Cadmium-regulated Genes from the Nematode Caenorhabditis elegans
IDENTIFICATION AND CLONING OF NEW CADMIUM-RESPONSIVE GENES BY DIFFERENTIAL DISPLAY*

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The transition metal cadmium is a pervasive and persistent environmental contaminant that has been shown to be both a human toxicant and carcinogen. To inhibit cadmium-induced damage, cells respond by increasing the expression of genes encoding stress-response proteins. In most cases, the mechanism by which cadmium affects the expression of these genes remains unknown. It has been demonstrated in several instances that cadmium activates gene transcription through signal transduction pathways, mediated by protein kinase C, cAMP-dependent protein kinase, or calmodulin. A codex is that cadmium should influence the expression of numerous genes. To investigate the ability of cadmium to affect gene transcription, the differential display technique was used to analyze gene expression in the nematode Caenorhabditis elegans. Forty-nine cDNAs whose steady-state levels of expression change 2–6-fold in response to cadmium exposure were identified. The nucleotide sequences of the majority of the differentially expressed cDNAs are identical to those of C. elegans cosmids, yeast artificial chromosomes, expressed sequence tags, or predicted genes. The translated amino acid sequences of several clones are identical to C. elegans metallothionein-1, HSP70, collagens, and mRNAs. In addition, C. elegans homologues of pyruvate carboxylase, DNA gyrase, β-adrenergic receptor kinase, and human hypothetical protein KIAA0174 were identified. The translated amino acid sequences of the remaining differentially expressed cDNAs encode novel proteins.

The transition metal cadmium is considered to be a serious occupational and environmental toxin. Cadmium was ranked number 7 on the Agency for Toxic Substances and Disease Registry/Environmental Protection Agency “Top 20 Hazardous Substances Priority List” in 1997 (1). In addition, it is a frequently found contaminant at Superfund sites (1). Cadmium is used primarily in metal coatings, nickel-cadmium batteries, and pigments (2, 3). It is also continuously introduced into the atmosphere through the smelting of ores and the burning of fossil fuels (2, 3). It has been suggested that increased industrialization has resulted in higher levels of accumulated cadmium in humans (4). The primary routes of nonoccupational exposure in humans are via inhalation and via ingestion of cadmium-containing food (5). Humans are continuously exposed to cadmium and accumulate the metal throughout their lives in liver, lung, and kidney tissue (3, 6). Toxicological responses of cadmium exposure include kidney damage, respiratory diseases such as emphysema, and neurologic disorders (5, 7). Cadmium has been classified as a group 1 human carcinogen (8). It induces lung, kidney, prostate, and testicular cancers in rats and mice (5). Human epidemiological data suggest that it causes tumors of the male reproductive system and induces respiratory tumors (5, 9).

Intracellular damage associated with cadmium exposure includes protein denaturation, lipid peroxidation, and DNA strand breaks. Proposed mechanisms by which cadmium induces this damage involve (a) metal binding to reduced cysteine residues and (b) the generation of reactive oxygen species, possibly by lowering reduced glutathione levels (10–12). To prevent cadmium-induced intracellular damage, cells respond to metal exposure by inducing the transcription of genes that encode defense and repair proteins. These proteins (a) chelate the metal to prevent further damage, (b) remove reactive oxygen species, (c) repair membrane and DNA damage, and (d) renature or degrade unfolded proteins. Cadmium has been shown to affect the steady-state levels of the mRNAs encoding metallothionein (13), heme oxygenase (14), γ-glutamylecysteine synthetase (15), low and high molecular weight heat shock proteins (16), and ubiquitin (17). In addition, increases in superoxide dismutase, catalase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase activities are observed following cadmium exposure in cultured cells and whole animals (18, 19). The mechanism(s) by which this metal modulates the levels of expression of most of these genes remains unknown.

Cadmium-activated transcription may occur through specific metal-responsive upstream regulatory elements found in the promoters of cadmium-responsive genes. These may include MRE sequences, found in most metallothionein genes (20–22), or cadmium-responsive elements, as found in the human heme oxygenase gene (23). Cadmium may also affect gene expression by influencing signal transduction pathways. Cadmium affects the activities of protein kinase C, cAMP-dependent protein kinase, and calmodulin (24, 25). It has been suggested that cadmium-induced transcription of the proto-oncogenes jun and fos is mediated via protein kinase C and calmodulin (25). Thus, cadmium can modulate the activities of complex signal transduction pathways that in turn can influence the expression of a myriad of genes. However, relatively few cadmium-responsive genes have been identified. In addition, there is a paucity of information on the influence of cell-specific and developmental factors on metal-inducible gene expression. We have used the reverse transcriptase-PCR protocol of differential display to

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‡ The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s); EST, expressed sequence tag; HSP, heat shock protein.
identify new cadmium-responsive genes from the nematode *Caenorhabditis elegans*.

The nonparasitic nematode *C. elegans* provides an excellent model system for obtaining an integrated picture of cellular, developmental, and molecular aspects of the regulation of cadmium-responsive gene expression. The adult hermaphrodite is composed of 959 somatic cells but contains highly differentiated muscle, nervous, digestive, and reproductive systems (26, 27). The developmental and cellular biology of *C. elegans* is thoroughly understood in exceptional detail (26, 27). High levels of evolutionary conservation between *C. elegans* and higher organisms are observed in many signal transduction, gene regulatory, and developmental pathways (28–30). In addition, homologues of many of the proteins induced as part of metal-activated stress responses in vertebrates have been identified in *C. elegans*. These include metallothionein (31, 32), superoxide dismutase (33, 34), ubiquitin (35, 36), heat shock protein 70 (37), glutathione S-transferase (38), and catalase (39). With the exception of metallothionein, the effect of cadmium on the transcription of these *C. elegans* genes remains unknown. *C. elegans* also contains homologues to many of the signal transduction proteins that have been implicated in modulating the cellular/molecular response to metal exposure (40–43).

One of the major advantages in using *C. elegans* as a model system to identify new metal-responsive genes is the magnitudes of cDNA and genomic DNA sequence data currently available. The nematode genome is relatively small (~10⁹ bp), and an abundance of information is available on the genetic and physical maps of its chromosomes (44). Currently, sequencing of the entire *C. elegans* genome is >80% completed, and >50,000 ESTs have been cloned and sequenced. Megabases of genomic and cDNA sequence data are readily available through GenBank™, the *C. elegans* Genome Project (45), and the *C. elegans* cDNA Sequencing Project.²

We have identified 53 differentially expressed DNA fragments from a mixed stage population (i.e. a population at all stages of development) of cadmium-exposed *C. elegans*. Subsequent analysis confirms that the steady-state level of expression of 48 of these clones increases 2–6-fold following cadmium exposure. In addition, a single clone was isolated whose level of expression decreased ~2-fold. Sequence analysis has identified *C. elegans* cosmids, predicted structural genes, and ESTs that are identical to the differentially expressed mRNAs. Furthermore, the cadmium-responsive cDNAs are the products of 32 independent genes.

**EXPERIMENTAL PROCEDURES**

**Growth and Isolation of *C. elegans*—**The N2 strain of *C. elegans* was grown in liquid S medium (0.1 mM NaCl, 50 mM potassium phosphate, pH 6.0, 5 mg/ml at a density of 10⁶ cells/ml, 10 mM potassium citrate, 3 mM CaCl₂, 3 mM MgCl₂, 50 µM EDTA, 25 µM FeSO₄, 10 µM MnCl₂, 10 µM ZnSO₄, and 1 µM CuSO₄) using Escherichia coli OP50 as a food source (46). In experiments where nematodes were exposed to cadmium, the medium was supplemented with 100 µM CdCl₂ (32). *C. elegans* were grown in the presence of metal for 8 or 24 h at ~20°C. Nematodes were then collected following centrifugation at 800 × g for 5 min. Pellets were suspended in 50 mM NaCl containing 35% sucrose (final concentration), and viable nematodes were collected from the top of the solution following centrifugation at 1000 × g for 5 min at 4°C. Nematodes were then washed three times by suspension in M9 buffer (22 mM KH₂PO₄, 42 mM Na₂HPO₄, 85 mM NaCl, 1 mM MgSO₄) followed by sedimentation at 800 × g. Washed nematode pellets were finally suspended in a small volume of M9 buffer, rapidly frozen in liquid nitrogen, and stored at ~80°C.

**RNA Isolation—**Total RNA was isolated from mixed stage populations of *C. elegans* exposed to 100 µM CdCl₂ for 8 and 24 h and control, nonexposed nematodes. Frozen worms were first ground into a fine powder using a liquid nitrogen-cooled mortar and pestle. Powdered *C. elegans* (200 mg) were then homogenized in 2 ml of TRIzol (Life Technologies, Inc.). RNA was then collected from the aqueous phase following the addition of chloroform, precipitated by adding isopropyl alcohol, and then air-dried. The dried RNA pellet was then dissolved in diethyl pyrocarbonate-treated water. For some experiments, poly(A⁺) RNA was subsequently isolated using the Poly(A) Tract® mRNA isolation system following the manufacturer's instructions (Promega).

**mRNA Differential Display—**Differential display was performed following the protocol of Liang and Pardee (47). Briefly, 50 µg of total RNA isolated from either of three populations of *C. elegans*, controls, or those grown in the presence of cadmium for 8 or 24 h, was treated with 10 units of RNase-free DNase I (Boehringer Mannheim) in 10 mM Tris-Cl buffer, pH 8.3, containing 50 µM KCl and 1.5 mM MgCl₂. The DNA-free RNA was precipitated with ethanol and dissolved in diethyl pyrocarbonate-treated water. First-strand cDNAs were generated in reverse transcriptase reactions containing 0.2 µg of DNA-free total RNA, reverse transcriptase buffer (25 mM Tris-Cl, pH 8.3, 38 mM KCl, 1.5 mM MgCl₂, 5 mM dithiobisriothreitol), a 5 µM concentration of each dNTP, and a 1 µM concentration of one of four 3′-degenerate anchored oligo(dT) primers. The 3′-degenerate anchored oligo(dT) primers have the following sequences: T₃⁻MG, T₃⁻MA, T₃⁻MT, and T₃⁻MC, where M is 3-fold degenerate for G, A, and C. Primers were annealed to the RNA template by incubating the reaction mixture for 5 min at 65°C and then for 10 min at 37°C. First-strand cDNA synthesis was achieved following the addition of 100 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies) and incubation at 37°C for 50 min. The reaction was terminated by heating at 95°C for 5 min, which inactivates the reverse transcriptase.

Amplification of cDNA fragments was performed in 20-µl reactions. Each PCR mixture contained 2 µl of the products from one of the four reverse transcriptase reactions above and 18 µl of a solution containing Taq-PCR buffer (10 mM Tris-Cl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin), a 1 µM concentration of the same 3′-degenerate anchored oligo(dT) primer used in the first-strand synthesis reaction, four dNTPs (2 µM each), 10 µCi of [α-³²P]dATP (Amersham Pharmacia Biotech), 1 unit of AmpliTag DNA polymerase (Perkin-Elmer), and a 0.2 µM concentration of one of 20 5′-arbitrary decamers. The sequences of the 5′-arbitrary primers used in these reactions are presented in Table I. Reaction mixtures were subjected to 40 cycles of the PCR using the following parameters: denature at 94°C for 30 s, anneal at 42°C for 2 min, elongate at 72°C for 30 s. All PCRs were performed in duplicate. The amplified cDNAs produced from duplicate reactions of RNA isolated from control, 8-h treated and 24-h treated *C. elegans* were size-fractionated in parallel by polyacrylamide gel electrophoresis in 6% acrylamide, 8% urea gels.

Following electrophoresis, gels were dried onto Whatman 3MM paper and exposed to Kodak X-AR film for 24 h. Differentially expressed cDNAs were visualized by autoradiography. To isolate differentially expressed cDNA fragments, regions of dried gels corresponding to the cDNAs were excised. Gel slices were rehydrated in 100 µl of distilled water, and T 5 mini-agarose columns (PE Applied Biosystems) were used to isolate DNA from the gel slices. The amplified DNA from the gel slices was then subjected to agarose gel electrophoresis to confirm that DNA from the gel slices was differentially expressed.

### Table I

| Primer designation | Sequence         |
|--------------------|------------------|
| AP-3              | AGGTGACCGT       |
| AP-4              | GGTACTCCAC       |
| AP-6              | GCCATCGTAC       |
| AP-7              | CGGAGGAGAT       |
| AP-8              | GAGGTGCGG       |
| AP-9              | CGTGGCAGATA      |
| AP-10             | TACGAACTGAC      |
| AP-13             | AGTGGACCGAC      |
| AP-15             | AGGCGCTGTTG      |
| AP-18             | CGTACGCTAGG      |
| RT-1              | TACACCGG        |
| RT-2              | TGGATTTGGTC      |
| RT-3              | CTTTCTACC       |
| RT-4              | TTCCTGCCTTCC    |
| RT-5              | GGAACCAACTC      |
| RT-6              | AACTCGGTC       |
| RT-7              | TCGATACAGG      |
| RT-8              | TGGAAAAAGG      |
| RT-9              | TCGGTCATAG      |
| RT-10             | GGTACTAA      |

² Sequence data and information about the *C. elegans* cDNA Project can be obtained on the World Wide Web at [http://www.ddbj.nig.ac.jp/C_elegans/html/CE_INDEX.html](http://www.ddbj.nig.ac.jp/C_elegans/html/CE_INDEX.html).
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H₂O following a 10-min incubation at room temperature. The RNA was then extracted from the rehydrated gels by incubating at 100 °C for 1 min in tightly capped microcentrifuge tubes. cDNA was recovered by extracting in the presence of 0.3 M sodium acetate and 50 μg of glycogen (Boehringer Mannheim). The eluted cDNA was reamplified in a PCR reaction with the identical pair of primers used in the mRNA differential display reaction. PCR reaction conditions were similar to those above, except the concentration of the dNTPs was increased to 20 μM, and the [α-35S]dATP was omitted. Amplified cDNA fragments were resolved by gel electrophoresis using 1.5% agarose gels and then purified using QIAEXII kit (QIAGEN).

**Southern and DNA Sequence Analysis**—Gel-purified cDNAs were directly inserted into the T-A cloning vector pGEM-T (Promega). DNA inserts were subsequently sequenced using T7 and SP6 primers by the dideoxynucleotide chain termination procedures of Sanger et al. (48) (U.S. Biochemical Corp. Sequenase Kit, version 2.0).

**Computer Analysis**—Analysis of cDNA sequence data including sequence comparisons, alignments, and assembly of cDNA sequences were performed using PC/GENE-Intelli-Genetics software. BLAST sequence comparisons, alignments, and assembly of cDNA sequences using the nonredundant, *C. elegans* genome and *C. elegans* EST data bases. For some sequence analysis, the "*C. elegans* data base" (CaeDB) software was used (50). Predicted *C. elegans* genes were identified by the *C. elegans* Genome Project using the GENEFINDER program (51).

**Northern Blot Analysis**—Samples of total RNA (20 μg) or poly(A⁺) RNA (2 μg) were denatured in a 2.2 M formaldehyde, 50% (v/v) formamide buffer and then subjected to denaturing gel electrophoresis on a 1.5% agarose gel. Size-fractionated RNAs were then transferred to Nytran membrane (Schleicher and Schuell). Membranes were probed with 32P-labeled cDNA fragments of the differentially expressed mRNAs. cDNAs to be used as probes were generated by the PCR from the cloned DNA fragments recovered from differential display gels, as described above. cDNAs were labeled with [α-32P]dCTP (Amersham Pharmacia Biotech) by random-primed labeling. Membranes were hybridized in 6× SSC (1× SSC: 0.15 M sodium chloride, 15 mM sodium citrate, pH 7.0, 1.25× Denhardt’s solution, 0.5% SDS, 300 ng of denatured sonicated salmon sperm DNA, and heat-denatured probe at 42 °C for 16 h. Following hybridization, membranes were washed at a high stringency of 0.3× SSC, 0.1% SDS. The amount of probe hybridizing to the DNA was determined by PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA). After autoradiography, the membrane was exposed to X-ray film (DuPont) at −70 °C. The intensity of the autoradiographic signal was determined by densitometric analysis with Gel Pro-Analyzer software (Media Cybernetics, Inc., Silver Spring, MD). Membrane hybridization intensities were determined by ImageQuant analysis (Molecular Dynamics). Steady-state levels of mRNA expression were all normalized to that of the constitutively expressed myosin light chain mRNAs (32, 52).

**Reverse Northern Dot-blot Analysis**—Changes in the steady-state levels of differentially expressed mRNAs in *C. elegans* following cadmium exposure were also determined by reverse Northern dot-blot analysis by the modified procedure of Zhang et al. (53). Brieﬂy, differentially expressed cDNAs that were previously cloned into pGEM-T were amplified using primers that anneal to the T7 and SP6 RNA polymerase binding sites, which flank the cDNA insert. cDNAs were amplified and subsequently purified using PCR spin columns (QIAGEN). Approximately 100 ng of each amplified cDNA was denatured with mixing by 0.1 N NaOH (final concentration) and incubating at 100 °C for 5 min. The solution was neutralized following the addition of 3× SSC (final concentration), and then the volume was adjusted to 700 μl with distilled H₂O. 200 μl of each sample was applied to one of three nitro membranes in a Bio-Dot microfiltration apparatus (Bio-Rad). Membranes were then baked for 30 min at 80 °C under vacuum. As positive and loading controls, 100 ng of mtl-1 cDNA and myosin light chain DNA were also applied to each membrane, respectively.

**RESULTS**

**Effect of Cadmium on Gene Expression**—The level of the *C. elegans* mtl-2 mRNA was measured by Northern blot analysis to confirm that the cadmium exposure protocol outlined above affects gene expression (32). A 32P-labeled oligonucleotide probe that is specific for the 3′-end of the mtl-2 mRNA was hybridized to a membrane that contained RNA prepared from control *C. elegans* or nematodes exposed to 100 μM CdCl₂ for 24 h (Fig. 1). The steady-state level of mtl-2 mRNA increased in response to cadmium exposure to that previously reported (32). This verified that the cadmium treatment protocol alters gene expression in *C. elegans* and can be used for the differential display analysis.

**Identification of Cadmium-responsive Genes by Differential Display**—mRNA expression patterns of nontreated *C. elegans* and those exposed to cadmium for 8 and 24 h were compared by mRNA differential display in order to identify new genes whose transcription is regulated by cadmium. A total of 20 5′-arbitrary decamers, including five that have sequences that are homologous to the mtl-1 cDNA, were used. Each of the 20 decamers was paired with one of four 3′-degenerate anchored oligo(dT) primers and used to amplify cDNAs prepared from control and cadmium-treated *C. elegans*. All amplification experiments were performed in duplicate using RNA prepared from independently treated populations of *C. elegans*. This generated a total of 480 separate reactions: three populations of cDNA amplified using 80 combinations of primers, in duplicate.

**To bias against isolating differentially displayed cDNAs that are "false positive" (54), only cDNAs whose level of expression were affected by cadmium in duplicate experiments were selected for further analysis. In addition, cDNA fragments that have altered levels of expression in both the 8- and 24-h cadmium-treated samples were selected. Representative data are presented in Figs. 2 and 3. A total of 75 differentially expressed cDNA fragments were identified and excised from the gels. Of the cDNAs that were selected for further evaluation, the majority showed an increase in band intensity as a result of cadmium treatment.**
of cDNA fragments were isolated from cadmium-exposed C. elegans and probed with 32P-labeled cDNA fragments. Northern blot analysis confirmed that as a result of cadmium treatment, the RNA and myosin light chain DNAs were used as controls in the reverse Northern dot-blot analysis (53). Each pair of cDNA products was, however, amplified using an identical pair of primers (Table II). Four clones, DDRT2, DDRT7, DDRT16, and DDRT26, are derived from the predicted gene F35E8.11. They were amplified using the same 3'-degenerate oligo(dT) primer; however, four different 5'-primers were used (Table II). In several cases, pairs of cDNA fragments were isolated that are products of the same gene: DDRT3 and DDRT4, DDRT19 and DDRT20, and DDRT25 and DDRT28. The lengths of the cDNA fragments in each pair are different. Each pair of cDNA products was, however, amplified using an identical pair of primers (Table II). Four cDNA fragments were isolated that have sequences that are homologous to the C. elegans rDNA tandem repeats in cosmid F31C3. The sequences of two of the clones, DDRT23 and DDRT38, are identical. The sequences of these rDNAs are homologous to a region in the cosmid between nucleotides 25265 and 25433. The sequences of clones DDRT48 and DDRT32 are not homologous to the other rDNA clones. They

**FIG. 2.** Representative mRNA differential display band patterns of control and cadmium-treated C. elegans. Total RNA was isolated from C. elegans exposed to cadmium for 0, 8, and 24 h, as indicated at the top, and analyzed by differential display. RNA from duplicate populations of treated and control C. elegans was reverse transcribed and amplified with the 3'-degenerate anchored oligo(dT) primer T12MA and the 5'-arbitrary decamer AP-13 (left panel) and T25MG and RT-10 (right panel). Amplified cDNA fragments were resolved by electrophoresis in a 6% denaturing polyacrylamide gel. cDNA fragments that were subsequently isolated (DDRT15, DDRT16, and DDRT25) are indicated by arrows.

Treatment compared with identically sized DNA fragments from the control sample. A single product, VL9, was identified that showed decreased band intensity in the cadmium-treated C. elegans cDNA compared with the control.

Fifty-three cDNAs were successfully extracted from the acrylamide gels, reamplified, and cloned. Because of the large number of cDNAs successfully isolated, no further attempt was made to clone the remaining fragments. The cloned cDNA fragments ranged in size from 141 to 326 bp (Table II). These cDNAs were subsequently sequenced, and changes in the in vivo steady-state level of expression of the cognate mRNAs following cadmium treatment were evaluated.

**Northern and Reverse Northern Blot Analyses with Differentially Displayed cDNA Fragments**—Northern blot analysis was initially used to confirm that the differentially expressed cDNA fragments, VL3, VL9, VL11, VL19, VL20, VL21, and DDRT16, represent mRNAs whose steady-state levels change following cadmium exposure in vivo. RNA blots were prepared with either size-fractionated total RNA or poly(A+) mRNA, which were isolated from control and cadmium-exposed C. elegans, and probed with 32P-labeled cDNA fragments. Northern blot analysis confirmed that as a result of cadmium treatment, the levels of expression of VL19 and DDRT16 increased 2.3- and 3.5-fold, respectively (Fig. 3). There is also a 2-fold increase in the level of VL21 mRNA. The mRNAs for VL3, VL9, VL11, and VL20 were not detected by Northern blots containing poly(A+) mRNA isolated from either control or cadmium-treated C. elegans.

Reverse Northern dot-blot analysis (53) was performed as an alternative to traditional Northern blots. In this analysis, all of the differentially expressed clones as well as positive and loading controls were simultaneously examined. Representative results are shown in Fig. 4 and Table II summarizes the quantitative analysis from three separate experiments.

**C. elegans mtl-1** and myosin light chain DNAs were used as controls in the reverse Northern dot-blot analysis. The mtl-1 mRNA is induced 5-fold after cadmium treatment (Fig. 4), while the level of expression of myosin light chain mRNA remained constant. These results are consistent with those previously reported (32).

Of the 53 cDNA fragments isolated, 46 of the clones corresponded to C. elegans RNAs whose level of expression increased 2-5-fold following an 8-h cadmium treatment and up to 6-fold after a 24-h exposure (Table II). Two differentially expressed mRNAs, VL3 and DDRT38, do not show any significant change in expression following an 8-h cadmium exposure, compared with control nematodes. After a 24-h exposure, however, there is an ~2-fold increase in their levels of expression. The level of expression for VL9 decreased ~2-fold in response to cadmium. These results confirm that the changes in the levels RNA observed by differential display analysis reflect the in vivo molecular response of C. elegans to cadmium. Four of the clones, DDRT24, DDRT36, DDRT37, and DDRT47, did not significantly change their level of expression after either 8- or 24-h cadmium exposure (Table II). These clones are defined as false positives.

**Nucleotide Sequencing and Homology Searching**—The nucleotide sequences of the differentially expressed DNA fragments were compared against the C. elegans genomic and EST data bases. Only seven of the cDNA fragments, DDRT12, DDRT15, DDRT21D, DDRT29, DDRT41, VL3, and VL21, did not show a >95% sequence identity to regions of the C. elegans genome (Table IV). Forty-four clones were identical to C. elegans cosmid and/or yeast artificial chromosome sequences. In addition, 39 clones were identical to C. elegans ESTs (26 of 39) or predicted genes (32 of 39).

The results of the BLASTN analysis showed that the differentially expressed cDNA fragments were derived from 32 independent genes (Table IV). Eight cDNA clones, VL1, VL5, VL7, VL8, VL10, VL12, VL13, and VL15, are identical to the mtl-1 cDNA sequence. This result is not unexpected, because five of the 5'-random decamer primers used in the amplification reactions are identical, or have a one-nucleotide mismatch, to regions in the mtl-1 cDNA. These primers were specifically selected to function as internal controls that amplified the mtl-1 cDNA, in order to confirm the efficacy of differential display analysis in identifying cadmium-responsive C. elegans genes.

Four clones, DDRT2, DDRT7, DDRT16, and DDRT26, are derived from the predicted gene F35E8.11. They were amplified using the same 3'-degenerate oligo(dT) primer; however, four different 5'-primers were used (Table II). In several cases, pairs of cDNA fragments were isolated that are products of the same gene: DDRT3 and DDRT4, DDRT19 and DDRT20, and DDRT25 and DDRT28. The lengths of the cDNA fragments in each pair are different. Each pair of cDNA products was, however, amplified using an identical pair of primers (Table II). Four cDNA fragments were isolated that have sequences that are homologous to the C. elegans rDNA tandem repeats in cosmid F31C3. The sequences of two of the clones, DDRT23 and DDRT38, are identical. The sequences of these rDNAs are homologous to a region in the cosmid between nucleotides 25265 and 25433. The sequences of clones DDRT48 and DDRT32 are not homologous to the other rDNA clones. They
Differentially expressed cDNAs are derived from the same gene (see analysis. Experiments. Values in parenthesis were determined by Northern blot of myosin light chain mRNA and are the average of three independent to nonexposed by reverse Northern blot analysis. -Fold change in expression is relative to nonexposed.

| Clone name  | Primers used in PCR | Size of PCR product (bp) | Fold change in mRNA levels following cadmium exposure* |
|-------------|---------------------|--------------------------|------------------------------------------------------|
| DDRT1       | T12MG/RT-4          | 255                      | 2.2 3.2                                              |
| DDRT2       | T12MG/RT-4          | 214                      | 3.1 3.9                                              |
| DDRT3       | T12MG/RT-5          | 162                      | 2.0 2.4                                              |
| DDRT4       | T12MG/RT-5          | 187                      | 2.5 2.8                                              |
| DDRT5       | T12MA/RT-7          | 254                      | 3.2 3.9                                              |
| DDRT6       | T12MC/RT-5          | 219                      | 4.7 5.0                                              |
| DDRT7       | T12MG/RT-6          | 217                      | 3.6 4.0                                              |
| DDRT8       | T12MA/RT-8          | 240                      | 3.5 2.8                                              |
| DDRT9       | T12MG/RT-10         | 212                      | 2.7 2.5                                              |
| DDRT10      | T12MC/RT-7          | 228                      | 2.2 2.9                                              |
| DDRT12      | T12MA/RT-7          | 226                      | 3.0 3.2                                              |
| DDRT15      | T12MG/RT-10         | 243                      | 2.8 3.1                                              |
| DDRT16      | T12MG/RT-10         | 240                      | 4.3 4.9 (3.5)                                        |
| DDRT17      | T12MA/RT-10         | 200                      | 3.7 5.7                                              |
| DDRT18      | T12MC/RT-10         | 228                      | 2.2 2.9                                              |
| DDRT19      | T12MT/AP-3          | 322                      | 2.7 3.9                                              |
| DDRT20      | T12MT/AP-3          | 213                      | 2.6 2.8                                              |
| DDRT21U     | T12MC/AP-4          | 284                      | 2.2 2.6                                              |
| DDRT21D     | T12MG/AP-4          | 188                      | 2.2 4.2                                              |
| DDRT22      | T12MC/AP-4          | 292                      | 2.5 3.3                                              |
| DDRT23      | T12MG/AP-4          | 277                      | 3.3 3.0                                              |
| DDRT24      | T12MC/AP-4          | 272                      | 1.3 1.5                                              |
| DDRT25      | T12MA/AP-13         | 228                      | 3.9 4.4                                              |
| DDRT26      | T12MG/AP-13         | 238                      | 4.4 4.7                                              |
| DDRT27      | T12MA/AP-13         | 308                      | 1.7 1.8                                              |
| DDRT29      | T12MG/AP-15         | 141                      | 3.9 4.4                                              |
| DDRT30      | T12MA/AP-15         | 289                      | 2.7 3.1                                              |
| DDRT32      | T12MT/AP-15         | 252                      | 2.6 2.4                                              |
| DDRT33U     | T12MG/AP-18         | 208                      | 3.0 3.5                                              |
| DDRT33D     | T12MA/AP-15         | 171                      | 3.1 3.5                                              |
| DDRT34      | T12MA/AP-18         | 189                      | 2.4 3.0                                              |
| DDRT35      | T12MT/AP-18         | 314                      | 1.6 1.9                                              |
| DDRT36      | T12MA/CP-18         | 292                      | 1.1 1.1                                              |
| DDRT37      | T12MC/CP-18         | 267                      | 1.0 1.2                                              |
| DDRT38      | T12MC/CP-18         | 238                      | 1.1 1.8                                              |
| DDRT40      | T12MC/CP-18         | 264                      | 1.9 2.3                                              |
| DDRT41      | T12MC/CP-18         | 154                      | 2.2 2.1                                              |
| DDRT47      | T12MC/CP-13         | 165                      | 1.5 1.4                                              |
| DDRT48      | T12MG/CP-13         | 232                      | 2.1 2.6                                              |
| DDRT50      | T12MT/CP-15         | 254                      | 2.2 2.2                                              |
| VL1         | T12MT/CP-15         | 217                      | 4.7 (5.2)                                            |
| VL3         | T12MT/CP-6          | 199                      | 1.4 2.4                                              |
| VL5         | T12MT/CP-7          | 216                      | ND* ND                                               |
| VL7         | T12MT/CP-7          | 217                      | ND ND                                                |
| VL8         | T12MT/CP-6          | 214                      | ND ND                                                |
| VL9         | T12MT/CP-6          | 356                      | 1.7 – 2.3                                            |
| VL10        | T12MT/CP-7          | 196                      | ND ND                                                |
| VL11        | T12MT/CP-7          | 142                      | 3.2 6.1                                              |
| VL12        | T12MG/CP-10         | 216                      | ND 5.2                                               |
| VL13        | T12MG/CP-9          | 216                      | ND ND                                                |
| VL15        | T12MT/CP-10         | 217                      | ND ND                                                |
| VL19        | T12MG/CP-9          | 285                      | 3.6 5.0 (2.3)                                        |
| VL20        | T12MT/CP-9          | 148                      | 2.8 5.0                                              |
| VL21        | T12MC/CP-10         | 326                      | 2.2 2.5 (2.0)                                        |

* Clones labeled with identical superscript numbers indicate that the differentially expressed cDNAs are derived from the same gene (see Table IV).

a Steady-state levels of differential expressed mRNAs were determined by reverse Northern blot analysis. -Fold change in expression is relative to nonexposed C. elegans. All values have been normalized to the level of myosin light chain mRNA and are the average of three independent experiments. Values in parenthesis were determined by Northern blot analysis.

The results of the BLASTX analysis are presented in Table IV. Cadmium exposure causes an increase in the steady-state levels of several C. elegans proteins that are homologous to proteins in the protein data bases. Clone VL21 corresponds to a mRNA that encodes the C. elegans HSP70F protein precursor. The expression of HSP70 has been shown to increase following cadmium exposure in mammalian cells (16, 55). This response, however, has not been reported in C. elegans. Cadmium exposure also induced the expression of a mRNA that encodes a DNA gyrase homologue, DDRT41. The metal caused a 3-fold increase in the levels of mRNAs DDRT30 and DDRT33D that encode two different DDRT homologues in C. elegans.

**DISCUSSION**

The survival of an organism is constantly challenged by exposure to chemical toxins. To inhibit the effects of these

![Fig. 3. Northern blot analysis showing differential expression of selected cadmium-responsive genes. C. elegans poly(A) RNA (2 μg), isolated from nematodes exposed to 100 μM CdCl₂ for 24 h (+) or control nematodes (−), was resolved by denaturing agarose gel electrophoresis. Northern blots were hybridized with 32P-labeled cDNA probes prepared from the differential display cDNA fragments VL19 (upper left panel) or DDRT16 (upper right panel). Following PhosphorImager analysis, the probes were removed, and the membrane was reprobed with a 32P-labeled DNA fragment homologous to myosin light chain mRNAs (lower panels).**
chemicals, cells increase the production of a variety of defense and repair proteins. These increases can be directly related to changes in gene transcription. Differential display analysis was used to identify genes whose levels of expression are affected by cadmium exposure in C. elegans. From this analysis, 49 cDNA fragments were identified, which are the products of 32 different genes, whose steady-state levels of expression are modulated by cadmium treatment in vivo. Differential display analysis has been previously used to identify mRNAs whose levels of expression are modulated by exposure to other stressors. Oxidative stress in cultured cells, induced with diethylmaleate, hydrogen peroxide, or ultraviolet light, increases the expression of multiple mRNAs derived from both genomic and mitochondrial genes (56–60). Sequence analysis of the differential display clones revealed that several of these mRNAs encode homologues of known proteins (e.g. vimentin, c-Fos, cytochrome oxidase, ribosomal protein L4); however, the majority encode novel proteins (56–60). Recently, three genes were identified by differential display from C. elegans that are also induced by oxidative stress, caused by paraquat exposure (61). Although the intracellular damage elicited by cadmium exposure is similar to that induced by oxidative stress, the nucleotide and amino acid sequences of the paraquat-inducible gene products, glutathione S-transferase, a zinc-finger/leucine zipper protein, and a novel protein, are not homologous to the cadmium-responsive products identified in the present study.

Two methods were employed to confirm that the cDNA fragments identified by differential display represent RNAs whose steady-state levels of expression change as a result of cadmium exposure in vivo: Northern and reverse Northern analyses. All of the isolated differentially displayed cDNA fragments were analyzed by reverse Northern, and seven fragments by traditional Northern analysis. The reverse Northern analysis confirmed that the level of expression of 49 of the cognate RNAs changed 2–6-fold following cadmium exposure. The magnitude of the change in the levels of expression for clones DDRT16, VL19, and VL21 were comparable when determined by either method (Table II). The reverse Northern analysis appears to be a more sensitive assay than traditional Northern blotting, because only the former detected mRNAs of VL3, VL9, VL11, and VL20. The lower sensitivity of Northern analysis for the confirmation of differentially expressed mRNAs has been attributed to the short lengths of the differentially displayed cDNA fragments (<360 bp; Table II), which usually fail to generate 32P-labeled probes with sufficient specific activities to detect low abundance mRNAs (53).

Thirty-one unique C. elegans gene products were identified by differential display whose levels of expression increase following cadmium exposure. Twenty-two of these mRNAs encode proteins whose amino acid sequences do not share significant homology with other proteins in the database. However, several mRNAs were identified that encode homologues of proteins that have been associated with cadmium-induced stress responses in cultured cells and other organisms. These include C. elegans HSP70F (hsp-6) and metallothionein-1 (mtl-1) and a C. elegans homologue of a protein that is associated with DNA repair, DNA gyrase (Table IV).

The ability of cadmium to induce metallothionein gene expression in a variety of species has been documented for many years (13). A detailed discussion of the effect of cadmium on C. elegans metallothionein gene transcription can be found in Freedman et al. (32). The second C. elegans metallothionein isoform, mtl-2, was not identified in this study (32). The 5’-arbitrary decamer primers used in this analysis were specifically selected that do not share sequence identity with the mtl-2 mRNA. This was done to prevent the repeated isolation of

![Figure 4](image-url)

**FIG. 4.** Representative reverse Northern dot blot of differentially expressed genes. Cloned DNA fragments were amplified, and ~100 ng of the amplified product was immobilized on triplicate membranes, as described under “Experimental Procedures.” The membranes were then hybridized with 32P-labeled cDNAs synthesized from poly(A)+ RNA prepared from either untreated C. elegans (top panel), or those exposed to cadmium for 8 h (middle panel) or 24 h (bottom panel). The location of each differentially expressed DNA fragment and the myosin light chain and metallothionein controls on the blots is presented in Table III.

| Table III |
| Location of each differentially expressed DNA fragment and the myosin light chain (MLC) and metallothionein (MTL-1) controls on the blots in Fig. 4 |

|   | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  |
|---|----|----|----|----|----|----|----|----|
| A | MLC | DDRT1 | DDRT2 | DDRT3 | DDRT4 | DDRT5 | DDRT6 | DDRT7 |
| B | DDRT9 | DDRT10 | DDRT12 | DDRT15 | DDRT16 | DDRT17 | DDRT18 | DDRT19 |
| C | DDRT20 | DDRT21U | DDRT21D | DDRT22 | DDRT23 | DDRT24 | DDRT25 | DDRT26 |
| D | DDRT28 | DDRT29 | DDRT30 | DDRT32 | DDRT33U | DDRT33D | DDRT34 | DDRT35 |
| E | DDRT36 | DDRT37 | DDRT38 | DDRT40 | DDRT41 | DDRT47 | DDRT48 | DDRT50 |
| F | VL3 | VL9 | VL11 | VL19 | VL20 | VL21 | MTL-1 |
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Table IV

Sequence analysis of cadmium-regulated, differentially expressed cDNAs

| Clone name | Cosmid | Sequence identity/homology | Gene product | GenBank™ accession no. |
|------------|--------|----------------------------|--------------|-----------------------|
| DDRT1      | T09B4  | T09B1.4, CELK00886         | AF071359     |
| DDRT2      | F35E8  | F35E8.11                   | AF071362     |
| DDRT3      | F35E12 | F35E12.7                   | AF071382     |
| DDRT4      | F35E12 | F35E12.7                   | AF071391     |
| DDRT5      | C56C10 | C56C10.12, CELK05910       | AF071396     |
| DDRT6      | W03C9  | W03C9.5, CELK06836         | AF071397     |
| DDRT7      | F35E8  | F35E8.11                   | AF071398     |
| DDRT9      | C35D10 | ND*                        | AF071399     |
| DDRT10     | C49C3  | ND                         | AF071353     |
| DDRT12     | ND     | ND                         | AF071354     |
| DDRT15     | ND     | ND                         | AF071355     |
| DDRT16     | F35E8  | F35E8.11                   | AF071356     |
| DDRT17     | C49A9  | C49A9.4, CELK02276         | AF071358     |
| DDRT18     | F13G3  | F13G3.4, CELK06645         | AF071360     |
| DDRT19     | ZK849  | ND                         | AF071361     |
| DDRT20     | ZK849  | ND                         | AF071363     |
| DDRT21U    | Y111B2 | ND                         | AF072438     |
| DDRT21D    | ND     | CELK05123                  | AF071364     |
| DDRT22     | F57G9  | ND                         | AF071365     |
| DDRT23     | F31C3  | C. elegans rDNA tandem repeats | AF071376 |
| DDRT24     | C56C10 | C56C10.8, CELK02788, human transcription factor BTF3 | AF071377 |
| DDRT25     | R119   | R119.5, CELK00686          | AF071378     |
| DDRT26     | F35E8  | F35E8.11                   | AF071379     |
| DDRT28     | R119   | R119.5; CELK00686          | AF071380     |
| DDRT29     | ND     | ND                         | AF071383     |
| DDRT30     | C27H5  | C27H5.5, CELK02088, C. elegans collagen (col-36) | AF071385 |
| DDRT32     | F31C3  | C. elegans rDNA tandem repeats | AF071386 |
| DDRT33D    | F31C3  | C. elegans cuticle collagen | AF071387     |
| DDRT34     | F20C5  | F20C5.1; CELK01295         | AF071388     |
| DDRT35     | R11D1  | R11D1.1; CELK02809; human hypothetical protein KIA0174 | AF071389 |
| DDRT36     | D2096  | D2096.8, CELK01725; human nucleosome assembly protein1 LIKE-1 | AF071390 |
| DDRT37     | K11H2  | K11H2.2; CELK02043; 80 S ribosomal protein | AF071391 |
| DDRT38     | F31C3  | C. elegans rDNA tandem repeats | AF071392 |
| DDRT40     | W02B3  | W02B3.2; bovine β-adrenergic receptor kinase | AF071393 |
| DDRT41     | ND     | S. pyogenes cit. DNA gyrase subunit B | AF071394 |
| DDRT42     | D2096  | D2096.8, CELK01725; human nucleosome assembly protein1 LIKE-1 | AF071395 |
| DDRT43     | F31C3  | C. elegans rDNA tandem repeats | AF071396 |
| VL1I       | K11G9  | K11G9.5; CELK3309; C. elegans metallothionein-1 (mtl-1) | AF071397 |
| VL3        | ND     | ND                         | AF071374     |
| VL5        | K11G9  | K11G9.5; CELK3309; C. elegans metallothionein-1 (mtl-1) | AF071375 |
| VL7        | K11G9  | K11G9.5; CELK3309; C. elegans metallothionein-1 (mtl-1) | AF071376 |
| VL9        | K11G9  | K11G9.5; CELK3309; C. elegans metallothionein-1 (mtl-1) | AF071377 |
| VL10       | K11G9  | K11G9.5; CELK3309; C. elegans metallothionein-1 (mtl-1) | AF071378 |
| VL11       | C60G3  | C60G3.8                    | AF071368     |
| VL12       | K11G9  | K11G9.5; CELK3309; C. elegans metallothionein-1 (mtl-1) | AF071369 |
| VL13       | K11G9  | K11G9.5; CELK3309; C. elegans metallothionein-1 (mtl-1) | AF071370 |
| VL15       | K11G9  | K11G9.5; CELK3309; C. elegans metallothionein-1 (mtl-1) | AF071371 |
| VL19       | D2023  | D2023.2; CELK00011; human pyruvate carboxylase | AF071372 |
| VL20       | B2028  | B2028.1                    | AF071373     |
| VL21       | CELK0200; C. elegans mitochondrial hsp70 protein F precursor | AF071374 |

a Clones labeled with identical superscript numbers indicate that the differentially expressed cDNAs are derived from the same gene.

b Analyzed with BLASTN using GenBank™ and C. elegans-specific data bases. The sequences have >80% nucleotide sequence identity.

c C. elegans genomic cosmids that have >90% nucleotide sequence identity.

d Predicted genes are designated by the cosmid name followed by the structural gene number (e.g. F35E8.11). C. elegans ESTs are denoted with the “CELK” designation.

* ND, not detected.

† Yeast Artificial Chromosome.

‡ Homologous proteins are presented that have a >60% amino acid sequence identity, based on BLASTX analysis.

mtl-2 cDNA fragments, as occurred with the mtl-1 mRNA (Table IV).

The C. elegans hsp-6 gene is both constitutively expressed and moderately inducible by heat shock (37). However, a cadmium-induced increase in the steady-state level of the C. elegans HSP70 mRNA has not been reported. Cadmium has been shown to activate the transcription of the C. elegans hsp-16 gene, which encodes for a low molecular weight HSP (62). In cultured cells, cadmium increases HSP70 protein and mRNA levels, as well as the levels of HSP28, -60, -68, -84, -90, and -100 (16, 55, 63). It also causes an increase in the binding of the heat shock transcription factor to HSP70 promoter (64). Cadmium may activate HSP70 transcription by several pathways. The metal can (a) directly denature proteins, which function as activators, (b) increase levels of reactive oxygen species, or (c) modulate the levels of second messengers.

An mRNA encoding the C. elegans homologue of DNA gyrase (topoisomerase) was isolated. The level of expression of this mRNA increases 2-fold in response to cadmium exposure (Table II). The ability of cadmium or other metals to modulate DNA gyrase mRNA or protein levels has not been reported for C. elegans or other organisms. Cadmium stimulates DNA damage in cultured cells, inducing chromosomal aberrations and strand breaks (65). It potentiates this damage by inhibiting DNA repair and enhancing oxidative stress (65, 66). Since (α) DNA gyrases/topoisomerases are integral components in DNA...
repair (67) and (b) DNA-damaging agents elevate the levels of topoisomerase in cultured cells and tumors (68, 69), increased expression of these genes would be a likely consequence of cadmium expression. The change in the level of DNA gyrase mRNA in cadmium-treated C. elegans may be the result of greater DNA repair activity. DNA in C. elegans is susceptible to damage from ionizing radiation and oxidative stress (70), although the effects of transition metals have not been examined. It is likely that cadmium exposure will lead to DNA damage in C. elegans and subsequent activation of DNA repair processes.

Several genes were identified whose products are not usually associated with a metal-induced or an oxidative stress response. The sequences of these gene products are homologous to pyruvate carboxylase, collagen, ribosomal RNA precursor, and β-adrenergic receptor kinase (Table IV). The levels of expression of the cognate RNAs increased 2–5-fold in response to cadmium exposure (Table II). These gene products have unrelated functions; however, they are connected in that calcium and/or cAMP has been shown to affect their expression in other organisms. In whole animals and cultured cells, cadmium stimulates the production of cAMP and increases intracellular free calcium concentrations (25, 71). Associated with the cadmium-induced changes in the levels of these second messengers is the activation of downstream targets: protein kinase C and calmodulin (25).

Chronic and acute exposure of rats to cadmium increases the activity of liver pyruvate carboxylase as well as other glycolytic enzymes (71, 72). It has been proposed that the increase in enzyme activity is due to elevated levels of cAMP caused by an increase in adenylate cyclase activity (71, 72). Elevated levels of cAMP in cultured cells, caused by forskolin treatment, also increase the levels of mRNA transcription factors and calcium synthesis (73, 74). It also increases the binding of the heat-shock transcription factors to HSP70 promoter (75). Thus, cadmium may affect the expression of the C. elegans genes by modulating cAMP levels.

Cadmium affects cellular calcium levels by inhibiting the calcium efflux system (76). In addition, it can substitute for calcium in protein kinase C and calmodulin to activate these proteins and subsequently affect downstream targets (24, 25). Elevation of cellular calcium levels or activation of protein kinase C has been shown to increase rDNA transcription and the mRNA levels of adrenergic receptor kinases 1 and 2 (77). The expression of the C. elegans genes responsive to calcium or cAMP has been shown to affect their expression in other organisms. In whole animals and cultured cells, cadmium stimulates the production of cAMP and increases intracellular free calcium concentrations (25, 71). Associated with the cadmium-induced changes in the levels of these second messengers is the activation of downstream targets: protein kinase C and calmodulin (25).

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