

Identification of stress-responsive genes in *Caenorhabditis elegans* using RT-PCR differential display

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ABSTRACT

In order to identify genes that are differentially expressed as a consequence of oxidative stress due to paraquat we used the differential display technique to compare mRNA expression patterns in *Caenorhabditis elegans*. A *C.elegans* mixed stage worm population and a homogeneous larval population were treated with 100 mM paraquat, in parallel with controls. Induction of four cDNA fragments, designated L-1, M-47, M-96 and M-132, was confirmed by Northern blot analysis with RNA from stressed and unstressed worm populations. A 40-fold increase in the steady-state mRNA level in the larval population was observed for the L-1/M-47 gene, which encodes the detoxification enzyme glutathione S-transferase. A potential stress-responsive transcription factor (M-132) with C₂H₂-type zinc finger motifs and an N-terminal leucine zipper domain was identified. The M-96 gene encodes a novel stress-responsive protein. Since paraquat is known to generate superoxide radicals *in vivo*, the response of the *C.elegans* superoxide dismutase (SOD) genes to paraquat was also investigated in this study. The steady-state mRNA levels of the manganese-type and the copper/zinc-type SODs increased 2-fold in the larval population in response to paraquat, whereas mixed stage populations did not show any apparent increase in the levels of these SOD mRNAs.

INTRODUCTION

The *Caenorhabditis elegans* model system, with its rapid life cycle and short lifespan, has been widely employed in the study of oxidative stress, aging and senescence (1–5). The free radical theory of aging postulates a correlation between free radical generation, environmental conditions, levels of antioxidant enzymes in the cell and the aging process (6,7). Substantial evidence supporting this theory has been obtained in studies involving long-lived *C.elegans* and *Drosophila* genetic mutant models (4,8–10).

Paraquat (1,1'-dimethyl-4,4'-bipyridinium) dichloride is a herbicide commonly used to generate oxidative stress *in vivo* by redox cycling. It is metabolically reduced to the stable paraquat

radical (PQ⁺) in a NAD(P)H-dependent reaction catalyzed by NADPH-cytochrome P-450 reductase. The paraquat radical in turn reduces molecular oxygen, producing the superoxide radical (O₂⁻) (11–13). Several components may contribute to the cytotoxicity of paraquat, which could result from depletion of cellular NAD(P)H leading to a shift to the oxidized forms of glutathione (11). However, there is strong evidence that the cytotoxicity of paraquat is mediated by reactive oxygen species (ROS), which inflict cellular injury by damaging biological macromolecules (14–17). Supporting this theory, elevated levels of antioxidant enzyme activity in response to paraquat have been demonstrated in several organisms, including bacteria (18–23), plants (24), *Drosophila* (25,26) and *C.elegans* (10).

Paraquat has been used in a variety of studies examining responses to oxidative stress and the mechanism of aging in *C.elegans*. Ishii *et al.* (27) isolated a paraquat-sensitive mutant of *C.elegans* (*mev-1*), which is hypersensitive to oxygen and possesses about half the SOD activity of the wild-type strain. Conversely, Vanfleteren (10) observed that wild-type *C.elegans* strains were less resistant to paraquat than long-lived *age-1* mutants, which have elevated levels of catalase and SOD in old age. Hartman *et al.* (28) demonstrated that *C.elegans* development is inhibited by paraquat at a rate inversely proportional to lifespan. These data all emphasize the important role of oxidative stress in the aging process. The responses of *C.elegans* to oxidative stress have therefore attracted considerable attention over past years (4,5,27–32).

The mRNA differential display RT-PCR method provides a sensitive and flexible approach to the identification of novel individual differentially transcribed genes in different sets of eukaryotic cells (33,34). With 8 × 3 primer combinations this method identifies ~1500 individual mRNA species (33,34). The *C.elegans* haploid genome contains 10⁸ base pairs, <4% of the human genome. Under optimal conditions the 16 000 genes in *C.elegans* can theoretically be screened using 80 × 3 different primer combinations.

To further characterize molecular stress responses this method was selected to identify proteins involved in cellular defense against oxidative stress. In order to identify genes that are responsive to paraquat a subset of *C.elegans* cDNA fragments was amplified by differential display RT-PCR using 8 × 3 primer combinations following induction with paraquat, in parallel with

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controls. In this study we also investigated the effect of paraquat on the mRNA levels of the Mn and Cu/Zn superoxide dismutases in two *C.elegans* populations.

MATERIALS AND METHODS

Nematode propagation

Wild-type *C.elegans* worms N2 (var. Bristol) were grown in 9 cm Petri dishes under atmospheric conditions at 22°C in nematode growth medium (NGM) and fed with *Escherichia coli* strain OP50 according to a standard protocol (35). Age-synchronous worm populations were initiated from eggs following alkaline hypochlorite treatment of gravid adults as described (36). Synchronized cultures in NGM plus OP50 were arrested 36 h following egg hatching, when L4 larval stages were predominant. Worm populations were generally cleaned of bacteria by 30–35% (v/v) sucrose flotation (36).

Treatment with paraquat

In an initial experiment to determine survival of *C.elegans* worms in the presence of paraquat within the time range cleaned worm cultures in NGM were incubated with serial dilutions of paraquat (methyl viologen; Sigma) in a 96-well microtitre plate (Greiner), with 10–20 worms/well. Survival, based on motility, was monitored (data not shown). In a final paraquat concentration of up to 200 mM <20% of the worms were viable after 1 h. In 100 mM paraquat, however, the survival rate after 2 h was >90% and this concentration and time were chosen as the experimental parameters for this study.

Following cleaning of worm cultures and prior to treatment with paraquat the worms were allowed to recover for 1 h at 22°C in NGM. This treatment also allowed gut bacteria to be digested. A sample of the worms ($\sim 3 \times 10^6$ in 25 ml NGM) was then treated with paraquat at a final concentration of 100 mM. Controls consisted of an equal number of worms in medium without paraquat. After incubation for 1 h worms were quickly harvested by pelleting at 1300 g and washed twice in cold 0.1 M NaCl. The samples were then snap frozen as pellets in liquid nitrogen for subsequent analyses.

Total RNA isolation and mRNA differential display

Treated and untreated worms were crushed under liquid nitrogen using a ceramic mortar and pestle. The fine particles were resuspended in TRIzol reagent (Gibco BRL) and total RNA was prepared according to the manufacturer's instructions.

Differential display was performed using the RNAimage Kit 1 (GenHunter) essentially according to the manufacturer's instructions, but with some modifications. Briefly, 50 µg total RNA were treated with DNase I using the MessageClean Kit (GenHunter). Following phenol extraction and ethanol precipitation first strand oligo(dT)-primed cDNA synthesis was performed in 20 µl reactions consisting of 0.2 µg freshly diluted total RNA, 25 mM Tris-HCl, pH 8.3, 37.6 mM KCl, 1.5 mM MgCl₂, 5 mM DTT, 20 µM dNTP mix and 0.2 µM one-base-anchored oligo(dT) primer (H-T₁₁M = HT₁₁G, AAGCTTT₉G; HT₁₁A, AAGCTTT₉A; HT₁₁C, AAGCTTT₉C). Reaction mixtures were incubated at 65°C for 5 min and 37°C for an initial 10 min, after which 100 U Moloney murine leukemia virus (MMLV) reverse

transcriptase were added. After an additional incubation for 60 min the reactions were heated at 75°C for 5 min, then cooled to 4°C.

For PCR amplification 2 µl cDNA template were used directly in a 20 µl reaction mixture consisting of 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 2 µM dNTP mix, 0.2 µM random (H-AP) primer (HAP1, AAGCTTGATTGCC; HAP2, AAGCTTCGACTGT; HAP3, AAGCTTTGGTCAG; HAP4, AAGCTTCTCAACG; HAP5, AAGCTTAGTAGGC; HAP6, AAGCTTCGACCAT; HAP7, AAGCTTAACGAGG; HAP8, AAGCTTTTACCGC), 10 µCi [α -³⁵S]dATP (Amersham Buchler), 1 U AmpliTaq DNA polymerase (Perkin Elmer) and 0.2 µM of the same anchored oligo(dT) primer used in first strand cDNA synthesis. Subsequent to an initial 30 s denaturation at 94°C, 40 amplification cycles were performed as follows: 30 s at 94°C, 2 min at 40°C, 30 s at 72°C, then a final 5 min at 72°C and a 4°C hold. All reactions were performed in thin-walled PCR tubes (BioGen) in a water bath thermocycler (Autogene II).

Gel electrophoresis, subcloning and sequencing

Following PCR amplification 2 µl formamide loading dye (95% formamide, 10 mM EDTA, pH 8.0, 0.09% xylene cyanol FF and 0.09% bromophenol blue) were mixed with 2.5 µl each sample. These were heated for 2 min at 95°C and loaded onto a 6% urea, TBE, polyacrylamide sequencing gel. Electrophoresis was carried out at 60 W constant power for 3.5 h. The gel was then recovered, transferred onto a piece of 3 MM Whatman paper and vacuum dried at 80°C for 90 min. After 24 h exposure to a BioMax autoradiographic film (Kodak) the film was developed and re-orientated on the gel. Fragments of interest were excised with a sharp scalpel and eluted by boiling in 20 µl sterile distilled water for 10 min. Eluted DNA (4 µl) was then re-amplified directly under identical conditions as in the initial PCR, with the corresponding H-T₁₁ and H-AP primers but in the absence of radioactivity.

Amplified PCR products were cloned using the TA™ Cloning Kit (Invitrogen) and sequences were determined by the dideoxy chain termination method (37) using the Sequenase Kit v.2.0 (US Biochemical). Six clones from each initial fragment were sequenced. Sequences which recurred at least four times were considered for further analysis. The GenBank was searched using the ACEDB and the *C.elegans* Blast server programs (38).

Northern blotting

Total RNA samples (30 µg) extracted from paraquat-treated and untreated worms were resolved by 1% agarose-formaldehyde gel electrophoresis and transferred onto a nylon membrane (Boehringer Mannheim) by capillary elution according to a standard protocol (39). In order to ensure an equivalent quantity of RNA on the blots from both treated and untreated samples a fragment of the *C.elegans* collagen cDNA (CeCoL-12, nt 139–569; GenBank accession no. X51622) (40) was labeled with [α -³²P]dATP (Amersham Buchler) by random primed labeling (Boehringer Mannheim) and used to probe the membranes. Hybridizations were at 42°C for 18 h in a formamide hybridization solution [50% formamide, 5× SSC (0.75 M NaCl, 0.075 M Na citrate, pH 7.0), 1% SDS, 5× Denhardt's reagent, 200 µg/ml denatured salmon sperm DNA]. Membranes were washed twice at 65°C in 2× SSC and twice in 1× SSC at 65°C, then subjected to autoradiography (BioMax autoradiographic film; Kodak) for 12–36 h. After developing the film the signals of treated and untreated worm

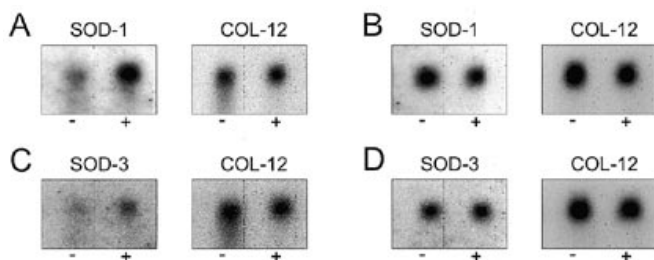


Figure 1. Northern blot analysis of *C.elegans* total RNA (30 µg) showing differential expression of genes in paraquat-treated (+) and untreated (-) worm populations. The left-hand panels (A and C) show autoradiograms of blots with larval (L) worm RNA. The right-hand panels (B and D) show autoradiograms of blots with RNA from a mixed (M) worm population. The blots labeled SOD1 (A and B) were probed with the CeCu/ZnSOD cDNA (GenBank accession no. L20135) (4) and those labeled SOD3 (C and D) with the CeMnSOD cDNA (DDBJ accession no. 12984) (41). Autoradiograms of the corresponding Northern blots probed with a *C.elegans* collagen cDNA (CeCoL-12, nt 139–569; GenBank accession no. X51622) (40) as a control are also shown in each panel.

RNAs were found to be of similar intensities. The membranes were then stripped of radioactivity by treating them with 0.1 N NaOH at room temperature for 2–5 min, followed by an extensive rinsing in 1× SSC.

Cloned cDNA fragments were excised from the vector by *Hind*III digestion, purified, radiolabeled with [α - 32 P]dATP and used as probes in Northern blots on the stripped membranes as described above. Following autoradiography the intensities of the hybridization signals from treated and untreated worms were compared visually and quantified by densitometry using a PhosphorImager (Molecular Dynamics).

Isolation of cDNA clones

cDNAs of the transcripts under investigation were isolated by RT-PCR. Based on the available sequences of cosmids encoding these cDNA fragments and the predicted coding regions two upstream sense and two downstream antisense primers were designed for each transcript. Oligo(dT) reverse transcribed cDNA was then amplified by PCR using the different primer combinations for each transcript. The cDNA clones were then blunt-end ligated into the *Eco*RV site of the pBluescript KS plasmid vector (Stratagene) and their complete double-stranded nucleotide sequences were determined.

RESULTS

Superoxide dismutase response to paraquat

Prior to the differential display experiments the response of *C.elegans* to paraquat was investigated with respect to SOD mRNA levels. The *C.elegans* cDNAs encoding Mn-containing superoxide dismutase (SOD3, CeMnSOD; DDBJ accession no. 12984) (41) and the Cu/Zn-type superoxide dismutase (SOD1, CeCu/ZnSOD; GenBank accession no. L20135) (4) were used as probes in Northern blot experiments with total RNA from paraquat-treated and control worms (Fig. 1). A 2-fold increase in the levels of mRNA from both genes was observed in larval but not in mixed stage populations (Table 1).

Table 1. Comparison of the steady-state mRNA levels in paraquat-treated and untreated worms

| cDNA | Fold induction | |
|-------|----------------|-------|
| | Larvae | Mixed |
| SOD1 | 2 | 1 |
| SOD3 | 2 | 1 |
| M-47 | 42 | 1.5 |
| M-96 | 3 | 5 |
| M-132 | 3.5 | 4 |

The relative intensities of signals (fold increase in signal) in autoradiograms of Northern blots, using SOD cDNAs or cDNA fragments isolated as a result of the mRNA differential display experiment as probes, are shown. Blots were subjected to densitometric analysis using a PhosphorImager and ImageQuant software (Molecular Dynamics).

Identification of paraquat-responsive genes by mRNA differential display

In an attempt to identify additional genes in *C.elegans* that are responsive to paraquat at a sublethal dose we performed the mRNA differential display (DD-RT-PCR) method of Liang and Pardee (33). Two independent differential display experiments were performed with RNA from a mixed stage population of control (-) and paraquat-treated (+) *C.elegans* worms, using three one-base-anchored oligo(dT) primers and eight random primers.

In previous studies on oxidative stress in *C.elegans* juvenile worms were shown to be more resistant to paraquat (10) and plumbagin (30) than older worms. Based on this observation we also performed differential display on RNA from a synchronized *C.elegans* larval population following exposure to 100 mM paraquat with identical primer sets.

Complex patterns, as exemplified by the autoradiograms (Fig. 2), were observed with the different primer combinations used. Under these conditions ~50 mRNA species detected on the differential display gel appeared to be reproducibly up-regulated in response to paraquat. The expression of other genes was either stable or appeared to be down-regulated. These were not considered for further analysis. Bands of interest, those with signals showing the greatest difference in intensity in control lanes relative to the test lanes, were eluted from the gel, re-amplified, ligated into a plasmid vector and their double-stranded sequences determined. Fragments as large as 800 bp were identified and those <200 bp were excluded from the study. In order to reduce redundancy sequences of the differentially displayed clones were compared amongst each other, since a single mRNA can be amplified by different primer pairs. One larval clone (designated L-1) and one clone from the mixed worm population (designated M-47) were amplified by the same primer pair and were identical. Also, only nucleotide sequences which recurred (at least four times out of six) were chosen for further analysis. Based on these criteria (band reproducibility on two differential display gels, fragment length and recurring nucleotide sequences of amplified cDNA fragments) the number of fragments to be analyzed was reduced to three and these candidates were used in subsequent experiments.

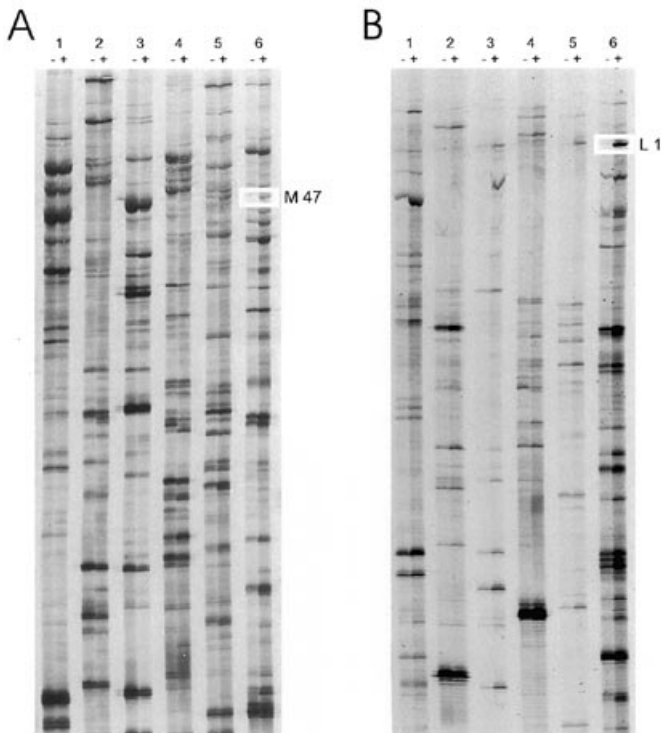


Figure 2. mRNA differential display patterns. (A) Representative pattern of a differential display autoradiogram of mRNA from a paraquat treated (+) or untreated (-) *C.elegans* mixed stage population (M), using the RNAImage Kit 1 (GenHunter) with six combinations of primer sets made up of H-T₁₁G, H-T₁₁A, H-T₁₁C and two arbitrary 12mers, H-AP2 and H-AP3. Heat-denatured cDNAs from control worms (-) and paraquat-treated worms (+) were resolved on a 6% polyacrylamide-urea sequencing gel, vacuum dried and autoradiographed. Lanes 1–3, H-AP2 with H-T₁₁G, H-T₁₁A and H-T₁₁C respectively; lanes 4–6, H-AP3 with H-T₁₁G, H-T₁₁A and H-T₁₁C respectively. The M-47 cDNA fragment is boxed. (B) Representative pattern of a differential display autoradiogram of RNA from paraquat-treated (+) and untreated (-) *C.elegans* larval stages (L) using the same primer pairs as in (A). The L-1 cDNA fragment is boxed.

Sequence comparisons with the *C.elegans* database

Over 75% of the *C.elegans* genome has been sequenced to date (42). This resource was exploited in our study to identify genes responsive to stress due to paraquat in *C.elegans*. The nucleotide sequences of our three selected differentially displayed cDNA

clones showed 100% identity to regions within characterized *C.elegans* cosmids (Table 2). All three cDNA fragments contain the 5'-primer used in the differential display experiment and are 3'-polyadenylated. Only one of these cDNA fragments (M-132) lacks a typical (AATAAA) polyadenylation signal sequence.

Nucleotide sequences of the differentially expressed cDNA clones

The nucleotide sequences of the entire coding and 3'-untranslated regions of the cDNAs corresponding to two of the differentially expressed clones (M-47 and M-132) were determined following PCR amplification and cloning. The M-47 cDNA was determined to be 726 bp, with a 207 amino acid open reading frame (Fig. 3A). The M-96 partial cDNA clone which was isolated is 683 bp and has a 101 amino acid open reading frame, with a 377 bp 3' non-coding region (Fig. 3B). The M-132 cDNA is 1070 bp and possesses a 293 amino acid open reading frame (Fig. 3C).

A BLAST search of the *C.elegans* database and GenBank indicated that the deduced amino acid sequence of the open reading frame of the L-1 (M-47) cDNA has a high level of homology to glutathione S-transferases (GSTs). Interestingly, based on the corresponding cosmid sequence, this gene lies on the complementary strand, downstream of two other genes encoding putative *C.elegans* GSTs which are in a tail-to-head orientation. This organization suggests that these three GST transcripts are possibly coordinately regulated from one promoter and are divergently transcribed. The size of the L-1 (M-47) transcript on the Northern blot (0.75 kb) corresponds to the size of the amplified full-length cDNA, confirming the predicted GST coding region in the corresponding cosmid Cek08f4. The nucleotide sequence also confirmed the predicted splicing profile of the gene. The M-132 clone overlaps a potential zinc finger protein coding region identified on *C.elegans* cosmid Cef40f8 and EST CEESC82F. Here also the size of the mRNA observed in the Northern blot (1 kb) and the sequence of the cDNA confirmed the predicted splicing pattern of the gene. Analysis of the deduced amino acid sequence of the open reading frame of the M-132 cDNA showed that the protein belongs to the C₂H₂ family of zinc finger proteins. This protein possesses three classical zinc finger motifs [C-x(2-4)-C-x(12)-H-x(3-5)-H]. In addition, it also possesses an N-terminal leucine zipper domain, with the classical configuration [L-(x)₆]₄. The deduced amino acid sequence of the open reading frame of the M-96 partial cDNA clone shows no significant homology with any known protein in the database.

Table 2. A comparison of the *C.elegans* cDNA sequences identified by the differential display method

| Fragment | Length of fragment isolated by differential display (bp) | <i>C.elegans</i> cosmid | Gene product | cDNA (bp) |
|-------------|--|---|------------------------------------|-----------|
| M-47 (=L-1) | 560 | k08f4 (+ strand) (21626–22485) | Glutathione S-transferase | 726 |
| M-96 | 480 | t05g5 (- strand) (6758–7886) | Unidentified | >683 |
| M-132 | 400 | k07c10/f40f8 (- strand) (132–894/25107–26385) | Zinc finger/leucine zipper protein | 1070 |

The lengths of the fragments isolated by the differential display method and their locations on the corresponding cosmids are shown, as well as the lengths of the cDNAs subsequently isolated from a cDNA library using the differential display fragments as probes. The corresponding gene products, based upon sequence homology, are also indicated.

| | | |
|----------|---|-----|
| A | M-47 (CeGST1) | |
| | M P N Y K L L Y F D A R A L A E P I R I M F A | 23 |
| | M L N V P Y E D Y R V S V E E W S K L K P T T | 46 |
| | P F G Q L P I L Q V D G E Q F G Q S M S I T R | 69 |
| | Y L A R K F G L A G K T A E E E A Y A D S I V | 92 |
| | D Q Y R D F I F F F R Q F T S S V F Y G S D A | 115 |
| | D H I N K V R F E V V E P A R D D F L A I I N | 138 |
| | K F L A K S K S G F L V G D S L T W A D I V I | 161 |
| | A D N L T S L L K N G F L D F N K E K K L E E | 184 |
| | F Y N K I H S I P E I K N Y V A T R K D S I V | 207 |
| B | M-96 partial cDNA | |
| | F S W N L K L L C S I R R T K V S E L I S K T | 23 |
| | S F S F L F S L Y L T I L L P M F A F F I M S | 46 |
| | S R S C I Q F P V F Y R S R I F S S S G L L W | 69 |
| | Y I N K A H E L Y A S H L T A N F G N N I Y I | 92 |
| | L V L C H L T V C * | 101 |
| C | M-132 | |
| | M S F L N N D F G S P P A T S S P P T T M P K | 23 |
| | L P T I Q D M L N N I G A S T V N ● M Q P N P | 46 |
| | Y ● M Q N Q I P ● P V P N L P ● N P F L H L N | 69 |
| | P A I S Q E I I Q Q F I A M S F N T P N V L A | 92 |
| | S I A N M G D D E G P S C N P K M R R G D L L | 115 |
| | K S V S M D S T E D P P S I T L D N N G D M I | 138 |
| | V P N N D K E G W C R N K K Y I E Q T E N G Y | 161 |
| | M C T V C R K V Y G R Y N S V S Y H V T I Y H | 184 |
| | R N P P I K C N V P N C Q F T T R E A R Y I H | 207 |
| | F H K N Y R H G I P L P E S I D Q G S R K C P | 230 |
| | H C R H V S K S P A M L E K H I R R H Q I K D | 253 |
| | G L S N I N E A I R E R T S T I C D E A M E I | 276 |
| | E P A E T E V D P I E T K P R S C T L * | 293 |

Figure 3. The deduced amino acid sequences of the three *C.elegans* transcripts up-regulated in response to paraquat, identified by the differential display RT-PCR method. (A) The deduced amino acid sequence of the *C.elegans* L-1/M-47 (GST) cDNA. (B) The *C.elegans* M-96 cDNA product partial deduced amino acid sequence. (C) The deduced amino acid sequence of the *C.elegans* M-132 cDNA product. The leucine residues in the N-terminal zipper domain are represented by shaded circles. The cysteine and histidine residues which comprise the three zinc fingers in the C-terminal domain are boxed and circled respectively.

Northern blot analysis using differentially displayed cDNA fragments

In order to confirm specific up-regulation of the transcripts identified and isolated by differential display Northern blot analyses of total RNA from both larval and mixed stage worm populations were performed. Figure 4 shows autoradiograms of Northern blots of total RNA from paraquat-treated (+) and control (-) *C.elegans* worms probed with the respective differential display fragments. All three fragments chosen (L-1/M-47, M-96 and M-132) showed a pronounced increase in mRNA levels following stress in the Northern blot experiments, confirming our differential display results. A >40-fold increase in the mRNA steady-state level of the L-1 (M-47) transcript was observed in the larval population in response to exposure to paraquat. However, in the mixed worm population this transcript was induced just

1.5-fold, as shown in Table 1. These results demonstrate that the L-1/M-47 transcript is expressed at very low levels in unstressed larval worms, whereas this gene is expressed at a higher level in adult worms prior to addition of paraquat. The M-96 and M-132 transcripts were induced >3-fold in both larval and mixed stage populations. This indicates that the basal level of constitutive expression as well as inducibility of these genes in response to paraquat are comparable in both the juvenile and mature stages.

DISCUSSION

With an interest in understanding how an organism responds to adverse environmental challenges we have studied the nematode response to paraquat. Stress results in changes in the cellular program, which involves significant transcriptional alterations aimed at increasing the chances of survival. Increased levels of

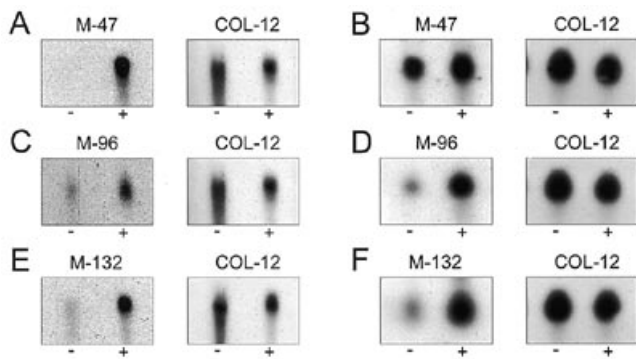


Figure 4. Northern blot analysis of *C.elegans* total RNA (30 μ g) showing differential expression of genes in paraquat-treated (+) and untreated (-) worm populations. The left-hand panels (A, C and E) show autoradiograms of blots with larval (L) worm RNA. The right-hand panels (B, D and F) show autoradiograms of blots with RNA from a mixed (M) worm population. The blots labeled M-47 (A and B), M-96 (C and D) and M-132 (E and F) were probed with the M-47, M-96 and M-132 cDNA fragments respectively. Autoradiograms of the corresponding Northern blots probed with a *C.elegans* collagen cDNA (CeCoL-12, nt 139–569; GenBank accession no. X51622) (40) as a control are also shown in each panel.

defense and repair mechanisms would be expected. Both prokaryotes and eukaryotes have been described as responding to oxidative stress by increased biosynthesis of defense enzymes. In prokaryotes transcriptional changes resulting from exposure to oxidative stress have been extensively characterized (18,43–46). Mechanisms controlling the responses of eukaryotic cells to oxidative stress have not been fully elucidated, but it has been suggested that a similar type of regulation exists which coordinately influences all of the enzymes with radical scavenging and repair activities which are required for defense against stress (30). This induction must be triggered either directly or indirectly by ROS and ROS-induced damage.

Research has begun to elucidate the eukaryotic antioxidant defense proteins (47–50) and possible transcription factors regulating this response, such as AP-1, NF- κ B and YAP-1/TRX2 (51–53). ROS have been shown to act as second messengers by influencing NF- κ B and AP-1 (54). In this study the *C.elegans* model system was used to investigate responses to paraquat-induced stress in order to identify stress-responsive genes encoding defense proteins. The RT-PCR differential display experiments resulted in identification of a detoxification enzyme and a transcription factor which are major components of this response. These observations also indicate that this experimental system is suitable for identification of specific genes activated in response to oxidative stress.

The GSTs are a family of essential detoxifying enzymes that catalyze conjugation of glutathione to a variety of electrophiles. GSTs have been shown to be involved in detoxification of a variety of xenobiotics which include paraquat, 3-methyl cholanthrene and plumbagin. Lipid hydroperoxides, products of peroxidation due to free radical attack on lipid membranes, are also substrates for these enzymes. The essential role of GSTs in the maintenance of cellular integrity has been clearly documented and therefore the induction of a potential GST (L-1/M-47) in response to paraquat, which was observed in these experiments, is a comprehensible consequence. Consistent with this, an increase in the level of GST enzyme activity following treatment with paraquat has been

shown in *Drosophila* (26) and cultured Vero cells (55). Induction of the L-1/M-47 cDNA in our experiments in *C.elegans* was dramatically age dependent. Larval worms appear to constitutively express very low amounts of this enzyme and are capable of increasing the mRNA levels >40-fold following stress. Adult worms, on the contrary, appear to constitutively express higher levels of this enzyme and are either not capable of or do not require induction. However, there remains a formal possibility that the transcripts are present at high levels in the developing embryos within the adult worms. Since *C.elegans* possesses a large GST gene family, it will also be interesting to determine whether induction in larvae is limited to this particular GST class or whether it involves interaction with other members of the *C.elegans* GST gene family. Interestingly, the induced GST (L-1/M-47) was found to lie within a cluster of putative GSTs in the *C.elegans* genome (cosmid Cek08f4).

Zinc finger proteins are cellular proteins which play a major role in transcriptional regulation by binding with high affinity to specific regions of DNA. In conjunction with leucine zipper domains these proteins may form hetero- or homodimers. In general these proteins activate transcription either constitutively or in a regulatory manner through post-translational modifications in response to external stimuli, although some may also be cell specific or developmentally regulated (56). Identification of a putative *C.elegans* transcription factor (M-132) with zinc finger and leucine zipper motifs and which is up-regulated in response to oxidative stress in these experiments implies that this factor may regulate further events in this stress-responsive cascade.

The superoxide dismutase (SOD) enzymes are a family of metalloenzymes responsible for quenching of the potentially deleterious effects of the superoxide radical. Three main types of superoxide dismutases catalyzing the same reaction and classified according to their metal content co-exist in aerobic organisms (57). Differential inducibility of these enzymes in response to oxidative stress has been demonstrated in several organisms (58,59). In this study induction of both the Cu/Zn- and the Mn-type SOD mRNAs was found to be age dependent. Similar results have been obtained at the enzyme activity level, rather than mRNA level, when lymphocytes from various age groups were treated with paraquat (60,61). Here SOD activity increased by 85% in lymphocytes from subjects 20–40 years old, whereas cells from subjects aged 65–79 showed only an 8% increase. Darr and Fridovich (30) also demonstrated an age-related increase in SOD activity in *C.elegans*. Young worms adapted to oxidative stress by increasing their SOD activity levels by up to 2-fold, whereas older nematodes did not induce SOD and suffered loss of viability. It appears, based upon our observations along with results from other investigators, that induction of SOD activity is due to changes in mRNA levels, either due to increased transcription initiation or mRNA stability.

This study allows a description of the program that is initiated following exposure of an organism to paraquat-induced stress. Changes in the levels of known antioxidant enzymes were documented and new and novel defense proteins, which are significantly induced in response to stress due to paraquat, were identified. Further studies on the kinetics and dose-responsiveness of these changes are required, as well as the effect of changes in oxygen tension. Recently developed methods, such as serial analysis of gene expression (SAGE) (62), will be applied. Also, the role of the M-96 gene product and other as yet uncharacterized defense proteins which have been identified by this RT-PCR differential display method require further investigation.

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