Acrylamide-Responsive Genes in the Nematode Caenorhabditis elegans

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As acrylamide is a known neurotoxin for many animals and potential carcinogen for humans, it came as a surprise when the Swedish National Food Agency and Stockholm University reported in 2002 that it is formed during the frying or baking of foods. We report here genomic and proteomic analyses on genes and proteins of Caenorhabditis elegans exposed to 500 mg/l acrylamide. Of the 21,120 genes profiled, 409 genes were more than twofold upregulated and 111 genes were downregulated. Upregulated genes included many that encode detoxification enzymes such as glutathione S-transferases (GSTs), uridine diphosphate-glucuronosyl/glucosyl transferases, and short-chain type dehydrogenases but only one cytochrome P450. Subsequent proteomic analysis confirmed the heavy involvement of GSTs. Because of their high expression levels and central roles in acrylamide metabolism, we analyzed the in vivo expression patterns of eight gst genes. Although all encoded GST and were more than twofold upregulated by acrylamide treatment, their expression patterns were varied, and their regulation involved the transcription factor SKN-1 (a C. elegans homolog of Nuclear factor E2-related factors 1 and 2). We then selected the gst-4::gfp-transformed C. elegans to study the detoxification rate of acrylamide and its metabolite glycidimide in living animals. This animal detects acrylamide as a green fluorescence protein (GFP) expression signal in a dose- and time-dependent manner and may prove to be a useful tool not only for rapidly and inexpensively detecting acrylamide, a harmful substance in food, but also for analyzing mechanisms of GST induction by acrylamide and other inducers like oxidative stresses.

Key Words: xenobiotics; Phase II enzymes; biomarkers; food safety; toxicogenomics; proteomics.

Acrylamide produced industrially by hydration of acrylonitrile is mainly used to synthesize polyacrylamides. Its neurotoxic, genotoxic, and carcinogenic effects have been studied in animals and humans for more than 40 years (Friedman, 2003). Polyacrylamide, which is nontoxic, has a multitude of uses ranging from a material in chemical papers and fibers, a flocculant in the treatment of drinking or waste waters, a soil stabilizer, and cosmetic additives to electrophoresis gels in research laboratories. In contrast, acrylamide monomer has been classified as a Group 2A carcinogen by the International Agency for Research on Cancer (IARC) (1994) and a Category 2 carcinogen, Category 2 mutagen, and Category 3 reproductive toxin by the EU (European Commission Council Directive 67/548/EEC [http://ec.europa.eu/environment/dansub/main67_548/index_en.html]). In our daily life, acrylamide is readily taken into the body by several routes: (1) inhalation by occupationally exposed people and cigarette smokers, (2) ingestion of drinking water from flocculent-treated water, and (3) skin absorption from cosmetics and laboratory experiments involving polyacrylamide gel electrophoresis (PAGE) (Carere, 2006).

A recent search for the cause of high background levels of acrylamide in people industrially unexposed revealed that it is formed during the frying or baking of foods by means of the Maillard reaction (Friedman, 2003; Tareke et al., 2002). Considering its presence in common foods like bread and baked cookies, we were prompted to understand the effects of acrylamide on animals. We used the model organism Caenorhabditis elegans, a whole animal system for toxicological research (Miwa et al., 1982; Tabuse et al., 1989) to analyze various biological parameters such as growth, reproduction, body movement, and lifespan. We observed no obviously deleterious consequences for either growth or fecundity when the animals were raised in the presence of acrylamide at concentrations of less than 500 mg/l (Hasegawa et al., 2004b). For lifespan, however, we found a curious biphasic response whereby acrylamide concentrations over the extremely low dose range of 0.5–50 µg/l reduced the animal’s lifespan severely, the moderate doses of 500 µg/l to 50 mg/l acrylamide had essentially no such effect, and the highest dose of 500 mg/l acrylamide again reduced the lifespan strongly (Hasegawa et al., 2004b). We surmised that there would be separate mechanisms operating in C. elegans to affect its lifespan differently at the very low and high acrylamide doses (Hasegawa et al., 2004a).

In this paper, we analyzed the action of 500 mg/l acrylamide on the whole sets of gene and protein expression in the animal to find useful molecules for establishing acrylamide detection methods and to understand the molecular mechanisms of acrylamide toxicity at higher concentrations.
MATERIALS AND METHODS

Nematode strains and culturing. C. elegans var. Bristol, strain N2 (Brenner, 1974), and unc-119 (ed3) (Maduro and Pilgrim, 1996) were obtained from the Caenorhabditis elegans Genetics Center (University of Minnesota, Minneapolis, MN). Four independent lines of synchronized L1-stage animals were prepared by the treatment of egg-containing adults with sodium hypochlorite (Stiemarke, 1999) and grown for 48 h at 20 °C on acrylamide (500 mg/l) or control (without acrylamide) plates (Hasegawa et al., 2004b) seeded with Escherichia coli OP50. By this time, the animals on each plate were at late L4 stage, and a little of the E. coli lawn remained on the plate. The animals were isolated by sucrose flotation (Johnstone, 1999), washed twice with M9 buffer, and then frozen in liquid nitrogen until all samples were collected.

DNA microarray. The frozen animals of four independent culture lines were transferred separately into a mortar precooled with liquid nitrogen, ground with a precooled pestle, and suspended in Gibco Trizol (Invitrogen, Carlsbad, CA) to isolate total RNA. Messenger RNAs (mRNAs) were isolated from each of the culture lines by the Poly A tract mRNA isolation system (Promega, Madison, WI) according to the manufacturer’s instructions. RNA labeling and microarray hybridization were performed by the Stuarr Kim Lab, Stanford University, Palo Alto, CA (Stuart Kim Lab Home Page, http://cmgm.stanford.edu/~kimlab/). The expression patterns of the mRNAs were analyzed four times for both the 500 ng acrylamide-grown animals and the controls. The quaduplicate data, downloaded from the Stanford Microarray Database (http://genome-www5.stanford.edu/), were analyzed by the two-sided t-test to identify significant differences in RNA expression levels.

Two-dimensional gel electrophoresis. About 50 mg of the frozen animals was transferred into a mortar precooled with liquid nitrogen, ground with a precooled pestle, and added into 200 μl of protein extraction buffer containing 7M urea, 2M thiourea, 5% Chaps, 2% Triton X-100, 2% Immobiline DryStrip precast gel buffer (Amersham Biosciences, Uppsala, Sweden), 1% nuclease mix (Amersham Biosciences), and Complete Protease Inhibitor Cocktail (Roche, Penzberg, Germany). After vigorous mixing, the homogenate was incubated for 30 min at room temperature, centrifuged for 10 min at 16,000 × g, and the supernatant was collected. Protein concentrations were determined by bichinonic acid protein assay (Pierce, Rockford, IL) with bovine serum albumin as the standard. Immobiline Dry Strip Gels (pH 4–7, 18 cm, Amersham Bioscience) were rehydrated with a mixture of 500 μg of sample protein in 190 μl of DeStreak Rehydration Solution (Amersham Bioscience) for 18 h at room temperature. The first dimension of the gel electrophoresis was performed with NA-1410 equipment (Nihon Eido, Tokyo, Japan) by a multistep incubation for 30 min at room temperature, centrifuged for 10 min at 16,000 × g, and the supernatant was collected. Protein concentrations were determined by bichinonic acid protein assay (Pierce, Rockford, IL) with bovine serum albumin as the standard. Immobiline Dry Strip Gels (pH 4–7, 18 cm, Amersham Bioscience) were rehydrated with a mixture of 500 μg of sample protein in 190 μl of DeStreak Rehydration Solution (Amersham Bioscience) for 18 h at room temperature. The first dimension of the gel electrophoresis was performed with NA-1410 equipment (Nihon Eido, Tokyo, Japan) by a multistep protocol (50 V/h, 100 V/h, 300 V/h, 1000 V/h, 1000 V/h, 100 V/h, 2000 V/h, 3000 V/h, 3500 V over night: total 56,000 Vh). Then, the gel was immediately equilibrated in buffer containing 0.5M Tris–HCl, pH 6.8, 6M urea, 30% glycerol, 1% sodium dodecyl sulfate (SDS), 2% bromophenol blue, and 25 mg/10 ml dithiothreitol for 1 h with slow rocking at room temperature. The second dimension of the gel electrophoresis was performed with 10% SDS-PAGE, 22 × 20 cm at 90 V for 16 h. After two-dimensional gel electrophoresis (2-DE), the gel was stained with Coomasie brilliant blue Stain One (Nacalai Tesque, Kyoto, Japan) according to the instruction protocol. The gel was scanned as a 16-bit tif-image with an Epson ES-8500 scanner. The images were analyzed by the software package Image Master 2D Platinum ver. 5.0 (Amersham Bioscience).

In-gel digestions were performed as described by Mäki et al. (2003), and digested peptides were extracted with 5 μl of extraction buffer (50% acetonitrile, 1% trifluoroacetic acid (TFA) in water) by sonication. One microliter of each sample was spotted onto the Anchor chip, mixed with about 0.2 μl of matrix solution (saturated 2-cyan-4-hydroxycinnamic acid in acetonitrile: 0.1% TFA = 2:1), and dried. Protein mass fingerprinting was performed on an Autoflex MALDI-TOF mass spectrometer (Bruker-Daltonics, Billerica, MA) with angiotenin I and II, substance P, bombesin, ACTH clip 1–17, ACTH clip 18–39, and somatostatin 28 as the peptide calibration standards (Bruker-Daltonics). The data were analyzed by using the MASCOT database search engine (Matrix Science) on the Swiss-Prot databases. Identified proteins were annotated from the WormBase (http://www.wormbase.org/).

GFP reporter constructs. To make green fluorescence protein (GFP) reporter constructs, PCR was performed with KOD Plus DNA polymerase (TOYOBO, Osaka, Japan) on N2 genomic DNA. PCR primers were designed to amplify each gene containing both its predicted promoter, about 0.5–1.5 kbp upstream from the predicted start site, and full coding regions without the stop codon. Primers are listed in Table 1. PCR-amplified DNA fragments were digested with proper restriction enzymes and then ligated into the gfp vector pPD95.77 (kindly provided by Fire, A., Stanford University) to obtain reporter constructs. One hundred micrograms per milliliter of each reporter construct so obtained was co-injected with an equal concentration of the rol-6(su1006)–containing plasmid pRF4 or the unc-119(is1+)–containing plasmid pDP#MM016B (kindly provided by Maduro, M., UC Riverside), respectively, into the gonadal arms of N2 wild type or unc-119 (ed3) adult hermaphrodites as described (Mello et al., 1991). Integration of transgenic extrachromosomal arrays into chromosomes was performed as described by Miitan (1995). GFP

TABLE 1

Primer Sequences for GFP Reporter Constructs

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>gst-24</td>
<td>5′—AATCTGGACAAATTTGGAATCCGCTGTTAATTTTAA—3′</td>
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<tr>
<td>GST-24Rev_BamHI</td>
<td>5′—CGGGATCCCAACAGAATCTGGTCTCGACGAGTT—3′</td>
</tr>
<tr>
<td>GST-30</td>
<td>5′—AATCTGGACAAATTTGGAATCCGCTGTTAATTTTAA—3′</td>
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<tr>
<td>GST-24Rev_BamHI</td>
<td>5′—CGGGATCCCAACAGAATCTGGTCTCGACGAGTT—3′</td>
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<td>GST-30</td>
<td>5′—AATCTGGACAAATTTGGAATCCGCTGTTAATTTTAA—3′</td>
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<tr>
<td>GST-24Rev_BamHI</td>
<td>5′—CGGGATCCCAACAGAATCTGGTCTCGACGAGTT—3′</td>
</tr>
</tbody>
</table>

Note. Underlined sequences indicate the restriction enzyme-cleaved sites.
expression patterns were observed with a ZEISS Axiovert 200 microscope equipped with a confocal laser-scanning module.

**SKN-1 binding sites.** Predicted SKN-1 binding sites were searched with TFSEARCH version 1.3 (Heinemeyer et al., 1998).

**RNA interference (RNAi).** Gene fragments of skn-1 or gst-4 were prepared by PCR amplification of *C. elegans* N2 complementary DNA with the following primers, respectively: Cskn1EcoRIFor, 5′—GAAGTCCGGCCAATCAATAATGATATGCTCC—3′; Cskn1EcoRIRev, 5′—GAAGTCCGGGCAACCTTTCTTTCCG—3′ or Cegst4RightEcoRI, 5′—ATGGAATTCTATAGTATGTATT—3′; Cegst4LeftEcoRI, 5′—ATGGAATTCTATACCTTTCTTGCTGC—3′. Both of the skn-1- or gst-4-derived PCR fragments were digested with EcoRI, and cloned into the EcoRI restriction site of the RNAi vector pPD129.36 (kindly provided by Fire, A., Stanford University). The PCR-fragment-ligated plasmids or the blank vector pPD129.36 were used to transform *E. coli* HT115 (Kamath et al., 2001).

For RNAi experiments, synchronized L1-stage transgenic animals were first cultured for 24 h at 20 °C with nematode growth medium (NGM) (containing 50 μg/ml ampicillin and 12.5 μg/ml tetracycline) plates seeded with *E. coli* HT115 transformed only with the blank vector. The animals were then collected, transferred onto NGM plates seeded with *E. coli* HT115 transformed with each different RNAi plasmid, and cultured for 24 h at 20 °C. Thereafter, the animals were again collected and transferred on NGM plates, with or without 500 mg/l acrylamide, seeded with each different *E. coli* HT115 RNAi bacteria. After 24 h of incubation, the animals were observed for GFP expression with both a Nikon SMZ1000 dissection microscope equipped with a fluorescence filter and a ZEISS Axiovert 200 microscope equipped with a confocal laser-scanning module.

**GST response.** Synchronized L1-stage transgenic animals, prepared by sodium hypochlorite treatment, were transferred onto NGM plates seeded with *E. coli* OP50 with or without 500 mg/l acrylamide and grown at 20 °C for 12, 24, and 48 h. At each time, animals were collected and washed three times with M9 buffer and resuspended in M9 buffer at 100 transgenic animals per 100 μl, which was put into each well (100 μl each of 8 to 12 wells) of a B & W IsoPlate-96. The GFP signal emitted from each well was measured three times with a Wallac 1420 ARVOSx multilabel counter (PerkinElmer, Waltham, MA).

**GFP signal kinetics.** Synchronized L1-stage transgenic animals were transferred onto NGM plates seeded with *E. coli* OP50 and grown until L4 stage at 20 °C for 48 h. One hundred synchronized L4 animals were collected and put into a well of a B & W IsoPlate-96 (PerkinElmer) containing *E. coli* OP50 (10^6^ cells) and acrylamide or its metabolite glycylidamide in S medium (Stiernagle, 1999), at a total volume of 100 μl per well, and incubated at 20 °C. To avoid drying up the medium, plates were sealed with optical adhesive covers (ABI Prism, Foster City, CA). The GFP fluorescence was measured at 1-h intervals with a Wallac 1420 ARVOSx multilabel counter (PerkinElmer). The GFP signal emitted from each well was measured three times, and the average value of the three was used as the value of that well. The mean value of the GFP signals for each acrylamide or glycylidamide concentration and the control was determined by averaging the values from 8 to 12 such wells.

**Statistical analysis.** Paired Student’s *t*-test (Microsoft Excel) was used to determine the significance of differences in the mean GFP signal values.

## RESULTS

### Genes Upregulated by Acrylamide

Our previous report (Hasegawa et al., 2004b) revealed the biphasic nature of the *C. elegans* lifespan in a dose-related response to acrylamide, whereby extremely low doses (0.5–50 μg/l) and a high dose (500 mg/l) of acrylamide shortened the *C. elegans* mean lifespan by about 15–19%, whereas moderate doses (500 μg/l to 50 mg/l) did not. To understand how acrylamide acts in *C. elegans*, we first analyzed the effects of the high dose of 500 mg/l acrylamide on *C. elegans* gene expression by using the full genome DNA microarray (Stuart Kim Lab Home Page, http://cimgm.stanford.edu/~kimlab/).

When animals were grown in the acrylamide concentration of 500 mg/l and compared with the control animals grown without acrylamide, 409 of the total 21,120 genes were upregulated more than twofold (*p* < 0.005).

Conspicuously, the 409 upregulated genes included a large number of genes encoding phase II detoxification enzymes, including 18 members of glutathione S-transferases (gst) and 6 members of uridine diphosphate-glucuronosyl/glucoyl transferases (ugt), as well as phase II enzymes, such as seven dehydrogenases, six of which are members of short-chain type dehydrogenases (sdh) (Lindblom and Dodd, 2006), and one cytochrome *P*450 (cy) (*cy-13A12*, *F14F7.3*), the mammalian homolog Cyp2E1 of which is believed to play a part in converting acrylamide to glycaldimide (Friedman, 2003). The changes in mRNA expression of these detoxification enzymes were generally greater than those for other genes (Table 2). The most highly upregulated gene was *gst-4*, which was 24.78-fold upregulated and encodes a human homolog of glutathione-requiring prostaglandin D synthase (Sigma class GST) (Table 2). Genes that encode enzymes necessary for glutathione metabolism such as systathione γ-synthase (*C12C8.2*), glutamine synthase (*gln-2*, *K03H1.1*), glutathione reductase (*C46F11.2*), glutathione peroxidase (*F26E4.12*), and glutamate-cysteine ligase catalytic subunit (*gcs-1*, *F37B12.2*) were also upregulated.

In addition to them, some stress response genes, such as those encoding superoxide dismutase (*C15F1.B*, sod-1) and a small heat shock protein (*ksp-16.49*, *T27E4.9*) were upregulated.

Also more than twofold upregulated were genes encoding such proteins as 54 major sperm proteins (MSPs) and 5 other sperm-related proteins, 18 members of 7-transmembrane chemoreceptors, 12 collagens, and 7 lectins. Many genes that encode signal transduction molecules such as 18 kinases and 15 phosphatases were upregulated as well. The microarray data for the genes described here and in Table 2 are listed in the Supplementary Data.

### Genes Downregulated by Acrylamide

A total of 111 genes were downregulated by 500 mg/l acrylamide treatment (*p* < 0.005). They included, rather unexpectedly, four CYP genes. Others of interest were one gene for metallothionein (*mtl-1*, *K11G9.6*), an enzyme active against heavy metal toxicity and oxidative stress, genes for 10 7-transmembrane chemoreceptors and 4 lectins. Many “housekeeping” genes encoding cell structure molecules, surface proteins, and proteins active in energy production, and lipid and fatty acid metabolism were also downregulated.
GSTs were also Upregulated at the Protein Level

Total proteins were extracted from animals treated with 500 mg/l acrylamide or without acrylamide as a control for 48 h at 20 °C (from L1 to late L4 stage), separated in the first dimension by pH gradient (pH 4–7) and in the second dimension by molecular weight (SDS-PAGE), and analyzed for their differences in expression. We detected 274 ± 31 (mean ± SD) spots from the control extracts and 334 ± 29 spots from the extracts with 500 mg/l acrylamide in four independent experiments. Among the latter, we identified 18 spots by MALDI-TOF mass spectrometer; of them, 14 spots were not affected by acrylamide but were identified as landmarks in the 2-DE, and four proteins were clearly upregulated by 500 mg/l acrylamide (Fig. 1 and Table 3). These were all identified as GSTs (Table 3). Just as in the microarray analysis, GST-4 was prominently upregulated at the protein level.

In Vivo GST Expression Patterns

Because of their high expression levels and the central roles they play for acrylamide metabolism in animals, we focused on these highly upregulated gst genes as suitable molecules for our further experiments. About 50 gst genes exist in the C. elegans

<table>
<thead>
<tr>
<th>Gene</th>
<th>LG</th>
<th>Average±SD</th>
<th>p value</th>
<th>Gene</th>
<th>LG</th>
<th>Average±SD</th>
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<td>UGT</td>
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<td>C23G10.6</td>
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<td>1.28±0.15</td>
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<td>&lt;0.005</td>
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</table>

Note. Microarray analysis was repeated four times.

LG, linkage group.

Average ± SEM represents log2 ratio.

Two-sided t-test was used to analyze significant differences.

GSTs were also Upregulated at the Protein Level

Total proteins were extracted from animals treated with 500 mg/l acrylamide or without acrylamide as a control for 48 h at 20 °C (from L1 to late L4 stage), separated in the first dimension by pH gradient (pH 4–7) and in the second dimension by molecular weight (SDS-PAGE), and analyzed for their differences in expression. We detected 274 ± 31 (mean ± SD) spots from the control extracts and 334 ± 29 spots from the extracts with 500 mg/l acrylamide in four independent experiments. Among the latter, we identified 18 spots by MALDI-TOF mass spectrometer; of them, 14 spots were not affected by acrylamide but were identified as landmarks in the 2-DE, and four proteins were clearly upregulated by 500 mg/l acrylamide (Fig. 1 and Table 3). These were all identified as GSTs (Table 3). Just as in the microarray analysis, GST-4 was prominently upregulated at the protein level.

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![FIG. 1. 2-DE gel images of Caenorhabditis elegans total protein. 500 μg of protein was separated in the first dimension by pH gradient (pH 4–7, 18 cm), and in the second dimension by molecular weight. (A) Proteins from animals grown on control plates without acrylamide; 277 ± 31 (mean ± SD) protein spots were detected from four independent experiments. (B) Proteins from animals grown on 500 mg/l acrylamide; 334 ± 29 (mean ± SD) protein spots were detected from four independent experiments. (C) Eighteen identified spots are indicated on a 2-DE gel image. The spot numbers correspond to the proteins listed in Table 3. Color images are available in the Supplementary Data.](https://academic.oup.com/toxsci/article-abstract/101/2/215/1641882/FIG.1)
TABLE 3  
Identified Proteins

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<tr>
<th>No.</th>
<th>Protein name Sequence Da</th>
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<td>Glutathione S-transferase 7 F11G11.2</td>
<td>23,128 6.30</td>
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<td>50</td>
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*Spot numbers correspond to spots on the 2-DE gel image (Fig. 1C). Intermediate filament protein has two isoforms (spliced variant, Spot Nos. 169 and 171) and is predicted to function as a structural component of the cytoskeleton.

SKN-1 induces GSTs expression

To analyze SKN-1 involvement in GST expression, we constructed the chromosomal-integrated line MJUCU017 {ksi17[constitutive GST-4::gfp, pDP#MM016B]}, which has an expression pattern the same as that for the MJUCU001 extrachromosomal line when treated with acrylamide (Figs. 3A–C). Knockdown of skn-1 by feeding skn-1 RNA prevented the GST-4 expression except for that in the pharynx and body wall muscle (Figs. 3D–F). Even gst-4 knockdown by feeding gst-4 RNA failed to prevent GST-4 expression in the pharynx (Figs. 3G–I). Thus, GST-4 expression induced by acrylamide appeared to be regulated by skn-1 in part body other than the body wall, where it is regulated by a far-up regulated mechanism. RNAi against either skn-1 or gst-4 did not work in the pharynx, which means either that feeding RNAi does not work in the pharynx or that GST-4 expression in the pharynx is independent of RNA inhibition. We searched for the position and orientation of predicted SKN-1 binding sites in GST genes and, similarly as was reported previously (Blackwell et al., 1994), found that the promoters of the GST-encoding genes examined bear two or more putative binding sites for the Nuclear factor E2-related factors 1 and 2 (Nrf1/2)-like C. elegans ortholog SKN-1 (data not shown).

GST response

Figure 4 shows the changes in GST response in the eight transgenic animals after 12, 24, and 48 h of exposure to 500 mg/l of acrylamide from the L1 stage. Data points indicate the mean values ± SEM of GFP signals at each time compared with that for the control (100%) from three independent experiments (or plates). Although the GFP signals were too weak for the expression changes in GST-24, -2, -37, and C02D5.3c to be detected by this experiment, we could detect them for GST-4, 7, 38, and 30 (Fig. 4). After 48 h, GST-4 expression was strongest, having continued to increase to 510 ± 24%.
gst-4 Expression Increases by Acrylamide in a Dose- and Time-Dependent Manner

Figure 5A shows the kinetics of GST-4 expression by acrylamide over concentrations from 50 to 500 mg/l. Columns indicate the mean values ± SEM of GFP signals at each acrylamide concentration compared with that for the control (100%) from three independent experiments (or plates). The GFP signal was not detected under these experimental conditions until 50 mg/l acrylamide, and increased in a dose- and time-dependent manner from 100 mg/l. After 3 h, the GFP signal was detected.

FIG. 3. Knockdown of skn-1 (RNAi) arrests GST-4 expression except for that in the pharynx and body wall muscle. (A–C) GST-4 expression patterns in MJCU017 treated with 500 mg/l acrylamide (blank RNAi). Strong GFP signal is detected from the whole body. (A) Whole body; Nomarski + fluorescence. (B) Whole body; fluorescence. (C) Head region; fluorescence. (D–F) GST-4 expression patterns of skn-1 RNAi in MJCU017 treated with 500 mg/l acrylamide. Expression was arrested except for that in the pharynx and body wall muscles. (D) Whole body; Nomarski + fluorescence. (E) Whole body; fluorescence. (F) Head region; fluorescence. (G–I) GST-4 expression patterns of gst-4 RNAi in MJCU017 treated with 500 mg/l acrylamide. GST-4::GFP expressed only in pharynx. (G) Whole body; Nomarski + fluorescence. (H) Whole body; fluorescence. (I) Head region; fluorescence. Scale bars (A, B, D, E, G, H) 200 μm; (C, F, I) 50 μm. Color photographs are available in the Supplementary Data.

FIG. 2. GST expression patterns in gst::gfp transgenic animals. (A–P) Photographs were taken through Nomarski optics with laser-scanned fluorescence microscopy (Nomarski + fluorescence). (a–p) Photographs were taken with laser-scanned fluorescence microscopy (fluorescence). Scale bars, (A–I, K–P and a–i, k–p), 200 μm; (J and j) 20 μm. MJCU008 for gst-24: (A, a) Control animals without acrylamide; GFP signal was constitutively expressed in the head and tail regions. (B, b) Animal treated with 500 mg/l acrylamide. GFP signal resembled that for the control. MJCU002 for gst-30: (C, c) Control animals without acrylamide. No GFP signal was detected. (D, d) Animals treated with 500 mg/l acrylamide. A GFP signal was induced in the head, vulva, and tail regions. MJCU003 for gst-7: (E, e) Control animals without acrylamide. GFP signal was constitutively expressed in the head and intestine region. (F, f) Whole animal treated with 500 mg/l acrylamide. GFP signal was from the hypodermis. MICU012 for C02D5.3c: (G, g) Control animal without acrylamide. Weak GFP signals were from the tail region. (H, h) Animal treated with 500 mg/l acrylamide. GFP signal was from the head, intestine, and tail regions. MJCU007 for gst-2: (I, i) Control animal without acrylamide. Weak GFP signal was from the head region. (J, j) Animal treated with 500 mg/l acrylamide. GFP signal in the head region resembled that for the control. MICU001 for gst-4: (K, k) Control animals without acrylamide. No GFP signal was detected. (L, l) Animal treated with 500 mg/l acrylamide. Whole body showed strong GFP signal. MICU006 for gst-37: (M, m) Control animals without acrylamide. No GFP signal was detected. (N, n) Animal treated with 500 mg/l acrylamide. Weak GFP signal was from head, gonad, and tail regions. MJCU009 for gst-38: (O, o) Control animal without acrylamide. GFP signal was constitutively detected from the intestine region. (P, p) Animal treated with 500 mg/l acrylamide. Stronger GFP signal detected from the intestine region. Color fluorescence photographs are available in the Supplementary Data.
signal at 500 mg/l acrylamide started increasing (105 ± 6%, normalized to the control signal, without acrylamide, as 100%), and after 12 h reached 187 ± 35%.

When treated with glycidamide, an epoxide metabolite of acrylamide and thought to be very reactive with electrophiles, GFP signals increased by 10, 12, and 14% within the first hour, respectively, at 0.5 l g/l, 500 l g/l, and 500 mg/l and, except for 500 mg/l, maintained the same % levels over 12 h. At 12 h, the GFP signal at 500 mg/l of glycidamide reached 133 ± 5%, which was 70% of that for 500 mg/l acrylamide (Fig. 5B). The glycidamide-driven induction profile of GST-4 appeared similar to that for microsomal GST1 from MCF7 cells (Clement et al., 2007), although the latter was measured for its RNA expression level.

DISCUSSION

In this report, we analyzed the whole gene expression pattern in C. elegans that were grown from the L1 hatchee-stage on 500 mg/l acrylamide plates for 48 h; whereas, previously we reported the chronic toxic effects of 500 mg/l acrylamide on the animals’ postembryonic development (Hasegawa et al., 2004b). Our DNA microarray data showed that genes for a large number of major detoxification enzymes, such as GST, UGT, and SDR, were upregulated more than twofold by the acrylamide exposure. They consist of phase I (SDR) and phase II (GST and UGT) enzymes, which act in the detoxification and excretion of endogenous products of oxidative stress and electrophilic xenobiotics (Lindblom and Dodd, 2006). These genes are abundant and form some clusters in the C. elegans genome (WormBase, http://www.wormbase.org/). Average changes in gene expression before and after exposure to 500 mg/l acrylamide were huge; five of six SDR, 13 of 18 GST, and two of six UGT genes were more than four times (> 22) upregulated (Table 1). In clear contrast, at the extremely low acrylamide concentration of 0.5 l g/l, no genes in the phase I and II detoxification pathways were included among 14 upregulated and 56 downregulated genes (Hasegawa et al., 2004a). This conspicuous difference in the gene profiles at high and low acrylamide doses would provide a basis for explaining the biphasicity we observed in the C. elegans lifespan (Hasegawa et al., 2004a,b).

In the main acrylamide detoxification pathway of vertebrates, acrylamide is metabolized by GST to mercapturic acid and excreted in urine; whereas in the alternate pathway, acrylamide is metabolized by CYP, a phase I enzyme, to glycidamide, which may be mainly responsible for acrylamide-caused DNA damage (Friedman, 2003). Although CYP genes also exist abundantly in the C. elegans genome, only one CYP gene was upregulated and four genes were downregulated. This finding of many upregulated SDRs suggests that SDRs, not
CYPs, are the main phase I enzymes involved in acrylamide metabolism in *C. elegans*. Genes for other enzymes upregulated more than fourfold were casein kinase (Y38H8A.3), glycogen synthase kinase (gska-3, C36B1.10), nicotinamide adenine dinucleotide (reduced) (flavin oxidoreductase/12-oxophytodienoate reductase) (ZK742.4), peptide methionine sulfoxide reductase (F43E2.5), permease of the major facilitator superfamily (C35A5.3), carbon–nitrogen hydrolase (*nir-1*, ZK1058.6), zinc-binding oxidoreductase (F39B2.3), and reductases with a broad range of substrate specificities (R05D8.7). It is expected that these enzymes should play important roles in the body’s response to acrylamide exposure and metabolism.

Genes encoding MSPs were also more than twofold upregulated by 500 mg/l acrylamide exposure. The MSPs are sperm-specific proteins that bind one another to form the sperm cytoskeleton required for sperm motility (Italiano et al., 1999) and are also emitted as signals to promote oocyte maturation and ovulation (Miller et al., 2003). A huge number of MSP genes highly cluster in the *C. elegans* genome (WormBase, http://www.wormbase.org/), whose expression is regulated by DAF-2, a *C. elegans* homolog of insulin/IGF-1 (Halaschek-Wiener et al., 2005). This is one of the most studied hormonal pathways for controlling dauer formation, aging, and stress responses (Baumeister et al., 2006). In contrast, several *C. elegans* MSP genes were downregulated by exposure to the herbicide atrazine (Reichert and Menzel, 2005). Thus, the relationships of the MSPs with xenobiotics are unclear, although they appear to have some as yet unknown function in the oxidative stress response. Oxidative damage is one major threat to the integrity of mammalian sperm (Drevet, 2006).

About 50 *gst* genes are identified in the *C. elegans* genome (WormBase, http://www.wormbase.org/), and we constructed transgenic animals expressing eight GST proteins. The GST expression patterns of these eight transgenic animals were different from each other (Fig. 2). Some GSTs were expressed constitutively and the others expressed inducively. GSTs form a superfamily of phase II enzymes and exist in every organism from prokaryotic bacteria to humans (Vuilleumier and Pagni, 2003). Among the 18 *gst* genes upregulated more than twofold by 500 mg/l of acrylamide (Table 2), *gst-4* showed the highest induction profile of all in acrylamide-treated animals (× 24.78 compared with the control). Not only did the rate of transcription increase, but GST-4::GFP was detected over the whole animal body when treated with acrylamide (Fig. 2L, I).

The expression of *gst-4* was dramatically induced by such oxidative stressors as paraquat, juglon, plumbagin, hyperbaric oxygen treatment, or the endocrine active substances diethylstilbestrol and progesterone (Cutodia et al., 2001; Leiers et al., 2003; Link and Johnson, 2002; Reichert and Menzel, 2005; Tawe et al., 1998). Overexpression of *gst-4* conferred resistance on nematodes against oxidative stresses (Leiers et al., 2003).

Interestingly, acrylamide upregulated the two other genes *gst-2* and -3 closely located on the same chromosome as *gst-4* (Table 2). The gene *gst-4* lies on the complementary strand in relation to *gst-2* and *gst-3* as it shares the same 726-bp sequence in head to head fashion with *gst-2* (WormBase, http://elegans.swmed.edu/), and *gst-2* and *gst-3* on the same strand in head to tail fashion with 204 bp in between. Therefore, this result apparently indicates that the 726-bp sequence functions as a promoter for both *gst-4* and *gst-2*, albeit in an opposite orientation. Their expression patterns were, however, somewhat different from each other, as *GST-2* was constitutively and weakly expressed only in the mouth region compared with the strong, whole body expression by *GST-4* (Fig. 2J, j).

*C02D5.3* is reported to form three isoforms (C02D5.3a, b, and c in WormBase, http://www.wormbase.org/). Our result suggests that C02D5.3c expression may be regulated by a promoter located in the third intron of C02D5.3a and that it is constitutive and increased by exposure to acrylamide (Fig. 2H, h). Differences or similarities of transcriptional regulation, expression patterns, or functions of the three isoforms remain to be investigated.

In mammals, GSTs are regulated by the bZIP transcriptional factor Nrf1/Nrf2 in response to oxidative or electrophilic stresses (Nguyen et al., 2003). Nrf2 is, in turn, negatively regulated by the oxidative stress sensor Keap1 (Kobayashi et al., 2006). In *C. elegans*, the transcription factor SKN-1, which functions in the specification of the EMS blastomeres during early embryogenesis (Blackwell et al., 1994), is found to function similarly to Nrf proteins by inducing GCS-1 expression against oxidative stresses (An and Blackwell, 2003). We found that the genes for superoxide dismutase, glutamate cysteine ligase catalytic subunit (GCS), and one zinc-binding oxidoreductase (F39B2.3) were upregulated by acrylamide treatment and all contained SKN-1 binding sites in their predicted promoters. Interestingly, all examined GST promoters also contained multiple copies of putative binding sites for SKN-1. We obtained evidence that GST-4 expression was induced by acrylamide partly via SKN-1 (Fig. 3). SKN-1 regulates the expression of the phase II enzyme GCS-1 in the intestine, but not in the pharynx (An and Blackwell, 2003). Because the knockdown of *skn-1* (RNAi) arrested the GST-4 expression in most parts of the body except for the pharynx and body wall muscle, we might suggest that neither the pharynx nor body wall muscle is regulated by SKN-1. Because the knockdown of *gst-4* (RNAi) did not prevent GST-4 expression
in the pharynx either, whereas it did prevent that for the body wall muscle (Figs. 3D–F), we suggest that GST-4 expression in the body wall muscle is SKN-1 independent. We have yet to know why the GST expression in the pharynx was not blocked, although one might think that RNAi itself does not work in the pharynx.

The short-chain type dehydrogenase SDZ-8 (C55A6.5) is a member of phase I detoxification enzymes (Lindblom and Dodd, 2006) and reported to be zygotically regulated by SKN-1 (Robertson et al., 2004). This SDZ-8 and another three SDRs (C55A6.3 to C55A6.6) were upregulated by acrylamide (Table 2) and constitute one operon (Wormbase, http://www.wormbase.org/). These genes are also good candidates for being regulated by SKN-1 in response to oxidative stresses.

Although we demonstrated here that the GFP signal response to acrylamide shows concentration and time dependency, the timing wherein we can distinguish the GFP signal from background in these experiments is by no means the time of actual GFP expression, because we can visually observe the GFP signal by fluorescence microscopy after 30 min in young adult gfp::gst-4 transgenic animals exposed to 500 µg/l, 50 mg/l, and 500 mg/l of acrylamide (unpublished results). Therefore, we would expect the GFP signals to be detected much earlier with more sensitive equipment.

The advantages and usefulness of C. elegans as an organism of choice for toxicity (or safety) testing and other environmentally-related evaluations have been amply demonstrated by many researchers over the years (Boyd et al., 2003; Dengg and van Meel, 2004; Guven et al., 1999; Hasegawa et al., 2004b; Lagido et al., 2001; Leiers et al., 2003; Miwa et al., 1982; Mutwakil et al., 1997; Reichert and Menzel, 2005; Tabuse et al., 1989). Therefore, we would like to propose that the transgenic animals with GST::GFP reporter constructs such as these reported here be used as biosensors to detect the presence of xenobiotics in our foods or the environment.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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