Molecular Characterization of a Novel, Cadmium-inducible Gene from the Nematode Caenorhabditis elegans

A NEW GENE THAT CONTRIBUTES TO THE RESISTANCE TO CADMIUM TOXICITY*

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Cadmium is an environmental contaminant that is both a human toxicant and carcinogen. To inhibit cadmium-induced damage, cells respond by increasing the expression of genes that encode stress-response proteins. We previously reported the identification of 49 cadmium-inducible mRNAs in the nematode Caenorhabditis elegans. Here we describe a new cadmium-responsive gene, designated cdr-1, whose rate and level of inducible expression parallel those of the C. elegans metallothioneins. The CDR-1 mRNA contains an open reading frame of 831 bp and encodes a predicted 32-kDa, integral membrane protein. Following cadmium exposure, cdr-1 is transcribed exclusively in intestinal cells of post-embryonic C. elegans. In vivo, the CDR-1 protein is targeted specifically to the intestinal cell lysosomes. cdr-1 transcription is significantly induced by cadmium but not by other tested stressors. These results indicate that cdr-1 expression is regulated by cadmium and in a cell-specific fashion. Inhibition of CDR-1 expression renders C. elegans susceptible to cadmium toxicity. In conclusion, cdr-1 defines a new class of cadmium-inducible genes and encodes an integral membrane, lysosomal protein. This protein functions to protect against cadmium toxicity.

The transition metal cadmium is considered a serious occupational and environmental health threat. It is continuously introduced into the atmosphere through the smelting of ores and the burning of fossil fuels and is commonly found in “Superfund” clean-up sites (1–3). Humans are exposed to cadmium primarily via inhalation and the ingestion of cadmium-containing foods (4). Toxicological responses of cadmium exposure include kidney damage, respiratory diseases, neurological disorders, and lung, kidney, prostate, and testicular cancers (4).

Cadmium induces intracellular damage via the (a) nonspecific inactivation/denaturation of proteins, by binding to free sulfhydryl residues; (b) displacement of zinc co-factors from a variety of proteins, including transcription factors; and (c) generation of reactive oxygen species, which ultimately oxidize DNA, proteins, and lipids. Although cadmium itself is not redox active in vivo and cannot directly catalyze the reduction of molecular oxygen, it has been suggested that the production of reactive oxygen species is a consequence of a cadmium-induced depletion of cellular glutathione or the inactivation of copper/zinc-superoxide dismutase (5, 6).

To attenuate the toxic effects of cadmium, cells respond by increasing the steady-state levels of a variety of proteins. The functions of these proteins can be broadly defined, because those that repair intracellular damage or those that remove the toxicants (e.g. cadmium, reactive oxygen species). To remove toxicants, cells activate the transcription of genes that encode proteins that are involved in scavenging reactive oxygen species, including heme oxygenase, γ-glutamylcysteine synthetase, superoxide dismutase, catalase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase (7–11). Cadmium is essentially removed from the cell through chelation by MT1 or exported by means of metal ion pumps. These pumps can transport the metal from the cytoplasm into lysosomes or out of the cell (12–15).

To identify new cadmium-responsive genes that are involved in intracellular defense, the reverse transcriptase-PCR protocol of differential display was used (16). Forty-eight differentially expressed mRNAs, which correspond to the products of thirty-two independent genes, were identified in the non-parasitic nematode Caenorhabditis elegans (17). C. elegans provides an excellent model system for obtaining an integrated picture of cellular, developmental, and molecular aspects of transition metal toxicity. The adult hermaphrodite is composed of 959 somatic cells. The developmental and cellular biology of C. elegans is thoroughly understood, and the nematode contains highly differentiated muscular, nervous, digestive, and reproductive systems (18). Furthermore, high levels of evolutionary conservation between C. elegans and higher organisms are observed in many of the proteins that are induced as part of a metal-activated stress response. These include MT (19), superoxide dismutase (20, 21), ubiquitin (22), heat shock protein 70 (23), glutathione S-transferase (24), catalase (25), and multidrug resistance-associated proteins (14). C. elegans also contains homologues to many of the regulatory proteins

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that have been implicated in modulating the molecular response to metal exposure (26–29).

In the collection of cadmium-responsive C. elegans ESTs identified by differential display, four were derived from an identical mRNA (17). Here we describe the isolation and characterization of the full-length mRNA and the gene, designated cdr-1 (cadmium-responsive gene family), from which the ESTs were derived. cdr-1 encodes a novel gene-specific primer VHCL4 that was purified and inserted into the pGEM-T vector, and sequenced. Sequence analysis confirmed that the 5'-end of cdr-1 was isolated using this protocol.

A cDNA clone that contained the full-length cdr-1 sequence was subsequently created by first digesting a plasmid that contained the 3'-end of CDR-1, pGEM-VHCL3 with Ncol and EcoRI. Following agarose gel electrophoresis, a 3377-bp fragment was isolated. This fragment contained the pGEM-T vector and 377 bp of the 3'-end of the cdr-1 cDNA, which includes the translation stop codon. A cDNA fragment that contained the 5'-end of the cdr-1 was obtained following the digestion of the plasmid pGEM-VHCL10 with Ncol and EcoRI. A 488-bp fragment, which includes the translation start site, 6'-untranslated region, and 15 bp of the pGEM multiple cloning site, was purified, inserted into the 3377-bp plasmid, and sequenced. Sequence analysis confirmed the presence of a full-length CDR-1 cDNA clone. This plasmid was designated pGEM5-CDR-1.

**Computer Analysis**—Analysis of nucleotide and amino acid sequence data, including assembly of continuous cDNA sequences, sequence comparisons, and data base searches were performed using PGOENE-IntelliGenetics software (IntelliGenetics, Mountainview, CA) and BLAST programs (31, 32). Genomic sequence data were obtained from the C. elegans Genome Project (available through the Wellcome Trust Sanger Institute). The location within the C. elegans genome of cdr-1 was determined using ACeDb (A C. elegans Database) (33).

**Isolation of the cdr-1 Gene**—BLAST nucleotide sequence analysis identified several cosmids that include the entire cdr-1 coding and potential 5'-regulatory regions. One of these cosmids, F35E8 (accession number: Z81529), was obtained from the C. elegans Genome Project.

To isolate a genomic fragment that contains cdr-1, F35E8 was digested with XhoI. Based on the sequence data, F35E8 contains two XhoI sites at 15,882 and 21,707, which flank the 5'– and 3' ends of cdr-1. Following XhoI digestion, a 5,825-bp fragment was purified and then inserted into pGEM3zf(—), which was cut with XhoI.

**Preparation of RNA and Northern Blot Analysis**—Total C. elegans RNA was prepared from washed nematodes as previously described (19). For some experiments poly(A)+ RNA was purified using the Poly (A) Tract System, following the manufacturer's instructions (Promega).

Northern blot analysis was performed using 32P-labeled EST clone DDR16 or full-length CDR-1 cDNA as probes, as previously described (19). PhosphorImager analysis and ImageQuant software (Molecular Dynamic System) were used to determine the amount of probe that hybridized to the RNA. After the amount of CDR-1 was determined, the blot was then rehybridized to a 32P-labeled C. elegans myosin light-chain probe, which served as a loading control. The steady-state level of CDR-1 mRNA was normalized to that of the constitutively expressed myosin light-chain mRNAs (~1150 and 880 nt) (5).

**Kinetics of CDR-1 mRNA Accumulation**—The rate of CDR-1 mRNA accumulation following cadmium exposure was determined by exposing C. elegans to 100 µM cadmium for different times (19). When cadmium was added, the culture contained nematodes at all stages of development and an adequate supply of food. After exposing C. elegans to cadmium for 1, 2, 4, 8, 16, and 24 h, the nematodes were isolated, total RNA was purified and then subjected to Northern hybridization performed. The steady-state level of CDR-1 mRNA at each time point was normalized to the levels of the two myosin light-chain mRNAs.

**Preparation and Analysis of Transgenic C. elegans**—A ~3.5-kbp fragment of genomic DNA that is immediately upstream from the initiator ATG in cdr-1 was prepared from the cosmids F35E8 by employing the PCR. The cdr-1 promoter/enhancer fragment was amplified by using Ffu polymerase and the primers VHCL11 and VHCL12. The 5' primer VHCL12 (5'-GGCGGTATCCCTAAGCCGATATGATTGACCC-3') precedes a BamHI site in F35E8 and corresponds to nt 13,809–13,828 in the cosmids. The 3' primer VHCL11 (5'-CCCGGATCCCTATTGATACCTGGACCCACCT-3') is the reverse complement of nt 17,357–17,338 in the C. elegans genome. CDR-1 mRNA fragment was inserted into pPD95.10, which was cut with the identical enzymes. Following BamHI digestion, a 5,825-bp fragment was purified and then inserted into the RC40 large T antigen nuclear targeting sequence. Thus, the 5'-promoter/enhancer region of cdr-1 will control the expression of a β-galactosidase reporter gene that is preceded by the SV40 large T antigen nuclear targeting sequence. The 3'-end of the reporter plasmid contains the dominant selectable marker gene rol-6 into the gonadal syncytium of young adult C. elegans as described previously (19, 35). Transgenic C. elegans were selected and maintained as indicated in previous studies (19).
These lines of transgenic nematodes contain the reporter transgene as a heritable extrachromosomal array, which can be lost after several generations (35). To permanently integrate the array into the C. elegans genome, transgenic nematodes were exposed to 300 J/m² of ultraviolet radiation. After several generations, C. elegans that contain the integrated transgene were isolated by identifying individual nematodes of which 100% of the progeny express the rol-6 phenotype. Integrated C. elegans were out-crossed four times with a wild type strain to remove any mutations that may have occurred because of the radiation treatment. The transgenic strain used in the subsequent studies is designated JF9 (mtIs7, cdr-1/lacZ).

Cell-specific, developmentally regulated patterns of cdr-1 transcription were determined in cadmium-exposed and non-exposed C. elegans as previously described (19). Transgenic C. elegans were out-crossed four times with a wild type strain to remove any mutations that may have occurred because of the radiation treatment. The transgenic strain used in the subsequent studies is designated JF9 (mtIs7, cdr-1/lacZ). These lines of transgenic nematodes contain the reporter transgene as a heritable extrachromosomal array, which can be lost after several generations (35).

Effects of Stressors on cdr-1 Transcription—C. elegans JF9 (mtIs7, cdr-1/lacZ) were used to investigate the effects of exposure to heat shock, transition metals, and oxidative stress on cdr-1 transcription. The effect of heat shock on cdr-1 transcription was determined using conditions previously described (19, 36). Briefly, transgenic C. elegans were incubated at 33°C for 1.5 h. The nematodes were allowed to recover by incubating at 20°C for 16 h. After the recovery period, nematodes were collected and the level of β-galactosidase activity was quantified from the amount of blue chromogen produced as previously described (37).

To examine the effects of metals and oxidative stress on cdr-1 transcription, nematodes were collected and then suspended in K medium (38). C. elegans were exposed to CdCl₂, CuSO₄, ZnSO₄, HgCl₂, or Pb(NO₃)₂ with constant, gentle agitation at 20°C for 24 h. The concentrations of added metals have been previously shown to induce the transcription of mtl-2 and hsp-16 (36, 37) and are below their respective LC₅₀ values (38, 39). To induce oxidative stress, C. elegans were exposed to either paraquat or juglane for 24 h at concentrations that are reported to induce hsp-16 and glutathione S-transferase tran.
Molecular Characterization of a Cadmium Resistance Gene

Table I
Putative upstream regulatory elements in cdr-1

| Element              | Eukaryotic consensus sequence | Sequence and location in cdr-1 |
|----------------------|------------------------------|------------------------------|
| Metal response       | TGC (A/G) CNCG               |                               |
| Heat shock           | NGAAANNTCCNGAAN              |                               |
| Antioxidant response | TGA (C/G) TCA               |                               |
| GATA                 | (A/T) GATA (A/G)            |                               |
| cAMP                 | TGAAGTCA                    |                               |
| TATA box             | TATAA                       | (−30 to −23)                  |

a Positions are relative to the transcription start site.

b Inverse complement of the consensus sequence.

In C. elegans (24, 36), the effects of these stressors on cdr-1 transcription were then determined from the levels of β-galactosidase activity.

In Situ Hybridization—A population of wild type C. elegans at all developmental stages were treated with 100 μM cadmium for 24 h. Nematodes were isolated and fixed and the external collagenous cuticle was permeabilized and prehybridized as previously described (40). Digoxigenin-labeled probes were then added to a suspension of fixed, permeabilized nematodes in 40% (v/v) formamide, 5% SSC, 0.1 mg/ml sonicated salmon sperm DNA, 50 μg/ml heparin, and 0.1% Tween 20. Hybridization was carried out at 48°C for 16 h.

To synthesize the digoxigenin-labeled probes, anchored PCR was employed using pGEM5-CDR-1 as a template. An antisense CDR-1 DNA probe was generated using plasmid DNA, which was linearized following NcoI digestion, and a SP6 primer. A CDR-1 sense probe was prepared from linearized pGEM5-CDR-1, which was digested with NoI, and a T7 primer. Binding sites for both T7 and SP6 primers are in pGEM5zf(+) and flank the CDR-1 cDNA insert.

Following the hybridization, C. elegans were washed and then incubated at 4°C for 20 h with a 1:2500 dilution of alkaline phosphatase-linked anti-digoxigenin antibody (Roche Molecular Biochemicals). Following several washes, antigen-antibody complexes were visualized by incubating the nematodes with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Roche Molecular Biochemicals) (40).

In Vivo Localization of CDR-1—To determine the intracellular location of CDR-1 in vivo, a C. elegans expression vector was constructed in which the expression of a CDR-1-eGFP fusion protein is regulated by the cdr-1 promoter. The eGFP contains the Ser-65 to Cys-65 substitution (22, and it adds a GTACCTTAAT consensus for the 5′-untranslated region, with a typical polyadenylation site (AUG—C) as previously described (40). The 930-bp CDR-1 cDNA fragment was isolated and inserted into pCDR-1, which was digested with identical enzymes. The plasmid pCDR-1 was digested with 

Cloning and Sequence Analysis of the CDR-1 cDNA—The full-length CDR-1 cDNA was derived from the EST DDRT16 using 5′-RACE. The full-length of CDR-1 was determined from the (a) size of the mRNA transcript, estimated by Northern blot analysis; (b) longest open reading frame; and (c) available C. elegans genomic sequence data.

A Northern blot that contained size-fractionated C. elegans poly(A)+ RNA was hybridized to a [32P]-labeled DDRT16 cDNA probe. A single mRNA that hybridizes to DDRT16 is present in cadmium-exposed C. elegans. This mRNA has a size of 900 nt and is not present at a significant level in mRNA prepared from non-exposed nematodes (results not shown).

To obtain the full-length sequence of CDR-1, twenty independent cDNA clones that contain the 5′-end of CDR-1 were sequenced. The length of the CDR-1 mRNA, determined as assembling overlapping sequences from DDRT16 and 5′-RACE products is 894 nt, including 3′- and 5′-untranslated regions (Fig. 1). This value is in good agreement with the size of the mRNA obtained by Northern blotting.

CDR-1 mRNA contains a single open reading frame of 831 nt. An initiator ATG codon (nt 16–18) lies within the context of a consensus C. elegans translation start site, (A/G)NNATGT (Fig. 1). The stop codon TGA (nt 847–849) is followed by a 46-nt, 3′-untranslated region, with a typical polyadenylation signal (AAATAA) that is 13–18 nt 5′ of the poly(A) tail.

The 5′-ends of many C. elegans mRNAs are shortened and covalently modified by trans-splicing reactions (47). The sequences of the 5′-ends of the RNA fragments obtained during the 5′-RACE analysis were 100% identical to the genomic sequence in the C. elegans cosmid F35E8 (see below). Thus, the cdr-1 transcript is not trans-spliced. Comparison of mRNA sequences, ascertained by RACE, to genomic sequences allows the transcription start site to be determined. cdr-1 transcription begins at a unique site (A, +1, Fig. 1) and contains a 15-nt
untranslated region, which is 24-bp downstream from a consensus TATA box sequence (Table I).

The full-length CDR-1 nucleotide sequence was compared with sequences in several of the GenBank® data bases using the BLASTN program. CDR-1 is 100% identical to the predicted C. elegans cDNA F35E8.11, which was identified from the F35E8 cosmid sequence data using the Genfinder program (48). The CDR-1 mRNA does not have any significant levels of homology to any non-Caenorhabditis nucleotide sequences currently in the data bases.

Structure and Organization of cdr-1—cdr-1 is located near the center of chromosome V in the C. elegans genome. The size of cdr-1, including the structural gene and the upstream regulatory region, is predicted to be 2475 bp. The predicted gene includes the region of F35E8 between (a) the end of the 3′-untranslated region of the CDR-1 mRNA, and (b) the translation start codon of the predicted mRNA F35E8.10, which is located 1016 bp upstream of the cdr-1 transcription start site. F35E8.10 is transcribed from the complementary DNA strand, relative to cdr-1. F35E8.10 is predicted to encode a novel protein; however, homologous C. elegans ESTs have been identified, suggesting that the mRNA is expressed in vivo.

Comparison between the CDR-1 mRNA and genomic sequences enabled the elucidation of the intron/exon organization of the cdr-1 structural gene. The primary CDR-1 transcript is derived from six exons that are contained within ~1.5 kbp (Fig. 2). All of the introns in this gene are small, ranging in size from 52 to 347 bp. The occurrence of small introns is a common characteristic of the C. elegans genome. The nucleotide consensus sequences adjacent to the intron/exon junctions in cdr-1 are

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**Fig. 2.** Schematic diagram and exon/intron organization of cdr-1. The top panel shows the intro/exon organization of cdr-1. Exons are labeled with numbers, whereas introns are labeled with lowercase letters. The left table describes the size and amino acid content of the exons. The right table presents the intron size and the nucleotide sequence adjacent to the intron/exon junctions.

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**Fig. 3.** Hydropathy profile of the CDR-1 amino acid sequence. A sliding window of 17 amino acids was used during the analysis (56). Hydrophobic regions are shown with positive values. The boldface bars define the predicted transmembrane domains.
similar to those described for other *C. elegans* genes (26, 49).

Potential UREs were identified by comparing the consensus sequences of UREs that are known to control the transcription of other eukaryotic metal/stress-responsive genes to the contiguous /H110111-kbp DNA, which flanks the 5'/H11032-end of *cdr-1* (Table I).

*cdr-1* contains sequences that are 100% identical to the consensus sequences for a TATA box, a single metal response element, two antioxidant response elements, and a heat shock element (50–52). In addition, *cdr-1* contains two potential GATA-transcription factor-binding sites (53, 54). A homologous cAMP-response element was also identified (55).

**Analysis of the *CDR-1* Protein Sequence**—The *CDR-1* mRNA is predicted to encode a protein of 277 amino acids with a molecular mass of 32,126 and an estimated isoelectric point of 7.60. Hydropathy analysis of the deduced amino acid sequence indicates that *CDR-1* is highly hydrophobic, with greater than 80% hydrophobicity over the entire protein (Fig. 3) (56). *CDR-1* is predicted to be an integral membrane protein, with two transmembrane spanning domains; one is located at the N terminus (residues 2–23), and the second is near the middle of the protein (residues 157–175) (Fig. 3). The protein may be targeted to one or more membranes of either the endoplasmic reticulum or secretory vesicles (57). *CDR-1*, however, lacks any of the common protein cleavage signal sequences.

**Fig. 4.** Amino acid sequence comparison of *CDR-1* with the failed axon connection protein from *D. melanogaster*. The derived amino acid sequence of *CDR-1* is aligned with that of the failed axon connection protein from *D. melanogaster* (FAX). Amino acids that are identical in the aligned proteins () and residues conserved (–) are marked.
C. elegans

1-h exposure to 100 μM CdCl₂, and then 20-μg RNA samples were subjected to Northern blot analysis. The membrane was probed with a CDR-1 cDNA probe. The amount of CDR-1 mRNA was normalized to the integrated intensities of the signals obtained with a myosin light chain DNA probe at each time point. The values obtained at the 16-h time point were defined as the maximal level of induction. The data presented are means from three independent experiments (± S.D.).

CDR-1 contains several potential phosphorylation sites. Prosite analysis reveals consensus motifs for two protein kinase C phosphorylation sites (residues 28–30 and 84–86), four casein kinase II phosphorylation sites (residues 123–126, 157–160, 198–201, and 227–230), and one tyrosine kinase phosphorylation site (residues 148–155). It also contains an myc-type, helix-loop-helix dimerization domain (residues 87–95). CDR-1 does not contain consensus motifs for glycosylation or myristoylation sites (58).

The CDR-1 amino acid sequence was compared with sequences in GenBank™ data bases using the BLASTP and TBLASTN programs. CDR-1 has the highest levels of homology (27% identity, 46% similarity) with the failed axon connection protein (FAX) from Drosophila melanogaster (accession number: SS8776). The Drosophila FAX protein is involved in neuronal development, and it was discovered as a genetic enhancer of a mutation in the tyrosine kinase abl (59). The alignment of their amino acid sequences is presented in Fig. 4. It should be noted that the regions that are adjacent to the predicted transmembrane-spanning domains have the highest levels of homology.

**Induction of CDR-1 mRNA by Cadmium**—CDR-1 mRNA is not evident in a Northern blot of total RNA prepared from C. elegans grown in the absence of added cadmium. Following a 1-h exposure to 100 μM CdCl₂, reporter transgene activity was not observed. The regions that are adjacent to the predicted transmembrane-spanning domains have the highest levels of homology.

**Cell-specific and Developmental Expression of cdr-1**—The ability of cadmium to affect the cell-specific, developmentally regulated pattern of cdr-1 transcription was investigated by exposing several independent lines of transgenic C. elegans, which contain the cdr-1lacZ reporter transgene, to 100 μM cadmium for 24 h. Following cadmium exposure, cdr-1 promoter activity is evident exclusively in the intestinal cells of C. elegans (Fig. 6). Transcription was observed in all post-embryonic stages of development, and not in developing embryos. In the absence of cadmium, reporter transgene activity was not observed.

Whole mount in situ hybridization analysis was used to monitor the level and cellular distribution of CDR-1 mRNA in larval and adult nematodes. When cadmium-exposed C. elegans were hybridized to an antisense CDR-1 cDNA probe, CDR-1 mRNA was observed throughout the intestine in all post-embryonic developmental stages (Fig. 6). In contrast, CDR-1 mRNA was not detected at a significant level in non-exposed nematodes (results not shown). As a negative control, cadmium-treated C. elegans were hybridized to a digoxigenin-labeled CDR-1 antisense probe as described under "Experimental Procedures." C shows that the CDR-1 transcript is expressed throughout the intestine of L1. "ph" marks the location of the pharynx in all panels. Nematodes were photographed using Nomarski optics as described previously (19).

**Dose Dependence of cdr-1 Transcription in Transgenic C. elegans**—Transgenic C. elegans that contain an integrated cdr-1lacZ transgene were used to monitor the effects of various concentrations of cadmium on cdr-1 transcription. C. elegans
strain JF9 (mtIs7, cdr-1::lacZ) was exposed to cadmium for 24 h and the level of cdr-1 transcription determined from the yield of blue chromogen (37). The inducible, cell-specific pattern of cdr-1 transcription was identical at all cadmium concentrations (i.e. only occurred in intestinal cells). However, the magnitude of the response was concentration dependent (Fig. 7).

cdr-1 transcription was induced at a minimal cadmium concentration of 1 μM. A maximal level of expression was observed following exposure to 25 μM cadmium. Using the data presented in Fig. 7, an EC50 value ~2 μM was derived. The EC50 is defined as the concentration of cadmium added to the growth medium that will induce cdr-1 transcription to a level that is 50% of the maximal level detected. The EC50 value for cdr-1 transcription is similar to that reported for mtl-1, and it is two to three orders of magnitude lower than the 24-h exposure LC50 for cadmium (37–39).

Effects of Various Stressors on cdr-1 Transcription—C. elegans strain JF9 (mtIs7, cdr-1::lacZ) was used to examine the effects of environmental stressors on cdr-1 transcription. These stressors included heat shock, oxidative stress, and other transition metals. The exposure conditions (i.e. toxicant concentrations and exposure times) used in these studies were identical to those previously described to induce the transcription of other stress response genes (Table II). The only stressor that was able to induce cdr-1 transcription was cadmium. This characteristic is unique to cdr-1. Typically, the transcription of stress-response genes can be induced by several of the stressors examined.

CDR-1 Protein Localization in Vivo—To determine the intracellular location of CDR-1, transgenic C. elegans were generated that contain CDR-1-eGFP fusion protein whose expression is regulated by the cdr-1 promoter. C. elegans strain JF13 (mtEx10, cdr-1::CDR-1-GFP) was exposed to 100 μM cadmium for 24 h. Initial observations of cadmium-treated JF13 (mtEx10, cdr-1::CDR-1-GFP) nematodes clearly demonstrated that the CDR-1-eGFP fusion protein was concentrated in small punctate structures in the intestinal cells (Fig. 8). The size and distribution of these structures were similar to those of lysosomes (42). In contrast, when a non-fusion form of eGFP is expressed in intestinal cells, it accumulates in the cytoplasm but not in vesicles.3 To confirm that CDR-1 was targeted to the lysosomes, JF13 (mtEx10, cdr-1::CDR-1-GFP) C. elegans were fed RITC-dextran, prior to cadmium exposure. In the absence of cadmium, CDR-1-eGFP and rhodamine signals on the light micrograph. Locations where the eGFP and rhodamine signals are coincident can be identified by the blue color. Black arrows indicate eGFP and rhodamine-containing lysosomes. White arrows indicate non-lysosomal gut granules.

RNA Interference—To determine the role of CDR-1 in the resistance to cadmium toxicity, wild type C. elegans were fed E. coli that were transformed with a plasmid that expresses CDR-1 dsRNA. This technique has been shown to phenocopy null mutations and to be as efficient as injecting dsRNA (46). The phenotype of C. elegans that were fed CDR-1 dsRNA, in

| Stressor | Treatment condition | β-Galactosidase activity |
|----------|---------------------|-------------------------|
| Lead     | 30.2 or 60.4 μM     | −                       |
| Mercury  | 7.4 or 18.4 μM      | −                       |
| Copper   | 31.5 or 62.6 μM     | −                       |
| Zinc     | 6.2 or 124 μM       | −                       |
| Cadmium  | 100 μM              | +                       |
| Paraquat | 1 or 10 mM          | −                       |
| Jugalone | 40 or 100 mM        | −                       |
| Heat shock | 33 °C               | −                       |
| Control  | None                | −                       |

3 J. H. Freedman, unpublished observation.

![Fig. 7. Dose dependence of cdr-1 promoter activity in transgenic C. elegans. A mixed-stage population of JF9 (mtIs7, cdr-1::lacZ) C. elegans were exposed to various concentrations of CdCl2 for 24 h. The nematodes were then fixed, permeabilized, and stained for β-galactosidase activity for 2.5 h. The blue chloroformindigo precipitate was extracted with dimethylformamide and quantified as previously described (37).](image1)

![Fig. 8. Intracellular location of the CDR-1 protein in vivo. Transgenic C. elegans, which contain a cdr-1::CDR-1-eGFP expression vector (JF13 (mtEx10, cdr-1::CDR-1-GFP)), were fed RITC-dextran and then exposed to cadmium as described under “Experimental Procedures.” Upper panels, JF13 (mtEx10, cdr-1::CDR-1-GFP) C. elegans L2 larva. “L” and “Ph” indicate the location of the intestinal lumen and the nematode pharynx, respectively. Left panel, the location of CDR-1 visualized by fluorescent microscopy. Right panel, RITC-labeled lysosomes visualized using a rhodamine filter set. Lower panel, composite, high magnification (1000×) view of an adult JF13 (mtEx10, cdr-1::CDR-1-GFP) C. elegans. The nematode is oriented with the gonad on the left and the intestine on the right. Fluorescence images obtained using eGFP and rhodamine filter sets were superimposed on the light microphotograph. Locations where the eGFP and rhodamine signals are coincident can be identified by the blue color. Black arrows indicate eGFP and rhodamine-containing lysosomes. White arrows indicate non-lysosomal gut granules.](image2)
the absence of cadmium, was similar to control nematodes. These animals reproduced and developed similar to C. elegans grown in the absence of dsRNA and cadmium. In several of the C. elegans, small vesicles were observed in the body cavity (Fig. 9). In contrast, C. elegans that were fed CDR-1 dsRNA and grown in the presence of 50 μM cadmium were small, sterile, and failed to develop into adulthood. Growth and reproduction were slightly inhibited in nematodes grown in cadmium-containing medium, in the absence of dsRNA. However, these nematodes developed into adulthood and successfully reproduced. Microscopic examination of CDR-1 null nematodes exposed to cadmium showed that they accumulated fluid in the pseudo-ceolomic space, which eventually filled the entire space (Fig. 9). In addition, when gravid adult C. elegans, which were maintained in the absence of cadmium and dsRNA, were transferred to plates containing cadmium and dsRNA-containing bacteria, fluid accumulated in the body cavity. JF13 (mtEx10, cdr-1/CDR-1-GFP) C. elegans that were grown in the presence of cadmium and CDR-1 dsRNA exhibited a decrease in the level of CDR-1-eGFP, compared with JF13 (mtEx10, cdr-1/CDR-1-GFP) nematodes grown in cadmium alone (results not shown). The RNA interference results confirmed that CDR-1 is essential for defense against cadmium toxicity.

**DISCUSSION**

The reverse transcriptase-PCR protocol of differential display was used to identify new cadmium-responsive genes in C. elegans (17). Here we report on the cloning and analysis of a novel cadmium-inducible gene, designated cdr-1. CDR-1 encodes a predicted 277-amino acid, 32-kDa protein (Fig. 1). Amino acid sequence analysis identified two transmembrane-spanning domains, and it predicts that CDR-1 will be an integral membrane protein, which is targeted to the secretory pathway. The later prediction is supported by in vivo studies where it was clearly demonstrated that CDR-1 co-localizes with lysosomes (Fig. 8).

BLAST amino acid sequence analysis of CDR-1 showed that it is a novel protein. However, it does have regions that are homologous to the Drosophila FAX protein. FAX is a 47-kDa protein that is expressed in the Drosophila embryonic mesoderm and axons of the central nervous system. It has been shown that fox is constitutively transcribed during embryogenesis and that the protein is targeted to the plasma membrane (59). There are no reports that address the ability of environmental toxicants to induce fox transcription. In contrast, cdr-1 is transcribed only in intestinal cells in response to cadmium exposure. The function of FAX has not been fully defined. However, genetic data indicate that the protein is involved in signal transduction cascades, which affect neuronal development. The involvement of CDR-1 in signaling pathways is currently unknown, although the protein contains several potential phosphorylation sites. Because FAX and CDR-1 show (a) different patterns of cell-specific expression, (b) intracellular targeting, and (c) transcriptional regulation, their functions may be dissimilar. Because both proteins are targeted to cellular membranes, the shared amino acid homology may reflect intracellular targeting and not a common function.

cdr-1 transcription is induced in response to cadmium exposure (Figs. 5 and 7). In addition, the steady-state level of CDR-1 mRNA rapidly increases in the presence of this toxicant (Fig. 5). These observations confirm that cadmium is a potent activator of cdr-1 transcription. Cadmium-inducible cdr-1 transcription may be controlled by one of several potential UREs, which include AREs, an HSE, and an MRE. Both HSEs and AREs mediate stress-inducible transcription of a variety of eukaryotic genes. Cadmium has also been shown to activate transcription via these UREs (60, 61). However, heat shock or oxidative stress did not induce cdr-1 transcription in vivo (Table II), which suggests that these elements may not regulate cdr-1 transcription. The MRE is an obvious candidate as a regulator for cdr-1 transcription. It has been shown that MREs play essential roles in controlling cadmium-activated MT transcription in both vertebrates and invertebrates (62, 63). In contrast, there is evidence to suggest that MREs do not function in C. elegans. First, the mtl-1 promoter does not contain a consensus MRE sequence (19). Second, the MRE sequence that is present in mtl-2 does not control metal inducibility. Finally, analysis of the C. elegans genome and predicted protein data bases has not identified a homologue of the MRE-binding transcription factor MTF-1 (64). The contribution of MRE, HSE, and ARE in regulating cdr-1 transcription remains to be confirmed by further analysis.

Transgenic C. elegans and in situ hybridization studies confirm that cdr-1 is transcribed only in intestinal cells. Intestinal cell-specific expression of many C. elegans genes, including those encoding the vitellogenins, gut carboxylesterase, metal-inducible mtl-1 and mtl-2, P-glycoprotein, GATA-binding transcription factor-2, and aspartic and cysteine proteases, is dependent on the presence of one or more copies of UREs designated GATA elements (65–71). These elements are binding sites for members of the GATA family of transcription factors (69, 72). In C. elegans, GATA elements that are responsible for controlling intestinal cell-specific transcription have the consensus sequence (A/T)(GATA)(A/G). Consistent with these observations, two consensus GATA elements are present in the promoter/enhancer region of cdr-1 (Table I).

GATA elements are also found in the promoters of the two
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*C. elegans* MT genes. This raises the possibility that GATA elements can regulate metal-activated transcription. However, with the exception of *cdr-1*, *mtl-1*, and *mtl-2*, metal-activated transcription of genes that contain GATA elements has not been reported. In addition, it has been shown that GATA elements are not responsible for controlling metal-activated transcription of *mtl-1* or *mtl-2* (67).

The magnitude and rate of mRNA accumulation following cadmium exposure, and the sensitivities (i.e., the minimal concentration of metal that can induce transcription) of *cdr-1* are similar to those of *mtl-1* and *mtl-2* (19, 37, 73). In addition, the EC_{50} for cadmium-inducible *cdr-1* transcription is significantly below the 24-h LC_{50} for cadmium (2 μM versus ~8000 μM) (38). These observations suggest that cadmium is a specific activator of *cdr-1* transcription and that *cdr-1* expression is not induced as a secondary response to metal exposure (i.e., activated in response to cell death). In addition, the parallel responses of *cdr-1*, *mtl-1*, and *mtl-2* to cadmium exposure suggest that they may share common regulatory pathways.

A unique property of *cdr-1* is that cadmium has been found to be able to significantly induce its transcription (Table II). Typically, the transcription of cadmium-inducible genes is activated by a variety of stressors. For example, the transcription of MT, heme oxygenase, p58, cadmium exposure, and the sensitivities (concentration of metal that can induce transcription) of genes that contain GATA elements has not been reported. Several observations, however, support the hypothesis that *cdr-1* may function in osmoregulation in *C. elegans*. Expression of *cdr-1* and its function in the absence of metal remains to be resolved. In conclusion, *cdr-1* is expressed in intestinal cells in response to cadmium exposure. The gene encodes a novel protein that is targeted to lysosomes and is required for resistance to cadmium toxicity. The relation between *cdr-1* expression, cadmium detoxification, and osmoregulation in *C. elegans* remains to be explored.

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