] Although there is considerable overlap between genes identified in the three analyses (Fig. 1*A*), a high proportion of genes is uniquely identified in each study.

Comparison of Daf-2 and Dauer-regulated Genes with Other Gene Sets—We compared changes in gene expression between *daf-2 versus daf-16; daf-2* (the *daf-2* gene set) with gene expression differences between dauer larvae and larvae 12 h after the onset of dauer recovery (the dauer gene set) (20). Despite the difference in the methods used to generate the dauer and *daf-2* gene sets, an overlap between gene sets is evident (Fig. 1*B*). For example, 21% of genes up-regulated in dauers are also up-regulated in *daf-2* adults (219 genes), and 11% of genes down-regulated in dauers are also down-regulated in *daf-2* adults (145 genes). It is probable that genetic determinants of longevity and aging are enriched among these 364 overlapping genes.

The relatively large number of changes in both dauer and *daf-2* gene sets means that identifying biologically relevant changes on a gene-by-gene basis would likely prove very difficult, if not impossible. To circumvent this problem and to identify potentially important biochemical processes in a statistically rigorous way, we have made use of the freely available software package EASE (david.niaid.nih.gov/david/ease.htm) (41). EASE is a user-customized application that performs a statistical analysis to identify significant over-representation of functional gene classifications within gene lists. Essentially, it compares the prevalence of particular gene categories within

[2] J. A. Powell-Coffman, personal communication.

[3] T. E. Johnson, personal communication.

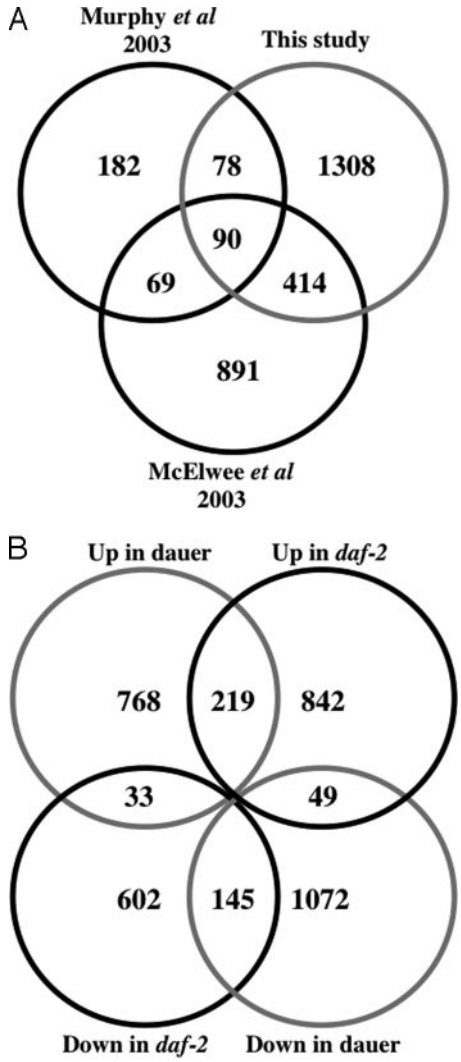

FIG. 1. *A*, comparison of genes identified as *daf-2* regulated in three microarray studies (see Refs. 16 and 17 and this study). *B*, overlap between genes differentially expressed in *daf-2* mutants and in dauer larvae.

our gene lists (for instance, cytochrome P450 genes), to the prevalence of that category in the genome as a whole. If transcriptional up- or down-regulation of a particular gene category or biological process is important for dauer or *daf-2* phenotypes, we would expect to find it over-represented within our lists of differentially expressed genes.

We have used EASE to examine our lists of differentially expressed genes for over-representation of functional categories from a number of biological data bases, including GO and Interpro. Additionally, we have created several customized data bases that contain microarray data and functional classifications from previously published studies, allowing us to compare our gene lists to other biological conditions that may show similar patterns of gene expression.

By using EASE, we have identified many gene classes that show significant over-representation in the dauer and *daf-2* gene sets. We first used EASE to examine the dauer and *daf-2* gene sets separately, and we identified gene groups that were over-represented ($p < 0.1$) in either the up-regulated or down-regulated genes. We then combined these separate analyses, and we identified gene groups that were similarly over-represented in both dauer and *daf-2* gene sets.

This initially identified 35 gene classes over-represented among genes up-regulated in dauer and *daf-2* gene sets and 26 gene classes over-represented among genes down-regulated in dauer and *daf-2* gene sets. Initial examination of these lists revealed that a number of lists contained identical or near identical members. Where redundant lists were identified, one representative member was selected for further analysis, and the rest were set aside. Excluding redundant lists, there remained 24 gene classes over-represented among genes up-regulated in dauer and *daf-2* gene sets, and 23 gene classes over-represented among genes down-regulated in dauer and *daf-2* gene sets (Table I). Gene classes showing over-representation in *daf-2* mutants alone ($p < 0.001$) are shown in Table II.

The dauer gene set was highly represented in the *daf-2* gene set; genes up-regulated in dauers were strongly over-represented among genes up-regulated in *daf-2* mutants ($p = 4.33e^{-26}$), and genes down-regulated in dauers were over-represented among genes down-regulated in *daf-2* ($p = 6.19e^{-08}$). There was also a weaker overlap between genes up-regulated in *daf-2* mutants and SAGE dauer tags (Table I). An earlier study of IIS-regulated genes identified sets of genes either up-regulated (class 1) or down-regulated (class 2) in long-lived IIS mutants (17). In that study, RNA interference tests confirmed the expectation that, overall, class 1 genes promote longevity, whereas class 2 genes promote aging. We observed a large overlap between class 1 genes and genes up-regulated in dauers, and class 2 genes and genes down-regulated in dauers (Table I). Thus, a strong dauer transcriptional signature is present in *daf-2* mutant adults. This is consistent with action of the same genes to control longevity in dauer larvae and *daf-2* mutant adults.

A number of gene expression topomountains were associated with dauer and *daf-2* gene sets. Mounts 6, 15, and 21 were over-represented in up-regulated dauer/*daf-2* gene sets and are therefore potentially longevity-associated. Mounts 19, 20, 24, 27, 30, and 31 were over-represented in down-regulated dauer/*daf-2* gene sets and are therefore potentially aging associated. Mt. 8 was over-represented in both up and down-regulated gene sets.

All genes in Mt. 15 are up-regulated in dauer larvae (20). Most interestingly, the overlap between *daf-2* up-regulated genes and this "dauer mountain" was the most significant of all the topomountains ($p = 6.16e^{-54}$), further evidence of a dauer transcriptional signature in *daf-2* adults. Mt. 15 is discussed in more detail below. (See Supplemental Data for further discussion of gene expression topomountains.)

The gene classes identified (Table I) provide indicators of varying informative value of processes altered in both dauer larvae and *daf-2* mutants. To identify such processes, we studied the contents of these gene lists, focusing on the individual genes within each gene category that were represented in both dauer and *daf-2* gene groups. Full lists of genes within each gene class that are up- or down-regulated in dauer larvae, in *daf-2* mutants, or in both contexts are available at haldane.biol.ucl.ac.uk.

Up-regulated Gene Groups—Among these gene groups is IPR002068: heat shock protein Hsp20. These are the small heat shock proteins (smHSP)/α-crystallin gene family of molecular chaperones. Six genes of this class are up-regulated in dauers and/or *daf-2* mutants (three in both). The six genes span the full size range of smHSPs; there are two *hsp-12*s (*hsp-12.3* and *hsp-12.6*), two *hsp-16*s (F08H9.3 and F08H9.4), *sip-1* (stress induced protein, F43D9.4, an Hsp20), and *hsp-43* (C14F11.5). The smHSPs are the only gene class where a role in longevity assurance in *C. elegans* is well demonstrated. Overexpression of *hsp-16.1* and *hsp-16.48* increases stress resistance and life span (42). Expression of smHSPs and other HSPs is regulated by the *hsf-1* (heat shock factor) transcription factor. *hsf-1* expression is required for the *daf-2* Age phenotype,

TABLE I
Gene groups where both the daf-2 and dauer gene sets are over-represented ($p < 0.1$)

Gene classes	*daf-2* gene set, score	Dauer gene set, score	Common overlapping genes[a]	Promoter element over-representation[b]				
				DBE	DAE	HSE	HSAS	HRE
Up-regulated genes								
Dauer-, *daf-2*-associated gene lists								
daf-2 up-regulated (this study)		$7.29e^{-29}$ (219/990)	219	[c]		[d]		
daf-2 up-regulated (16)	$5.15e^{-73}$ (213/489)	$3.51e^{-38}$ (158/513)	86	[c]				
Class 1 genes	$2.13e^{-52}$ (109/186)	$2.03e^{-21}$ (72/201)	47	[d]				
2-Fold up in dauer	$4.33e^{-26}$ (219/916)			[c]	[d]	[c]	[d]	[e]
SAGE dauer-specific tags	$3.00e^{-02}$ (47/291)	$4.85e^{-08}$ (63/293)	18			[d]		
Gene expression topomountains								
Mount 06	$3.67e^{-20}$ (125/807)	$6.44e^{-116}$ (250/803)	52	[d]		[e]	[d]	[e]
Mount 08	$9.19e^{-35}$ (139/685)	$5.37e^{-22}$ (114/708)	38		[c]			
Mount 15	$6.16e^{-54}$ (94/214)	$1.23e^{-57}$ (97/224)	42	[e]				
Mount 21	$4.46e^{-02}$ (15/131)	$1.41e^{-19}$ (44/140)	8		[c]			
Gene classes								
IPR002401: E-class P450, group I	$1.15e^{-08}$ (20/67)	$1.29e^{-04}$ (15/74)	6					
IPR002198: short-chain dehydrogenase/ reductase	$6.97e^{-05}$ (17/87)	$8.41e^{-06}$ (18/83)	6					
IPR000379: esterase/lipase/thioesterase, active site	$4.52e^{-03}$ (15/104)	$9.32e^{-06}$ (20/101)	6					
IPR001534: transthyretin-like	$1.44e^{-03}$ (10/45)	$4.75e^{-15}$ (23/47)	4	[e]				
IPR000010: cysteine protease inhibitors	$6.74e^{-03}$ (4/7)	$4.73e^{-02}$ (3/6)	1					
IPR002068: heat shock protein Hsp20	$8.67e^{-03}$ (5/14)	$7.01e^{-02}$ (4/16)	3					
IPR002213: UDP-glucuronosyl/glucosyltransferase	$2.29e^{-02}$ (10/68)	$6.86e^{-07}$ (18/70)	6					
IPR003703: acyl-CoA thioesterase	$3.34e^{-02}$ (3/5)	$2.06e^{-03}$ (4/5)	2					
IPR000873: AMP-dependent synthetase and ligase	$3.45e^{-02}$ (6/30)	$4.97e^{-05}$ (10/30)	3					
IPR002018: carboxylesterase, type B	$4.65e^{-02}$ (7/43)	$9.60e^{-04}$ (10/43)	2					
GO:0008152: metabolism	$5.49e^{-09}$ (59/427)	$1.15e^{-15}$ (74/406)	20		[d]			
GO:0016491: oxidoreductase activity	$1.45e^{-06}$ (35/229)	$8.09e^{-08}$ (37/213)	12					
GO:0006118: electron transport	$2.67e^{-04}$ (44/403)	$2.02e^{-03}$ (42/385)	13					
GO:0003824: catalytic activity	$7.57e^{-04}$ (36/322)	$1.90e^{-07}$ (45/299)	11		[d]			
GO:0016787: hydrolase activity	$1.06e^{-02}$ (19/161)	$3.55e^{-02}$ (18/163)	5					
Down-regulated genes								
Dauer-, *daf-2*-associated gene lists								
daf-2 down-regulated (this study)		$4.04e^{-11}$	(145/667)		[c]	[d]	[d]	
daf-2 down-regulated (16)	$7.73e^{-99}$ (277/881)	$1.69e^{-22}$ (220/916)	80		[c]			
Class 2 genes	$1.95e^{-17}$ (56/185)	$2.24e^{-3}$ (42/205)	22		[c]			
2-Fold down in dauer	$6.19e^{-08}$ (145/1123)							
Gene expression topomountains								
Mount 08	$4.88e^{-13}$ (74/685)	$7.45e^{-7}$ (88/708)	15		[c]			
Mount 19	$3.25e^{-48}$ (69/171)	$6.13e^{-23}$ (58/176)	21		[c]			
Mount 20	$4.46e^{-3}$ (15/147)	$8.7e^{-27}$ (57/146)	10					
Mount 24	$1.16e^{-30}$ (47/128)	$1.52e^{-07}$ (29/130)	17		[c]			
Mount 27	$2.56e^{-17}$ (27/75)	$1.96e^{-14}$ (30/76)	13		[c]			
Mount 30	0.0182 (6/36)	0.012 (8/35)	3					
Mount 31	$1.82e^{-5}$ (8/21)	0.0267 (6/24)	3					
Gene classes								
IPR003366: protein of unknown function DUF141	$2.70e^{-22}$ (28/50)	$7.34e^{-6}$ (16/51)	11					
IPR000560: histidine acid phosphatase	$2.79e^{-3}$ (6/20)	0.043 (5/17)	2					
IPR006025: neutral zinc metallopeptidases	0.0278 (11/98)	0.0388 (14/94)	3					
IPR000859: CUB domain	0.0344 (7/48)	0.0154 (10/49)	5		[c]			
IPR003582: Metridin-like ShK toxin	0.0396 (11/104)	$3.16e^{-5}$ (23/106)	2					
GO:0005529: sugar binding	$1.21e^{-3}$ (23/207)	0.0911 (21/207)	7		[c]			
GO:0006508: proteolysis and peptidolysis	0.0186 (30/371)	$3.81e^{-03}$ (46/337)	7					
GO:0016491: oxidoreductase activity	0.0559 (19/229)	$7.67e^{-03}$ (26/213)	6					
GO:0003824: catalytic activity	0.0810 (24/322)	$1e^{-5}$ (43/299)	8		[d]			
GO:0006730: one-carbon compound metabolism	0.093 (3/10)	0.0126 (5/11)	1					
GO:0006865: amino acid transport	0.096 (5/34)	0.0469 (7/30)	2					
WI5 Y2H NW132	0.0141 (7/40)	$2.04e^{-4}$ (14/37)	1					

[a] This is the number of genes in common between the differentially expressed genes in the columns *daf-2* gene set and Dauer gene set.
[b] DBE, *daf-16*-binding element; DAE, *daf-16*-associated element; HSE, heat shock element; HSAS, heat shock-associated site; HRE, *hif-1* (hypoxia-induced factor 1)-response element.
[c] $p < 0.001$.
[d] $0.05 > p > 0.01$.
[e] $0.01 > p > 0.001$.

and overexpression increases longevity (27), whereas loss of *hsf-1* results in progeria (43). RNAi studies demonstrate that smHSPs are important for the Age phenotypes resulting from either mutation of *daf-2* or overexpression of *hsf-1* (27). Thus, the presence of the smHSP gene class in Table I demonstrates that our analysis is capable of identifying gene classes linked to longevity. This suggests that other gene groups represented may also be linked to longevity.

TABLE II
Gene categories where only the daf-2 gene set is over-represented ($p < 0.001$)

Gene classes	Score	Promoter element over-representation[a]				
		DBE	DAE	HSE	HSAS	HRE
Gene groups up-regulated in *daf-2* mutants, not dauers						
Mount 01	$9.06e^{-06}$ (136/1456)	[b]	[b]	[c]	[b]	[b]
Mount 17	$5.32e^{-12}$ (40/174)					
Mount 22	$9.55e^{-05}$ (21/121)					
IPR004045: glutathione *S*-transferase, N-terminal	$7.35e^{-08}$ (15/41)					
Gene groups down-regulated in *daf-2* mutants, not dauers						
Mount 21	$4.21e^{-15}$ (32/131)		[b]			
Mount 22	$2.16e^{-04}$ (16/121)					
IPR002213: UDP-glucuronosyl/UDP-glucosyltransferase	$3.44e^{-08}$ (18/68)					
IPR005071: protein of unknown function DUF274	$6.83e^{-09}$ (11/19)		[b]			
IPR005828: general substrate transporter	$5.16e^{-06}$ (17/85)		[c]			
IPR004119: protein of unknown function DUF227	$7.48e^{-06}$ (9/22)					
GO:0006810: transport	$1.00e^{-04}$ (45/472)					
Stanford: lipid metabolism genes	$4.08e^{-04}$ (41/274)		[c]			

[a] DBE, *daf-16*-binding element; DAE, *daf-16*-associated element; HSE, heat shock element; HSAS, heat shock-associated site; HRE, *hif-1* (hypoxia-induced factor 1) response element.
[b] $p < 0.001$.
[c] $0.01 > p > 0.001$.

Three other gene classes in Table I are potentially linked to stress resistance: the cytochrome P450s (CYPs), the short-chain dehydrogenase/reductases (SDRs), and the UDP-glucuronosyltransferases (UGTs). There are 77 genes in the *C. elegans* genome that encode putative CYP proteins (correctly, heme thiolate protein P450s) (44). This number is typical of metazoan species (45); by contrast the *Saccharomyces cerevisiae* genome contains only three. In humans, many CYPs are monooxygenases (mixed function oxidases), catalyzing Reaction 1,

$$RH + O_2 + 2e^- + 2H^+ + NADPH \text{ or } NADH \rightarrow ROH + H_2O$$

REACTION 1

They metabolize a range of endobiotic lipophilic substrates, including steroids, fatty acids, prostaglandins, and lipophilic xenobiotics (46). In *C. elegans*, the DAF-9 CYP is believed to metabolize a steroid hormone critical to the regulation of dauer larva formation and with some influence on life span (47, 48). Six genes encoding CYPs showed increased expression in both dauer larvae and *daf-2* adults, 12 in *daf-2* mutants alone, and 3 in dauers alone. In 4/12 of the CYP genes up-regulated only in *daf-2* mutants, there is also a trend toward increased expression in dauer larvae ($p < 0.05$). Expression changes in all 77 CYP genes are summarized in supplemental Table II.

What biochemical and cellular processes might these CYPs be influencing? Unfortunately, little is known about the biochemical function of most *C. elegans* CYPs. The possible role of CYP genes in xenobiotic detoxification has been examined by screening for induction of transcription in response to a range of xenobiotic compounds (44). 15 CYP genes showed some degree of induction, four of which are among the dauer and/or *daf-2* up-regulated genes. These are B0213.15 (CYP34A9), which is up-regulated in *daf-2* mutants and dauer larvae, and C49C8.4 (CYP33E1), K07C6.3 (CYP35B2), and K07C6.4 (CYP35B1), which are up-regulated in *daf-2* mutants only. Expression of 35B1 and 35B2 was induced by a range of xenobiotics; for example, both were induced by ethanol (44). Notably, K07C6.4 (CYP35B1) was the most strongly up-regulated CYP gene in *daf-2* mutants (9.4-fold increase). Most interestingly, RNAi of this gene leads to partial suppression of the *daf-2* Age phenotype (17); this is also the case for B0213.15 (34A9)(xenobiotic induced) and T10B9.1 (13A4)(not xenobiotic induced). This suggests the possibility that processes that ensure xenobiotic resistance also promote longevity. Increased expression of CYPs accounts for the presence of the *GO:0006118 electron transport* gene category.

29 genes encoding putative short-chain dehydrogenase/reductases are up-regulated in dauers and/or *daf-2* adults (6 in both). SDRs function in xenobiotic detoxification, typically catalyzing the reduction of carbonyl groups in aldehydes and ketones, consuming energy from NADH or NADH in the process (49, 50).

A total of 22 genes encoding predicted UGTs are up-regulated in dauers and/or *daf-2* adults (6 in both). UGTs act in the smooth endoplasmic reticulum to glucuronidate (or glucosylate in insects) (51) small lipophilic molecules, thereby rendering them water-soluble and enabling their excretion (52). The substrates for UGTs are numerous, including diverse dietary constituents such as fatty acids and steroids, as well as xenobiotics and unwanted endobiotics (53). In mammals, this occurs mainly in the liver. Up-regulation of UGT expression has been noted previously in *daf-2* mutants (17) and dauer larvae, where it has been suggested that they might affect longevity via xenobiotic detoxification, or affect dauer formation by glucuronidating a dauer steroid hormone (20).

A total of 29 genes encoding transthyretin-like proteins are up-regulated in *daf-2* adults and/or dauer larvae (4 in both). In vertebrates, transthyretin is a transport protein found in extracellular fluids, which carries both thyroid hormones as well as vitamin A complexed with retinol-binding protein. However, the *C. elegans* transthyretin-like proteins that have been studied are members of a related but distinct protein family, the transthyretin-related proteins (TRP) (54). TRPs are found in vertebrates, invertebrates, prokaryotes, and plants. Although their function is unknown, the predicted ligand-binding site is almost entirely conserved, suggesting that, like transthyretin, they may function as carriers of lipophilic moieties.

A further clue as to the function of TRP is that all four TRP genes up-regulated in both dauers and *daf-2* mutants (C40H1.5, H14N18.3, Y51A2D.10, and Y51A2D.11) are in Mt. 8, which is associated with intestinal function and enriched for UGTs. Most TRPs are cytoplasmic (54); we tested for the presence of a predicted signal peptide (signifying probable secretion) by using the signal version 2.0 program (www.cbs.dtu.dk/services/SignalP-2.0/) (55). All four of these TRP genes are predicted to be secreted. We postulate that these TRPs play an active role in transporting unknown lipophilic substances from the intestine. None of these genes have close homologues outside the Nematoda. For commentary on other up-regulated gene classes and on down-regulated gene classes, see the Supplemental Material.

While studying these gene lists, we noticed several individual genes that were up-regulated in *daf-2* mutants that are worthy of note. For example, there was a 2.8-fold increase in transcript levels of C50F7.10, one of two *C. elegans* putative glycosyl hydrolases, homologous to mammalian klotho. Loss of function of this gene results in a progeroid phenotype in mice (56). Expression of *ges-1* was also increased (3.7-fold). The *ges-1* promoter has been used in a number of previous studies (57) to force expression of transgenes in the nematode intestine, for example, *daf-16*(+) in a *daf-16; daf-2* mutant background. Potentially, this could have resulted in a positive feedback loop of *daf-16* expression.

Gene Groups Altered in daf-2 Mutants Exclusively—A number of gene groups overlapped only with the *daf-2* gene group ($p < 0.001$, Table II). Gene groups altered in *daf-2* mutants but not dauers might reflect life span determinants unique to adults. After removal of redundant groups, there were four groups up-regulated only in *daf-2* mutants and eight groups down-regulated.

In *daf-2* adults, there was up-regulation of genes in Mts. 1 (muscle, neuronal), 17, and 22 (collagen). The most significant overlap was with Mt. 17. This topomountain is enriched with collagen genes (28); however, only 3/40 Mt. 17 genes up-regulated in *daf-2* encode collagens. There was also up-regulation of 16 GSTs (Table II). These proteins act in the cytosol, catalyzing the addition of the tripeptide glutathione to endogenous and xenobiotic electrophilic substrates, affecting the detoxification and metabolism of a range of compounds. The resulting glutathione adducts are more water-soluble. In this respect their function is similar to that of UGTs. GSTs also act in detoxification through their peroxidase activity and by passive binding of toxins. Additionally, GSTs play a role in oxidative stress resistance. GST-p24 (K08F4.7) is up-regulated in response to oxidative stress (58), and overexpression of K08F4.7 increases resistance to oxidative stress (59). We find K08F4.7 transcript levels to be increased in *daf-2* mutants (3.5-fold) but not in dauer larvae. GSTs also play a role in transport of specific hydrophobic ligands.

In *daf-2* adults there was down-regulation of genes associated with Mt. 21 (lipid metabolism) and Mt. 22 (collagen), as observed previously (16). There was also down-regulation of UGTs; thus there is over-representation of this gene class both in genes up-regulated and down-regulated in *daf-2* mutants. There was also down-regulation of two overlapping gene groups linked to transport (*IPR005828: General substrate transporter*; *GO:0006810: Transport*). One possible interpretation is that nutrient uptake is reduced in *daf-2* mutants. This is consistent with the down-regulation of several gene groups linked to protein degradation in dauers and *daf-2* mutants (Table I).

Potential Concerted Regulation of Highly Orthologous Gene Groups—Because EASE analysis requires a one-to-one correspondence between microarray probes and gene targets, it cannot be used to analyze probes that are predicted to hybridize to multiple genes. Although both the spotted array and oligonucleotide arrays described here were designed to reduce promiscuity, a small percentage (~7–10%) of the differentially regulated probes for both arrays cannot be unambiguously assigned to a single gene. There are at least two reasons for this; a probe may be poorly designed and cross-hybridize to unrelated genes, or the probe may be specific for a gene family in which members are highly homologous. In the latter case, these probes can be used to assay the expression of homologous gene families as a whole. We find that two such gene families, transposases and histones, are potentially differentially expressed in both dauers and *daf-2* adults.

A large number of promiscuous probes for transposase genes on both arrays are highly up-regulated (11 probes in the dauer data set and 13 probe sets in the *daf-2* data set). This may indicate general activation of transposition within the *C. elegans* genome in dauers and *daf-2* adults. Whereas increased transposition would not be expected to result in increased longevity, there are two possible explanations for this observation. First, the chromatin architecture in long-lived worms may differ from that of normal lived worms. In addition to activated transposases, we also find a large number of differentially expressed promiscuous probes for histone genes, another highly homologous gene family. In *daf-2* adults, 7 probes for histone H2 family members are down-regulated, and in both dauers and *daf-2* adults, probes for histone H4 are up-regulated (1 in *daf-2* and 5 in dauers). Notably, increased activity of *sir-2.1*, a putative histone deacetylase, increases life span in *C. elegans* in a *daf-16*-dependent fashion (60). Additionally, *daf-16*-dependent alterations in chromatin content has been observed previously in dauers, caloric restriction, and IIS mutants,[4] and it may be a that altered chromatin packing in these conditions leads to a permissive environment for transposase expression. The second possible explanation for the increased expression of transposase genes is that both dauers and *daf-2* adults show increased levels of heat shock gene induction, and we find that the promoters of transposase genes are highly enriched for heat shock elements (described below).

Gene Regulatory Elements in daf-2- and Dauer-regulated Genes—We have shown the presence of a dauer transcriptional signature in *daf-2* mutant adults. This indicates that similar transcriptional control factors are at work in each milieu, perhaps involving specific gene promoter elements. To explore this, we examined the frequency in *daf-2*- and dauer-regulated genes of five promoter elements potentially associated with IIS: the DAF-16-binding element (DBE) (37), the *daf-16*-associated element (DAE) (17), the HSE, the HSAS (38), and a potential hypoxia induction factor (*hif-1*)-response element (HRE).[2]

To do this, we first identified genes that contain these regulatory elements using DNAmotif Searcher, which uses a PSSM to identify potential regulatory motifs throughout the genome. These can then be matched against predicted coding regions to identify motifs within specified regulatory regions (such as promoters). By using this approach, we have identified regulatory motifs in promoter regions (0–1000 bp upstream of the predicted translational start sites) as well as in the first intron, which in *C. elegans* often contain regulatory sequences (for full lists of genes and PSSM matrices, see haldane.biol.ucl.ac.uk). By using these lists of genes containing particular regulatory motifs, we again used EASE to identify significantly over-represented functional categories which these transcription factors may regulate.

The DAF-16 transcription factor binds to the DBE, identified from 29 random oligonucleotides that bound to DAF-16 protein (37). These defined a core sequence, TTGTTTAC, which is common to all forkhead-binding sites (61, 62). DBE-containing gene groups that overlap with the *daf-2* gene set are listed in Tables I and II ($p < 0.05$). The 14 gene classes with a strong overlap ($p < 0.01$) with the DBE gene set are listed in supplemental Table II. These include genes up-regulated in *daf-2* mutants and dauers, plus two other gene groups that are enriched among genes up-regulated in dauers and *daf-2* mutants, including Mt. 15 ("mount dauer"). This provides strong evidence for the importance of the DBE in DAF-16-regulated gene expression and suggests that these gene classes are directly regulated by DAF-16. Some of the remaining gene groups may be over-represented because of possession of binding sites

[4] O. Ilkaeva, C.-K. Ea, and J. A. Waddle, personal communication.

for other classes of forkhead transcription factor. Most interestingly, none of the groups were those identified as enriched with genes *down*-regulated in dauers or *daf-2* mutants. This implies that DBEs are more important for DAF-16-dependent up-regulation of gene expression. Thus, the DBE appears to be a longevity-associated promoter element.

The *daf-16*-associated element (CTTATCA) was identified from a comparison of promoter sequences of *daf-2*-regulated genes (17). This sequence resembles the reverse complementary GATA-binding element (TTATCAGT). Of 29 gene classes strongly enriched ($p < 0.01$) for DAE-containing genes, 14 were also enriched for genes differentially expressed in dauers and/or *daf-2* mutants (supplemental Table II). Most interestingly, 13/14 classes show some down-regulation. These include Mts. 8 and 21, which are enriched with both *daf-2* up-regulated and *daf-2* down-regulated genes (Tables I and II). Moreover, the sets of genes down-regulated in *daf-2* mutants are themselves enriched for DAE-containing genes. These results strongly imply that presence of the DAE leads to DAF-16-dependent down-regulation of transcription. Thus, DAE appears to be an aging associated promoter element.

The HSE (TTCTAGAA) and the HSAS (GGGTGTC) are both associated with increased expression of chaperonin genes in response to heat shock (38). *daf-16*-dependent increases in expression of heat shock proteins are important for the IIS-associated Age phenotype (27, 42). This suggests that these promoter elements might be important for the transcriptional response to altered IIS. Several dauer/*daf-2*-associated gene groups, all of them up-regulated, did prove to be enriched for these elements (Tables I and II and supplemental Table I). Mt. 6 (neuronal gene enriched) was enriched for both HSE- and HSAS-containing genes. The most strongly HSE gene-enriched group was *IPR001888: Transposase*, type 1. This suggests that transposons might be activated by heat shock; the presence of an HSE was previously noted in the *Tc2* transposase gene (63).

hif-1 regulates the transcriptional response to oxygen starvation (hypoxia). A sequence resembling the mammalian HRE was recently identified as an enriched motif among the promoter regions of *C. elegans* genes whose expression is up-regulated by *C. elegans* HIF-1.[2] Because *daf-2* mutants are resistant to hypoxia (64), we predicted that genes with this HRE-like sequence might be regulated by *daf-2*. We detected over-representation of HRE genes among genes up-regulated in dauers but not in *daf-2* mutants. Mt. 6 (dauer/*daf-2* up-regulated) was also enriched with HRE genes. There was also a slight enrichment of *hif-1* genes in *daf-2* down-regulated genes. Potentially, the HIF-1 and DAF-16 proteins act together on genes in these clusters to affect the hypoxia resistance of DAF-2 mutants (64). In conclusion, this analysis shows that overall the DBE, HSE, HSAS, and HRE elements are enriched in genes up-regulated in dauers and/or *daf-2* mutants, and therefore represent potential longevity-associated promoter elements. By contrast, the DAE is associated with down-regulated genes and therefore represents a potential aging associated promoter element.

Poor Evolutionary Conservation of Mt. 15—Several observations suggest a possible link between Mt. 15 and the longevity of dauer larvae and *daf-2* adults. First, all genes in Mt. 15 are up-regulated in dauer larvae (20). Second, it is by a long shot the topomountain with the strongest over-representation of genes was up-regulated in *daf-2* adults ($p = 6.16e^{-54}$); the next most over-represented is Mt. 08 ($9.19e^{-35}$). Third, it is the only dauer/*daf-2* regulated topomountain that shows enrichment of DBEs (supplemental Table I). Analysis of microarray data from orthologous genes in a range of species has demonstrated evolutionary conservation of a number of topomountains (65). To explore the possibility that Mt. 15 might show evolutionary conservation of genes associated with longevity assurance, we compared the frequency in each *C. elegans* topomountain of genes with orthologues in *Drosophila*.

The topomountains over-represented in *daf-2* mutant-associated gene lists ($p < 0.001$) or both *daf-2* mutants and dauers ($p < 0.1$) are listed in supplemental Table III. Percent orthology varied among topomountains from 0 to 88%, mean ~18%. With the exception of Mt. 1, all of the potential longevity-associated mountains showed lower than average orthology, with Mt. 15 showing the lowest value (6.1%). Thus, evolutionary conservation of Mt. 15 is unlikely. Overall, the aging associated mountains showed a higher degree of orthology: mean, 31%, compared with 9% for the longevity-associated mountains. This implies that there is better evolutionary conservation among genes that promote aging than among those that promote longevity.

DISCUSSION

Microarray analysis has shown that large numbers of genes show differential transcript abundance in dauers (20) and *daf-2* mutant adults (see Refs. 16 and 17 and this study). Both dauers and *daf-2* mutant adults exhibit increased longevity. Thus, by comparing the transcription profiles of dauers and *daf-2* mutants, we have identified a number of gene groups and processes potentially contributing to longevity and aging.

A Dauer Transcriptional Signature in daf-2 Adults—We observed over-representation of dauer up-regulated genes among genes up-regulated in *daf-2* mutant adults, and of dauer down-regulated genes among genes down-regulated in *daf-2* mutants (Table I). This provides support for the idea that *daf-2* mutant longevity reflects expression of a dauer longevity program in the adult (2). It also provides some validation for our *daf-2* mutant/dauer comparative approach. Furthermore, the up-regulation in *daf-2* mutants and dauers of small heat shock proteins, which have a demonstrated role in longevity assurance, demonstrates that our analysis is capable of identifying life span-associated gene groups.

Increased Expression of Detoxification Genes in daf-2 Adults and Dauer Larvae—We observed up-regulation of genes encoding CYPs, SDRs, and UGTs in *daf-2* adults and dauer larvae (Table I). In mammals these three enzyme classes act in concert to dispose of toxic endobiotic or xenobiotic compounds (*e.g.* toxins, drugs, and carcinogens). Moreover, in *daf-2* adults (but not dauer larvae) we saw up-regulation of a fourth gene group linked to detoxification, the GSTs (Table II).

In pharmacological parlance, drug metabolism entails two successive phases: phase 1 (functionalization reactions) and phase 2 (conjugative reactions) (Fig. 2) (51). Phase 1 reactions result in chemically reactive functional groups, which are often (but not always) required for phase 2 reactions, the addition of side groups which increase solubility, aiding excretion. Phase 2 reactions represent the actual detoxification reactions.

Both CYPs and SDRs play a role in phase 1 metabolism in mammals. CYPs are the major effectors, acting mainly as mixed function oxidases, bioactivating xenobiotics or stable metabolites, usually by hydroxylation. The major effectors of phase 2 metabolism are the UGTs, but GSTs, sulfotransferases, and acetyltransferases are also important (51). Both CYPs and UGTs act in the smooth endoplasmic reticulum, whereas GSTs act in the cytosol, and SDRs in both locations. Taken alone, the significance of the up-regulation of many CYP genes in dauers and *daf-2* mutants is difficult to interpret, because CYPs function not only in drug metabolism but also in a range of biosynthetic processes (*e.g.* of steroids, fatty acids, prostaglandins, and vitamin D). Yet several observations suggest that CYP action in phase 1 metabolism is activated in dauers/*daf-2* mutants. First is the concurrent up-regulation of

SDRs, UGTs, and GSTs. Second, a number of the up-regulated CYP genes have been shown previously to be up-regulated in response to xenobiotics (44) (supplemental Table II).

A New Theory of Aging—The up-regulation of these four gene groups strongly implies that the capacity for phase 1 and phase 2 metabolism is up-regulated in *daf-2* mutants and dauer larvae. In the case of three CYPs and one UGT, a role in longevity assurance has been demonstrated by RNAi (17). These findings suggest a new picture of the biology of aging: a major cause of the molecular damage that gives rise to aging is a wide range of toxic compounds (endobiotic and perhaps also xenobiotic), particularly those that are lipophilic. We suggest that the enzymes involved in phase 1 and phase 2 detoxification metabolism are a major mechanism of longevity assurance (Fig. 3).

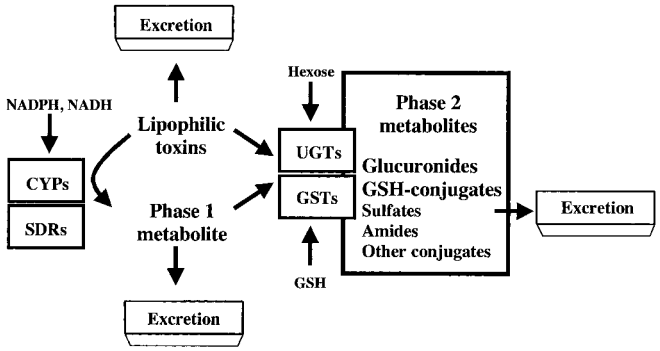

FIG. 2. **Phase 1 and phase 2 drug detoxification (adapted from Ref. 51).** CYPs, SDRs, UGTs, and GSTs act in concert to dispose of unwanted lipophilic and electrophilic molecules. See text for further explanation.

This theory provides a possible explanation for a number of other gene groups found in this study to be linked to *daf-2* mutant and dauer states. For example, in the gene class *IPR000379: Esterase/lipase/thioesterase*: a range of esterases play a role in reductive drug metabolism, *e.g.* the hydrolysis of the anesthetic procaine by plasma esterase (51). Likewise, the TRPs seem likely to be carriers of lipophilic compounds; possibly they also play a role in disposal of toxic lipophilic moieties. An alternative possibility is that TRPs act as carriers for lipophilic hormones involved in regulation of dauer formation and life span, for example the predicted steroid hormone associated with the DAF-9 CYP (47, 48).

Our analysis detected GST up-regulation and down-regulation of UGTs in *daf-2* mutants but not dauers. This could reflect differences in the targets of detoxification in each milieu, more lipophilic moieties in dauers and more electrophilic ones (perhaps xenobiotic) in adults. Such differences in gene expression may reflect differences in gene regulation by signaling pathways, such as IIS; alternatively, they may reflect differential induction of gene expression due to the presence of different detoxification target molecules.

Detoxification Is Energetically Costly; Links to the Disposable Soma Theory of Aging—The potential role of phase 1 and phase 2 metabolism in longevity assurance may be linked to the evolutionary theory of aging. According to Kirkwood's disposable soma theory, natural selection does not usually favor non-aging organisms because somatic maintenance processes that ensure longevity are energetically costly (66). A notable feature of both phase 1 and phase 2 metabolism of toxins is that they are energetically costly (in contrast to the action of SOD and catalase). The mixed function oxidase reactions of phase 1

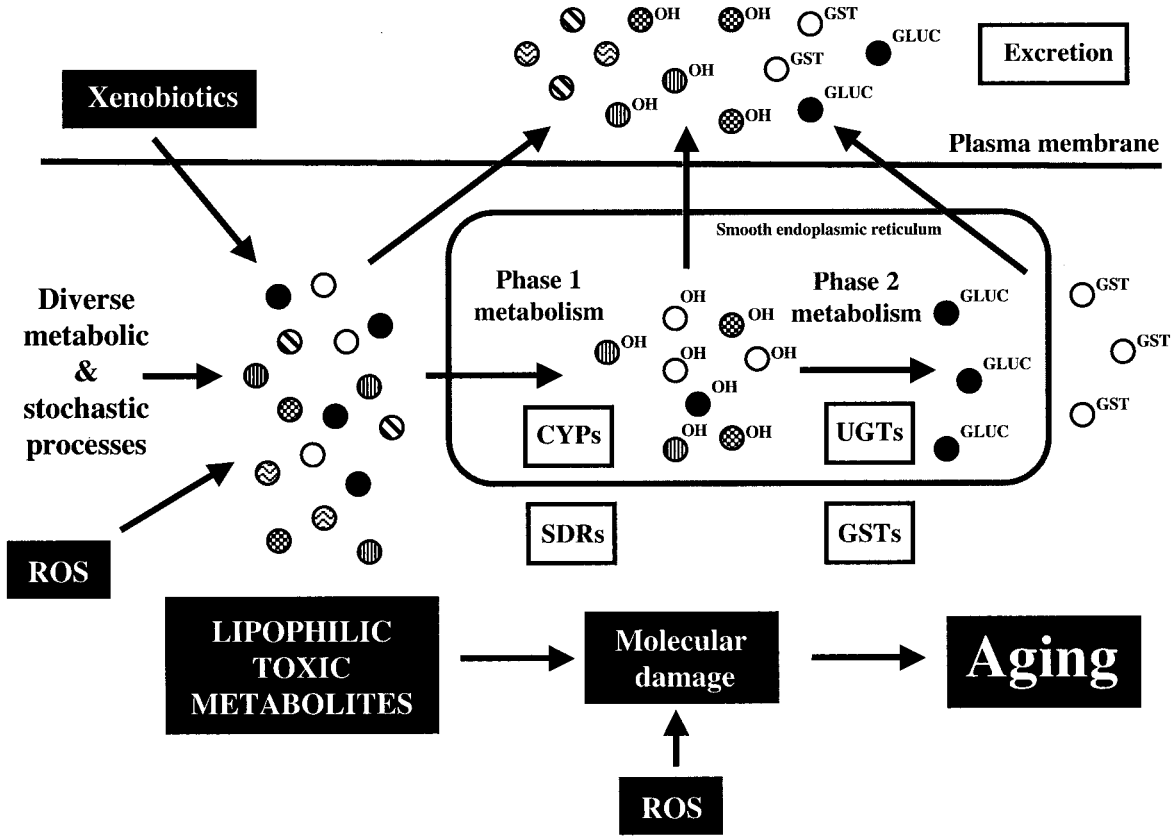

FIG. 3. **A new theory of aging.** We postulate that a major contributor to the aging process is molecular damage resulting from toxins (mainly endobiotic and lipophilic), which are targets of the phase 1/phase 2 drug detoxification system. According to this view, the smooth endoplasmic reticulum functions as a longevity organelle, derivatizing and excreting lipophilic toxins. However, this process is energetically costly, and most cells make the energy-saving step of down-regulating the detoxification system and dumping such toxins within lipofuscin depots. CYPs are mainly oxidases; *GCE*, glutathionyl; *Gluc*, glucuronosyl/glucosyl.

metabolism, catalyzed by CYPs, consume NADPH or NADH, as do SDR reactions. Similarly, each UGT glucuronidation reaction consumes a molecule of glucose (an extraordinary expense), and glutathionylation by GSTs consumes a molecule of glutathione. Potentially, increased phase 1 and 2 metabolism in *daf-2* mutant adults consumes energy that could otherwise be expended on reproduction, thus reducing fitness. The implications of this theory are explored in more detail elsewhere (67).

Insulin/IGF-1 Signaling Regulates Genes Linked to Nutrient Uptake—A number of gene classes linked to transport are down-regulated in dauers and *daf-2* mutant adults (Table I). These include the gene *nhx-2* (B0495.4), which is expressed in the apical membranes of intestinal cells and appears to prevent intracellular acidification by catalyzing the electroneutral exchange of extracellular sodium for an intracellular proton. Such acidification of intestinal cells occurs as the result of proton gradient-driven uptake of nutrients, *e.g.* by the oligopeptide transporter PEP-2 (OPT-2)(KO4E7.2) (68). Most interestingly, reduced expression of *nhx-2* slows development and extends life span in *C. elegans*, probably by inducing dietary restriction (68). Deletion of the *pep-2* gene does not increase life span *per se*, but enhances the *daf-2*(*e1370*) Age phenotype (69). We find that in *daf-2* mutants, *nhx-2* and *pep-2* show 5.6- and 7.7-fold reductions in transcript levels, respectively. Reduced expression of *pep-2* with reduced IIS was noted previously (17). In dauer larvae *nhx-2* and *pep-2* also show reduced transcript abundance, by 2.0- and 3.7-fold, respectively. This suggests that dietary restriction due to reduced nutrient uptake in the intestine contributes to the *daf-2* Age phenotype.

In conclusion, the aim of this work was to generate new ideas about mechanisms of aging from microarray data by using a novel approach: comparison of transcript profiles of dauer larvae and *daf-2* mutants. This approach was successful, in that it generated a number of new hypotheses about classes of genes and biochemical processes that determine life span, for example, the determinative role in life span of the detoxification system. Whether or not this theory is true is currently being tested in our laboratory.

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