J. Biochem. **139**, 967–979 (2006)
doi:10.1093/jb/mvj114

Identification and Reverse Genetic Analysis of Mitochondrial Processing Peptidase and the Core Protein of the Cytochrome bc_1 Complex of *Caenorhabditis elegans*, a Model Parasitic Nematode

Hiroyuki Nomura[1], Senarath B. P. Athauda[1,2], Hidenori Wada[1], Yumiko Maruyama[1], Kenji Takahashi[1] and Hideshi Inoue[1,*]

[1]Laboratory of Molecular Biochemistry, School of Life Science, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392; and [2]Department of Biochemistry, Faculty of Medicine, University of Peradeniya, Peradeniya and Institute of Fundamental Studies, Kandy, Sri Lanka

Received October 31, 2005; accepted March 26, 2006

Mitochondria could be a good target for anti-parasitic drugs. The α and β subunits of mitochondrial processing peptidase (MPP) and the core subunits of the cytochrome bc_1 complex, UCR-1 and UCR-2, are homologous to one another and are important for mitochondrial functions. However, our knowledge of these proteins in nematodes is very limited. *Caenorhabditis elegans*, a free-living nematode, has six genes coding for proteins homologous to these subunits. On primary structure comparison, and immunochemical and enzymological analyses, the gene products were assigned as follows: Y71G12B.24, α-MPP; ZC410.2, β-MPP; F56D2.1, UCR-1; VW06B3R.1, T10B10.2; and T24C4.1, UCR-2. The primary structures of β-MPP and UCR-1 from *Brugia malayi*, a parasitic nematode causing human filariasis, were deduced from their cDNA structures. Phylogenetic analysis showed that the UCR-1s from both *C. elegans* and *B. malayi* were less related to mammalian UCR-1s than to MPPs from various organisms. MPP and the bc_1 complex are essential for the life cycle of *C. elegans*, because their reverse genetic inhibition is lethal. This suggests the possibility that these proteins are also essential for the viability of *B. malayi* and other parasitic nematodes, and are potential targets for anti-parasitic agents.

Key words: *Caenorhabditis elegans*, metalloprotease, mitochondria, parasite, respiratory chain.

Abbreviations: BN-PAGE, blue native PAGE; dsRNA, double-stranded RNA; IPTG, isopropyl-β-D-thiogalactopyranoside; MPP, mitochondrial processing peptidase; ORF, open reading frame; PMSF, phenylmethanesulfonyl fluoride; UCR, ubiquinol cytochrome *c* reductase.

Lymphatic filariasis (elephantiasis), caused by the parasitic nematodes *Brugia malayi* and *Wuchereria bancrofti*, is one of the serious public health problems in tropical countries (*1*, *2*). Transmission of the disease occurs through the bite of a mosquito harboring infective larvae at the third larval stage (L3). The infective larvae migrate into the lymph glands and mature into adult worms within three to twelve months. The adults can survive for five to ten years, producing millions of microfilariae that circulate in the blood.

Proteolysis plays a major role in cellular catabolic and anabolic processes, and is essential for the survival of parasites. Moreover, proteolysis is essential for the parasitic life cycle including tissue penetration and evasion of host immune responses (*3*). Thus, some proteases are thought to be excellent potential targets for the design of novel anti-parasitic drugs. Indeed, aspartic proteases and cysteine proteases of *Plasmodium falciparum*, *etc.* are being studied for the development of anti-parasitic agents (*4*, *5*). In order to find potential targets for anti-filarial nematodes, we have screened the proteases of *Caenorhabdits elegans*, a free-living nematode, as a model parasitic nematode, for those whose reverse genetic inhibition causes inviable phenotypes (*6*–*9*). As a result, some proteins of zinc-metallopeptidase family M16B (*10*) have been found to be such candidates.

The subunits of mitochondrial processing peptidase (MPP) are representative members of peptidase family M16B. MPP plays an important role in the process of maturation of mitochondrial proteins as follows. The vast majority of mitochondrial proteins are encoded by nuclear genes and are synthesized on cytoplasmic ribosomes as larger precursors with an N-terminal extension peptide for targeting to mitochondria. The precursors are imported into the mitochondrial matrix with the aid of translocase complexes of the outer and inner membranes. After their import, their presequences are proteolytically removed by MPP (*11*, *12*). Many proteins mature through one-step cleavage by MPP.

MPP is located in the matrix of fungal and mammalian mitochondria, and in the inner membrane of plant mitochondria. Matrix-localized MPP is a heterodimeric protein with a molecular mass of ~100 kDa composed of two structurally related subunits, α-MPP and β-MPP, which exhibit 20–30% identity within any given species. Hetero-dimerization of these subunits is essential for the processing activity as demonstrated by the following fact;

*To whom correspondence should be addressed. Phone: +81 42 676 7256, Fax: +81 42 676 7256, E-mail: hinoue@ls.toyaku.ac.jp

neither recombinant α-MPP nor β-MPP alone shows proteolytic activity, but a mixture of the two proteins exhibits the activity (*13–15*).

The β-MPP subunit has a metal-binding motif, H*XX*EH(X)$_{72–76}$E, whose H*XX*EH is an inversion of the thermolysin metal-binding motif HE*XX*H, and serves as a catalytic subunit (*16*). In the zinc-binding motif of β-MPP, both His residues and the distal Glu residue participate in metal binding, and the first Glu residue is involved in water activation for hydrolysis of the peptide bond. This motif is present in all known β-MPP subunits but is less conserved in α-MPP subunits (*12*), in many of which the motif is conserved as H*XX*(E/D)(K/R). The α-MPP subunit is not directly involved in catalysis, but is implicated in substrate recognition (*17–21*). The α-MPP subunit has a characteristic Gly-rich segment in its middle portion, which is essential for binding and cleavage of the precursor proteins (*19*).

Based on the primary structure, the core 1 (UCR-1) and core 2 (UCR-2) subunits of mitochondrial ubiquinol-cytochrome *c* oxidoreductase (UCR), also known as the cytochrome bc_1 complex or complex III, which is a component of the respiratory chain in the mitochondrial inner membrane, are also classified into peptidase family M16B. UCR-1 and UCR-2 are structurally related to β-MPP and α-MPP, respectively. In *Saccharomyces cerevisiae* and mammals, MPP is similar but not identical to the core complex of UCR and is located in the matrix. In *Neurospora crassa*, however, β-MPP is identical to UCR-1 and a large fraction of it is incorporated into UCR (*22*). In plants, MPP is completely integrated into UCR, UCR-1 and UCR-2 being identical to β-MPP and α-MPP, respectively (*23–26*).

Interestingly, bovine heart UCR after Triton X-100 treatment was determined to have MPP-like activity (*27*, *28*). Also, mixing of the purified recombinant bovine UCR-1 and UCR-2 proteins restored the activity (*28*). Thus, the core complex has MPP-like peptidase activity. The UCR-1 subunit, however, has a Y*XX*EHX_{75}E motif instead of the metal-binding H*XX*E$X_{72–76}$E motif of the β-MPP subunit. The Tyr residue in this motif can be replaced with His, Phe, or Trp, but not with Thr, without loss of activity. The fact that bovine UCR-1 and its derivatives, which have a (Y/F/W)*XX*EX_{75}E motif instead of H*XX*EHX_{75}E, have protease activity suggests the possibility that the core proteins of other organisms also have MPP activity. It is unknown, however, whether or not it is a common feature of the core proteins of various organisms that they possess peptidase activity.

In the *C. elegans* genome, six genes, ZC410.2, F56D2.1, T24C4.1, T10B10.2, VW06B3R.1, and Y71G12B.24, were found to code for proteins homologous to the MPPs and core protein subunits from other organisms. Our knowledge of the family M16B proteins in nematodes, however, is very limited. Only a partial cDNA that encodes a polypeptide homologous to the *C. elegans* F56D2.1 gene product has been isolated from *Ostertagia circumcincta* (*29*), and the encoded protein is regarded as an unusual member of peptidase subfamily M16B because of the difficulty in assigning it to any particular subunit group of MPP or UCR. In this study, we attempted to assign the six gene products of *C. elegans*. Next, analysis involving site-directed mutagenesis was performed on the metal-binding motif of *C. elegans* β-MPP to investigate the structure/function relationship of the motif. Additionally, cDNAs for β-MPP and UCR-1 from *B. malayi* were identified. Because both MPP and the core protein of UCR were demonstrated to be essential for the *C. elegans* life cycle on reverse genetic analyses, those in the parasitic nematode are also thought to be essential and hence a good target for novel antifilarial drugs.

MATERIALS AND METHODS

Materials—The *B. malayi* EST clones, SW3ICA2657 and SWMFCA1058, and a cDNA library from third stage larvae of *B. malayi*, SAW94WL-BmL3, were obtained from Dr. S. A. Williams (Smith College). The *C. elegans* EST clones, yk811h12, yk97h7, yk1189e02, and yk103g6, were obtained from Dr. Y. Kohara (National Institute of Genetics, Japan). The EST clone, cm18g6, was obtained from Dr. R. H. Waterston (Washington Univ.). A plasmid vector for RNAi, pPD129.36, was obtained from Dr. A. Fire (Carnegie Institution of Washington). The *C. elegans* strains with mutant alleles, ZC410.2(tm1326) and F56D2.1(tm1181), were obtained from Dr. S. Mitani (Tokyo Women's Medical University). Polyclonal goat anti-rabbit immunoglobulin/HRP was purchased from Dako Cytomation. Other reagents used were of the highest grade available.

Cloning and Sequencing of cDNAs for C. elegans Family M16B Proteins—On sequencing, EST clones yk811h12, yk97h7, and yk1189e02 were found to include the complete open reading frames (ORFs) for the Y71G12B.24, F56D2.1, and VW06B3R.1 proteins, respectively. For ZC410.2, T10B10.2, and T24C4.1, the predicted ORFs were amplified by PCR from reverse-transcripts, which were prepared with a TimeSaver cDNA Synthesis Kit (Amersham Biosciences) from the RNA extracted from *C. elegans* with an RNA Isolation Kit (Stratagene). PCR was performed using a thermal cycler GeneAmp PCR system 2400 (Perkin-Elmer Applied Biosystems), and synthetic primers ZC410N3 and ZC410C for ZC410.2, T10N and T10C for T10B10.2, and T24N and T24C for T24C4.1 (Table 1). DNA sequencing was performed by the dideoxy chain termination method with a Dye terminator Cycle Sequencing Kit and a DNA sequencer ABI PRISM^{TM} 377 (Applied Biosystems).

Cloning of Bm-MPP-β and Bm-UCR-1—Clones SW3ICA2657 and SWMFCA1058 were found to code for proteins homologous to peptidases of the M16B family on a BLAST search (*30*) of the *B. malayi* EST database. On sequencing, SWMFCA1058 was found to have a complete ORF (*Bm*-MPP-β), while SW3ICA2657 was incomplete in the 5′ region. A DNA fragment was amplified from SW3ICA2657 by PCR using a pair of primers, T3 and M4A, which recognize the T3 promoter on the vector DNA and a sequence in the inserted DNA, respectively (Table 1). The amplified DNA was used as a probe to screen the SAW94WL-BmL3 cDNA library for a clone containing an entire ORF (*Bm*-UCR-1).

Preparation of Polyclonal Antibodies Against C. elegans Family M16B Proteins—The complete or partial ORF sequence was amplified from each cDNA clone by PCR using the following pairs of synthetic primers; ZC410NM and ZC410C for ZC410.2; Y71NM and Y71C for Y71G12B.24; F56NM and F56C for F56D2.1; VW06NM

Table 1. **PCR primers.**

Primer	Direction	Sequence (designed restriction site[a])
F56C	rev	CGGCGGGATCCATTCCTCCACCACGACAT (*Bam*HI)
F56C2	rev	GCGCGTTAATTAACTATTACATTCTCCACCACGAC (*Pac*I)
F56NM	fwd	CGCGCCCATGGTCTCCGTGAAGGACGT (*Nco*I)
F56NM2	fwd	GCGCGCATATGGTCTCCGTGAAGGACGTGC
M4A	rev	GAGATGTCATGGCGTTCAC (*Nde*I)
T3	fwd	AATTAACCCTCACTAAAGGG
T10C	rev	CGCGGAATTCAGAGTATCAACATATGGG (*Eco*RI)
T10N	fwd	CGCGCCATATGCTTAGCCGCAACATTG (*Nde*I)
T10NM	fwd	TATATGCGCGCCATGGGCACGACAAAACCAGTCGAAAAAGTG (*Nco*I)
T24C	rev	CGCGCCCATGGAAAGCTCGTCGGCATATG (*Nco*I)
T24N	fwd	CGGCGCATATGAGATCATCGTTTGTGTC
T7	fwd	TAATACGACTCACTATAGGG (*Nde*I)
VW06C	rev	GCGCCTCGAGTAGCTCGTCGAGATATGGG (*Xho*I)
VW06C2	rev	GCGCGCCATGGATAGCTCGTCGAGATATGG (*Nco*I)
VW06NM	fwd	GCGCGCCATGGTCTCGGCTGCAGCCAAATC (*Nco*I)
Y71C	rev	CCCAAGCTTATTGAACAAATATTTCAAATC (*Hin*dIII)
Y71C2	fwd	GCGCGTTAATTAATTATCAATTGAACAAATATTTCAAATC (*Pac*I)
Y71C3	rev	CGCGCGTCGACCTATCAATTGAACAAATATTTCAAATC (*Sal*I)
Y71NM	fwd	CATGCCATGGCGAGGGTTACTAGACTTCCG (*Nco*I)
Y71NM2	fwd	GCGCGCATATGTCGAGGGTTACTAGACTTC (*Nde*I)
Y71NM3	fwd	CGCGCGGATCCTCGAGGGTTACTAGACTTC (*Bam*HI)
ZC410C	rev	GCGCCTCGAGTTGCATTCTGATCAATCTTC (*Xho*I)
ZC410C2	rev	GCGCGCCATGGATTGCATTCTGATCAATCTTC (*Nco*I)
ZC410H100F	rev	TGAACGCCATATGCTCTAGAAAAAAAGCTGTTCC (*Nde*I)
ZC410H100N	rev	TGAACGCCATATGCTCTAGAAATTAAGCTGTTCC (*Nde*I)
ZC410H104Rs	fwd	TTTCTAGAGCGTATGGCGTTC
ZC410H104Ra	rev	GAACGCCATACGCTCTAGAAA
ZC410H100Y	rev	TGAACGCCATATGCTCTAGAAAATAAGCTGTTCC (*Nde*I)
ZC410H100YL102V	rev	TGAACGCCATATGCTCTACAAAATAAGCTGTTCC (*Nde*I)
ZC410N3	fwd	GCGCGCCATGGACAGAAGACTAGCC (*Nco*I)
ZC410NM	fwd	GCCGCCATATGCAAGTTCAGCCGAAATCAG (*Nco*I)

[a]The restriction sites used for the construction of plasmids are underlined.

and VW06C for VW06B3R.1; T10N and T10C for T10B10.2; and T24N and T24C for T24C4.1 (Table 1). After each amplified DNA had been digested at the restriction sites designed in the PCR primers, it was inserted into the pET22b vector (Novagen) in the cases of ZC410.2 (pET22b-ZC410.2), Y71G12B.24, F56D2.1 and VW06B3R.1, or into the pET21d vector in the cases of T10B10.2 and T24C4.1 for fusion with an $(His)_6$-tag at the C-termini. *Escherichia coli* BL21-CodonPlus^{TM}(DE3)-RIL (Stratagene) cells were transformed with the resulting plasmids. The transformed cells were cultured in M9ZB medium containing 50 μg/ml ampicillin and 30 μg/ml chloramphenicol with vigorous shaking at 37°C until the turbidity at 600 nm reached 0.6. Then isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and the culture was continued for an additional 3 h. The cells were harvested by centrifugation, and suspended in 50 mM Tris/HCl buffer, pH 8.0, containing 1 mM EDTA, 0.1 M NaCl, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1 mg/ml lysozyme. The suspension was kept on ice for 30 min, sonicated for 20 s × 10 times, and then centrifuged at 2,600 × *g* for 10 min. The precipitated inclusion bodies were washed with 50 mM Tris/HCl, pH 8.0, containing 0.1 M NaCl, 0.5% Triton X-100, and 1 mM PMSF, and additionally containing 2 M urea for ZC410.2, Y71G12B.24, and F56D2.1, 3 M urea for T10B10.2, and 4 M urea for T24C4.1, with stirring at room temperature for 2 h. Each suspension was centrifuged at 12,000 × *g* for 30 min. The recombinant protein was extracted from the inclusion bodies into 20 mM sodium phosphate buffer, pH 7.8, containing 8 M urea and 0.5 M NaCl (the urea buffer) by stirring overnight at room temperature. The suspension was centrifuged at 18,000 × *g* for 30 min and the supernatant was applied to a column of Ni^{2+}-immobilized chelating Sepharose Fast Flow (Amersham Biosciences). The proteins were eluted by a stepwise elution with urea buffers containing 50, 100, 200, 350, and 500 mM imidazole. Fractions containing the recombinant protein were concentrated using an Amicon Ultra-15 PLTK Ultracel-PL Membrane (Millipore) and then subjected to SDS-PAGE with a Disk Preparative Electrophoresis System (Nihon Eido). The fractions giving a single band of the recombinant protein on SDS-PAGE were pooled. After acetone precipitation, the pellet was suspended in 20 mM sodium-phosphate buffer, pH 7.4, containing 0.9% NaCl, mixed with three volumes of complete Freund's adjuvand (Wako), and then injected into rabbits to raise antisera. The antibodies raised were purified on a column of rProteinA Sepharose Fast Flow (Amersham Biosciences).

Blue Native (BN)-PAGE—Mitochondria were isolated from the *C. elegans* N2 (Bristol) strain as described (*31*). The mitochondria extracts (200–400 μg protein) were centrifuged at 200,000 × *g* for 5 min at 4°C. The pellets were suspended in 40 μl of 50 mM imidazole/HCl buffer, pH 7.0, containing 50 mM NaCl, 5 mM 6-aminohexanoic acid and 1.5% dodecyl-β-D-maltoside, and then allowed to stand on ice for 30 min. After centrifugation at 17,000 × *g* for 20 min at 4°C, 0.35% Serva blue G-250 (dissolved in 500 mM 6-aminohexanoic acid) was added to the supernatant, which was then subjected to BN-PAGE. BN-PAGE was performed essentially according to Schagger (*32*). Blotted proteins were detected with the antibodies raised against the family M16B proteins, polyclonal goat anti-rabbit immunoglobulin/HRP and ECL Western Blotting Detection Reagents (Amersham Bioscience).

Preparation of a Recombinant Protein by Coexpression—The cDNA sequences encoding the mature ZC410.2, Y71G12B.24, F56D2.1 and VW06B3R.1 proteins were amplified from each cDNA clone by PCR using the following pairs of primers: ZC410NM and ZC410C2 for ZC410.2; Y71NM2 and Y71C2 for Y71G12B.24; F56NM2 and F56C2 for F56D2.1; and VW06NM and VW06C2 for VW06B3R.1 (Table 1). For co-expression, the amplified DNAs of Y71G12B.24 and ZC410.2 or VW06B3R.1 and F56D2.1 were digested with appropriate restriction enzymes (Table 1) and then cloned into multi-cloning sites 1 and 2, respectively, of the pETDuet-1 vector (Novagen). The resulting plasmid pETDuet-Y71G12B.24/ZC410.2 was co-transformed with pG-KJE8 (Takara), carrying the dnaK, dnaJ, grpE, groES, and groEL chaperone genes, into *E. coli* BL21(DE3), while pETDuet-VW06B3R.1/F56D2.1 was transformed into *E. coli* BL21-CodonPlus™(DE3)-RIL (Stratagene). A pre-culture was performed overnight at 37°C in M9ZB containing 50 μg/ml ampicillin and 30 μg/ml chloramphenicol. The preculture (1% by volume) was added to fresh M9ZB containing 50 μg/ml ampicillin and 30 μg/ml chloramphenicol for VW06B3R.1/F56D2.1, or to M9ZB containing 50 μg/ml ampicillin, 30 μg/ml chloramphenicol, 2% arabinose, and 10 μg/ml tetracycline for Y71G12B.24/ZC410.2. The culture was performed at 37°C with vigorous shaking until OD_{600} reached 0.4. Then IPTG was added to a final concentration of 0.1 mM. After the culture had been continued at 15°C for 24 h, the cells were harvested by centrifugation at 6,000 × *g* for 10 min and suspended in sodium phosphate buffer, pH 7.0, containing 300 mM NaCl (lysis buffer), then lysozyme was added to a final concentration of 1 mg/ml. The suspension was kept on ice for 30 min, sonicated, and then centrifuged at 180,000 × *g* for 35 min. The supernatant was applied to a column of TALON™ Metal Affinity Resin (Clontech) equilibrated with the lysis buffer, and washed with 20 volumes of the buffer. The proteins were eluted by stepwise elution with the lysis buffer containing 50, 75, 100, and 200 mM imidazole. The fractions containing the recombinant protein were concentrated with an Amicon Ultra-15. The concentrated solutions were diluted with 100 volumes of 20 mM HEPES/KOH, pH 7.5, containing 0.1% Tween-20, and then concentrated again. The protein solution obtained was stored at –30°C.

Preparation of Recombinant Y71G12B.24 and ZC410.2 Subunits—The putative mature region of Y71G12B.24 amplified by PCR using a pair of primers, Y71NM3 and Y71C3 (Table 1), was inserted into the *Bam*HI/*Sal*I site of the pGEX-6P-3 vector (Amersham Biosciences), which was designed to express the recombination protein with a GST-tag fused at the N-terminus. For expression, *E. coli* BL21 transformed with the resulting plasmid was cultured in M9ZB containing 50 μg/ml ampicillin at 37°C with vigorous shaking. When OD_{600} reached 0.4, IPTG was added to a final concentration of 0.1 mM and the cells were cultured at 15°C for 24 h. The cells were harvested by centrifugation and suspended in PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.3) containing 1 mg/ml lysozyme. The suspension was kept on ice for 30 min and then sonicated. After the addition of 0.1% Tween-20, the lysate was stirred at 4°C for 20 min, and then centrifuged at 180,000 × *g* for 30 min. The supernatant was applied to a column of glutathione Sepharose 4 Fast Flow (Amersham Biosciences) equilibrated with PBS. By using a protease buffer (50mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20, and 1mM DTT) containing PreScission Protease (Amersham Biosciences), the recombinant Y71G12B.24 protein with the GST-tag removed was obtained. The fractions containing the recombinant protein were concentrated with an Amicon ultrafree-15 and then dialyzed against 20 mM HEPES-KOH, pH 7.5, containing 0.1% Tween-20. After the addition of 20% glycerol, the sample was stored at –30°C. To prepare the ZC410.2 recombinant protein, *E. coli* BL21(DE3) was co-transformed with expession plasmid pET22b-ZC410.2 and pG-KJE8. Expression and preparation of the recombinant protein were performed as in the case of Y71G12B.24/ZC410.2 co-expression.

MPP Activity Assay—As a substrate, the N-terminal 21 residues of mouse malate dehydrogenase precursor MDH1-21 (MLSALARPVGAALARSFSTSA) (*33*) were chemically synthesized using a solid phase peptide synthesizer, PSSM-8 (Shimadzu). A peptide comprising the C-terminal 5 residues of the presequence and the N-terminal 20 residues of the mature form of the subunit V of bovine UCR, $_{-5}V_{+20}$ (ASVRYSHTDIKVPDFSDYRR-PEVLD) (*27*, *28*), was also synthesized. 3 μg of the Y71G12B.24/ZC410.2 or VW06B3R.1/F56D2.1 co-expression protein was mixed with 5 nmol of MDH1–21 or 9 nmol of $_{-5}V_{+20}$ in a reaction buffer (20 mM HEPES/KOH, 0.1% Tween-20, pH 7.5) containing 1 μM $ZnCl_2$, and then incubated at 25°C (*33*). In the case of VW06B3R.1/F56D2.1, an alternative procedure was also used, as follows; 5 nmol of MDH1-21 or 9 nmol of $_{-5}V_{+20}$ was added after freezing of the enzyme in the reaction buffer at –80°C for more than 6 h (*28*). The total volume of the reaction mixture was 0.2 ml in every case. The reaction was stopped by the addition of trifluoroacetic acid to a final concentration of 0.05%. Each mixture was chilled on ice for 20 min and then centrifuged at 17,000 × *g* for 15 min. The supernatant was subjected to HPLC on a TSKgel-ODS-120T column (150 × 2.2 mm, Tosoh). Elution was performed with a linear gradient of 0–50% acetonitrile in 0.1% trifluoroacetic acid. The substrate and product peptides were monitored at 215 nm. The cleavage site of the substrate was determined by analyzing the product peptides using an HPLC apparatus (1100 series, Agilent Technologies) with a TSKgel-ODS-120T column (150 × 2.2 mm, Tosoh) eluted with a gradient of acetonitrile (0–60% in 60 min), connected to an LCQ™ DUO mass

spectrometer (ThermoQuest). To reconstruct MPP with the independently expressed ZC410.2 and Y71G12B.24 proteins, 0.1 μg of each protein was mixed and incubated with 5 nmol of MDH1-21 in the reaction buffer containing 2 μM $ZnCl_2$ at 25°C. The total volume was 0.1 ml.

RNAi—RNAi was performed according to the soaking method (*34*). The DNA templates for in vitro synthesis of double-stranded RNAs (dsRNAs) were prepared as follows. The 749-bp *Sac*I/*Apa*I fragment from cm18g6 for ZC410.2, the 897-bp *Nco*I/*Xho*I fragment from yk97h7 for F56D2.1, and the 1233-bp *Xho*I/*Xho*I fragment from yk1189e02 for VW06B3R.1 were subcloned in the corresponding respective cloning sites of pPD129.36. For Y71G12B.24, cDNA was amplified with a pair of primers, Y71NM and Y71C (Table 1), and its 1,388-bp *Nco*I/*Hin*dIII fragment was cloned into pPD129.36. For T10B10.2, cDNA was amplified with a pair of primers, T10NM and T10C, and its 729-bp *Cla*I/*Eco*RI fragment was cloned into pPD129.36. For T24C4.1, DNA was amplified with a pair of primers, T24N and T24C, and its 898-bp *Nco*I fragment was cloned into pPD129.36. From the resulting plasmids, the DNA templates for RNA synthesis were amplified by PCR using the T7 primer (Table 1).

Site-Directed Mutagenesis of ZC410.2—Four mutant structural genes for ZC410.2, H100Y, H100Y/L102V, H100F, and H100N, were prepared by PCR. Mutant DNA was amplified from cDNA using the ZC410NM primer and each synthetic primer, ZC410H100Y, ZC410H100YL102V, ZC410H100F, or ZC410H100N (Table 1). Each amplified DNA was digested with *Nco*I and *Nde*I, and then inserted into the *Nco*I/*Nde*I sites of the pET22b-ZC410.2 plasmid. The H104R mutant gene was prepared by PCR as follows. The 5′-half of ZC410.2 was amplified with a pair of primers, ZC410NM and ZC410H104Ra, and the 3′-half was amplified with ZC410H104Rs and ZC410C. The amplified products were purified and mixed, and then PCR was performed on the mixture using a pair of primers, ZC410NM and ZC410C. The amplified DNA was inserted into the *Nco*I/*Xho*I sites of the pET21d vector. Recombinant proteins were prepared as described above.

RESULTS

Assignment of C. elegans α/β-MPP and UCR-1/2 Genes by Primary-Structural Comparison—In the *C. elegans* genome database, six genes, Y71G12B.24, ZC410.2, F56D2.1, T10B10.2, T24C4.1, and VW06B3R.1, were found to code for proteins homologous to the subunits of MPP or the UCR core protein in a BLAST search. On sequencing of the complete ORFs of their transcripts, we deduced their amino acid sequences, which were consistent with the predicted structures in the database (Fig. 1).

The *C. elegans* protein most homologous to human α-MPP is the Y71G12B.24 protein (Table 2), and the Y71G12B.24 protein is also most homologous to α-MPP (Table 3). Additionally, only the Y71G12B.24 protein among the six gene products has the Gly-rich segment characteristic of α-MPP (Figs. 1 and 2A). Therefore, the Y71G12B.24 gene is predicted to code for α-MPP. On the other hand, the *C. elegans* protein most homologous to both human β-MPP and UCR-1 is the ZC410.2 protein. Because the ZC410.2 protein is most homologous to β-MPP (Tables 2 and 3), and because only the ZC410.2 protein among the six gene products contains the complete metal-binding H*XX*EH(*X*)$_{72–76}$E motif characteristic of β-MPP (Fig. 1), it is predicted to be β-MPP. In addition, Glu191 and Asp195 that are important for the proteolytic activity of rat β-MPP (*33*) are conserved in the ZC410.2 protein (Fig. 2B). Thus, Y71G12B.24 and ZC410.2 were predicted to be the MPP subunits.

The protein encoded by F56D2.1 is also most homologous to β-MPP, but β-MPP is less homologous to the F56D2.1 protein than to the ZC410.2 protein (Table 1). Moreover, the F56D2.1 protein has an Arg residue in place of the second His residue in the H*XX*EH$_{72–76}$E motif that is conserved in all known β-MPPs (Fig. 2B). Therefore, the F56D2.1 protein is not thought to be β-MPP. Since the F56D2.1 protein is also homologous to UCR-1, it is thought to be a candidate for UCR-1, although the possibility that the ZC410.2 protein plays dual roles as β-MPP and UCR-1 similar to in the case of *N. crassa*, in which the β-MPP and UCR-1 molecules are identical, remains. The T10B10.2, T24C4.1, and VW06B3R.1 proteins are most homologous to UCR-2, so they are candidates for UCR-2 (Table 1). It is uncertain, however, whether or not all of them actually serve as UCR-2. Thus, reliable assignments were not possible only through primary structural comparison of the UCR subunits.

B. malayi cDNAs Coding for Proteins Homologous to β-MPP and UCR-1—*B. malayi* cDNAs homologous to *C. elegans* ZC410.2 and F56D2.1 were obtained as described under "MATERIALS AND METHODS," and the proteins encoded were named *Bm*-MPP-β and *Bm*-UCR-1, respectively. *Bm*-MPP-β has a conserved Zn-binding motif, H*XX*EH*X*$_{75}$E, characteristic of β-MPP, but *Bm*-UCR-1 has N*XX*EH*X*$_{75}$E at the corresponding position (Figs. 1 and 2B). The motif in *Bm*-UCR-1 is different from H*XX*ER*X*$_{75}$E in the *C. elegans* F56D2.1 protein and also from Y*XX*EH*X*$_{75}$E in mammalian UCR-1 proteins.

Phylogenetic Relationship—Phylogenetic analysis was performed on the family M16B proteins including those from *C. elegans* and *B. malayi*. In the phylogenetic tree, the Y71G12B.24 protein is in the same subgroup as the α-MPPs from other organisms, and the VW06B3R.1, T10B10.2, and T24C4.1 proteins are in the subgroup of UCR-2 proteins (only those from *C. elegans* and man are shown in Fig. 3). The ZC410.2 protein and *Bm*-MPP-β belong to the same subgroup as the β-MPPs from other organisms (Fig. 3). This is consistent with the assignment mentioned above. Interestingly, however, the *C. elegans* F56D2.1 protein and *Bm*-UCR-1 as well as UCR-1 from *Saccharomyces cerevisiae* are not in the same subgroup as the mammalian UCR-1 proteins. The mammalian UCR-1s as well as putative UCR-1s from zebrafish and frog are more related to the β-MPPs from various organisms than to the F56D2.1 protein and *Bm*-UCR-1.

The F56D2.1, VW06B3R.1, T10B10.2, and T24C4.1 Proteins Were Immunochemically Detected in the UCR Complex—To experimentally identify the core subunits of UCR, immunochemical analyses were performed. Polyclonal antibodies were raised against each of the six gene products using the recombinant proteins expressed in *E. coli*. Anti-T10B10.2, anti-T24C4.1, and anti-VW06B3R.1 antibodies showed some cross-reactivity to their authentic antigens, but no cross-reactivity was

Fig. 1. **Sequence alignment of the peptidase family M16B proteins from *C. elegans* and *B. malayi*.** The glycine-rich region conserved in α-MPP is underlined. The His and Glu residues conserved in the metal binding H*XX*EH$(X)_{72-76}$E motif of β-MPP are indicated by #, and the Glu and Asp residues corresponding to those that are important for the activity of rat β-MPP are indicated by *. *Ce, C. elegans*; *Bm, Brugia malayi*.

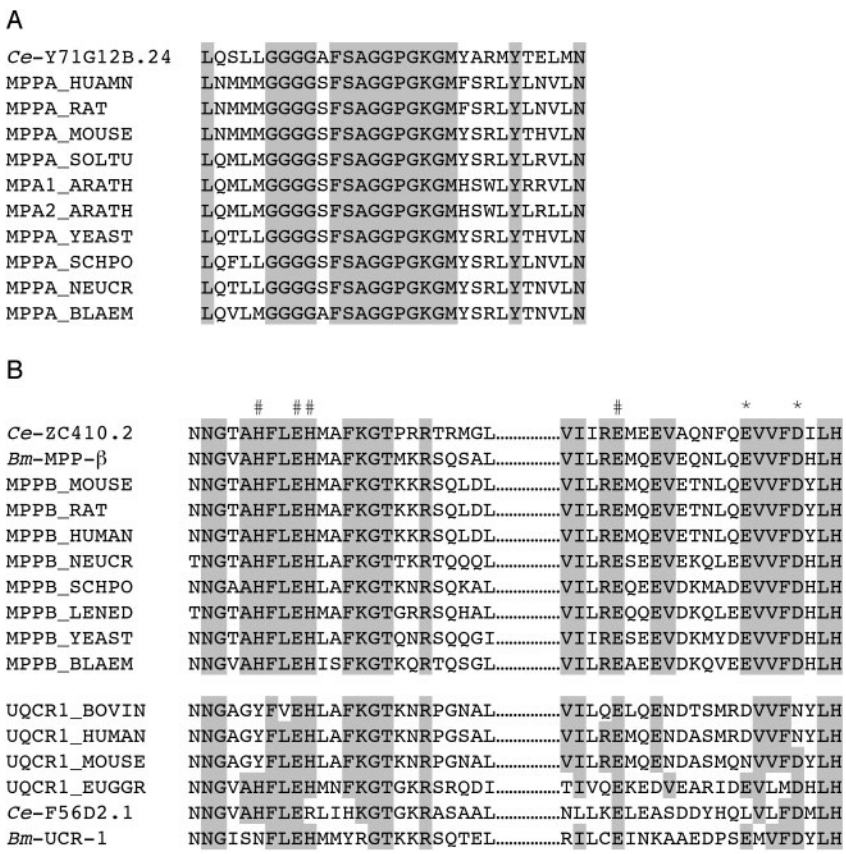

Fig. 2. **Alignment of the glycine-rich region of α-MPP (A), and the metal binding regions of β-MPP and UCR-1 (B).** (A) The residues completely conserved among the α-MPPs are shadowed. (B) The residues completely conserved among the β-MPPs are shadowed. The conserved residues in the metal-binding H*X*XEH(X)$_{72–76}$E motif are indicated by #. The Glu and Asp residues corresponding to those important for the activity of rat β-MPP (*33*) are indicated by *. The sequence data other than those for nematode proteins were obtained from the Swiss-Prot database.

Table 2. **The *C. elegans* genes that code for family M16B proteins, and the known proteins most homologous to them**.

Gene	Amino acid residue	Homologue	Amino acid residue	BLAST Expect	Identity
ZC410.2	458	Rat β-MPP	489	e-118	211/433 (48%)
Y71G12B.24	514	Human α-MPP	525	3e-93	171/455 (37%)
F56D2.1	471	Human β-MPP	489	1e-87	166/448 (37%)
T10B10.2	422	Human UCR-2	453	3e-35	110/414 (26%)
T24C4.1	427	Rat UCR-2	452	1e-30	121/441 (27%)
VW06B3R.1	410	Bovine UCR-2	453	1e-20	105/413 (25%)

Table 3. **The *C. elegans* proteins most homologous to the subunits of MPP and of the core protein of UCR from man.**

Human proteins	Amino acid residue	*C. elegans* homologue	Amino acid residue	Identity
α-MPP	525	Y71G12B.24	514	37%
β-MPP	489	ZC410.2	458	47%
UCR-1	480	ZC410.2	458	41%
UCR-2	453	T10B10.2	422	26%

observed among the other antibodies. Using the antibodies, Western blotting analysis was carried out on extracts from *C. elegans*. Although none of the gene products could be detected in the extracts from the whole animals, the F56D2.1, VW06B3R.1, and T10B10.2 proteins were detected in the mitochondrial membrane fraction. In addition, the T24C4.1 protein was detected faintly. Neither the ZC410.2 protein nor the Y71G12B.24 protein, however, could be detected in any fraction. On Western blotting after separation of the mitochondrial membrane protein complexes by BN-PAGE according to their size, one of the bands was found to react with both the anti-F56D2.1 and anti-VW06B3R.1 antibodies, and very faintly with the anti-T10B10.2 antibody (Fig. 4B, lanes 4, 5, and 7). When a higher concentration of the anti-T10B10.2 or anti-T24C4.1 protein antibody was used, immunochemical staining was also observed for the same band (Fig. 4B, lanes 8 and 9). Neither the anti-ZC410.2 nor the anti-Y71G12B.24 protein antibody stained any of the bands with high molecular masses. These facts suggest the possibility that the VW06B3R.1, T10B10.2, and T24C4.1 proteins form a complex with the F56D2.1 protein, and are probably involved in UCR (complex III) (*35*).

Association of the Recombinant ZC410.2 and Y71G12B.24 Proteins Restores MPP Activity—To confirm the assignment of the MPP genes, reconstitution of MPP from the recombinant proteins was examined. Although

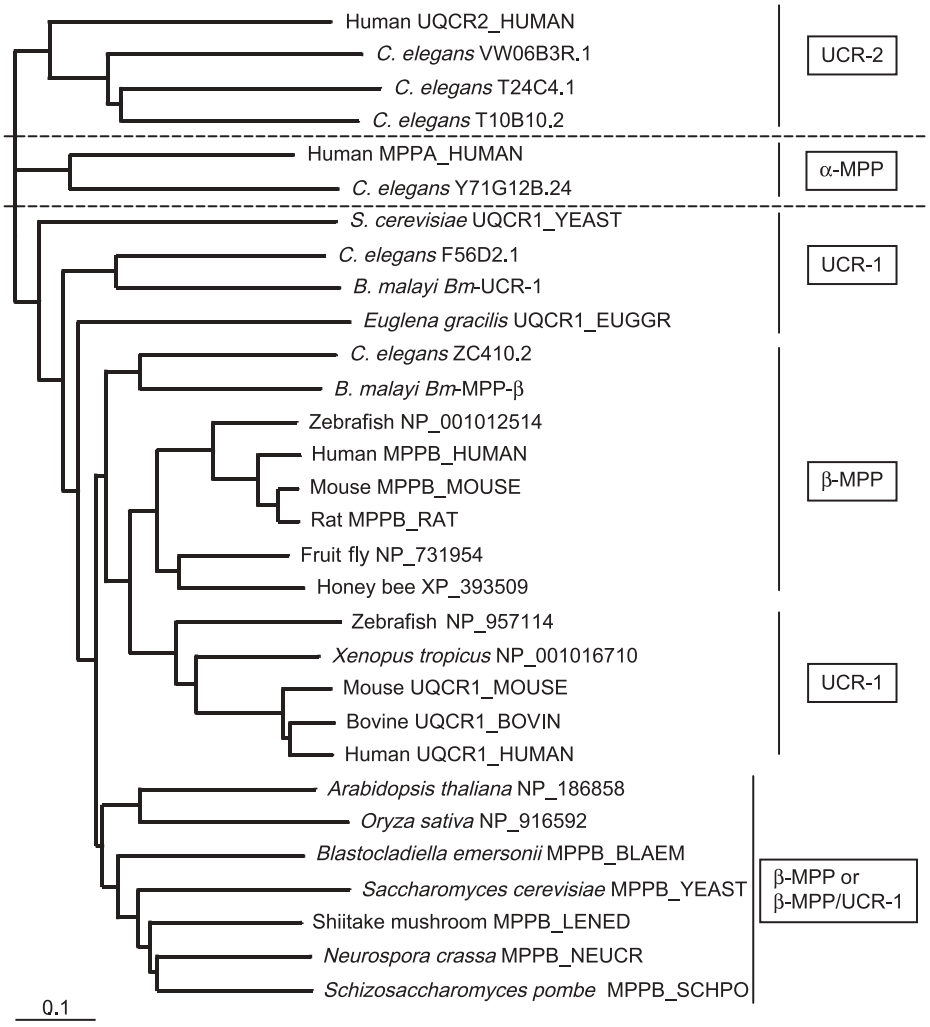

Fig. 3. **Phylogenetic relationship of β-MPP and UCR-1 proteins.** An unrooted neighbour-joining phylogenetic tree was drawn based on the results obtained with Clustal W (*39*).

neither the ZC410.2 nor the Y71G12B.24 protein showed any proteolytic activity independently (Fig. 5, A and B), a mixture of the two proteins was found to cleave a substrate peptide, MDH1-21, at the predicted processing site into two fragments, MLSALAPRVGAALRRS and FSTSA, which were identified on LC-MS analysis (Fig. 5C). This proteolytic activity was inhibited by EDTA, a metallopeptidase inhibitor (Fig. 5D). Therefore, the metallopeptidase activity of MPP is suggested to depend on the association of the ZC410.2 and Y71G12B.24 proteins.

The ZC410.2 and Y71G12B.24 proteins were also co-expressed in *E. coli* as soluble forms. In this case, the ZC410.2 protein was expressed as a fusion protein with a $(His)_6$-tag, and the Y71G12B.24 protein was expressed without any tag. On affinity purification on Ni^{2+}-immobilized chelating Sepharose, the Y71G12B.24 protein was co-purified with the ZC410.2-$(His)_6$ protein (data not shown), and equivalent amounts of the two proteins were detected on SDS-PAGE. This suggests that the ZC410.2 and Y71G12B.24 proteins form a 1:1 complex. The purified complex was also found to cleave MDH1-21 at the predicted site (Fig. 5E), which was inhibited by EDTA (Fig. 5F). These results are consistent with that Y71G12B.24 and ZC410.2 are α-MPP and β-MPP, respectively.

The Recombinant F56D2.1 and VW06B3R.1 Proteins Form a Complex but Show No Peptidase Activity—Among the three UCR-2 candidates, the VW06B3R.1 protein is the major one, as shown on Western blot analysis (Fig. 4). To confirm that the F56D2.1 protein forms a complex with the VW06B3R.1 protein, they were co-expressed in *E. coli*, a $(His)_6$-tag only being fused to the F56D2.1 protein. Upon purification on Ni^{2+}-immobilized chelating Sepharose, an equivalent amount of the VW06B3R.1 protein was copurified with F56D2.1. This fact suggests the formation of a 1:1 complex between the F56D2.1 and VW06B3R.1 proteins, consistent with that they are the UCR-1 and UCR-2 proteins, respectively. Using the purified complex, peptidase activity toward synthetic peptide MDH1–21 and also $_{-5}V_{+20}$ as substrates was assayed. No significant activity, however, was detected (data not shown). In addition, no peptidase activity was detected in the mixture of the independently expressed recombinant F56D2.1 protein with any of the recombinant VW06B3R.1, T10B10.2, and T24C4.1 proteins (data not shown).

Site-Directed Mutagenesis Shows That Most Mutations in the Metal-Binding Motif in ZC410.2 Are Unacceptable for MPP Activity—The phylogenetic tree (Fig. 3) suggests that mammalian UCR-1 diverged from an ancestral β-MPP or from β-MPP/UCR-1. The bovine UCR core protein has

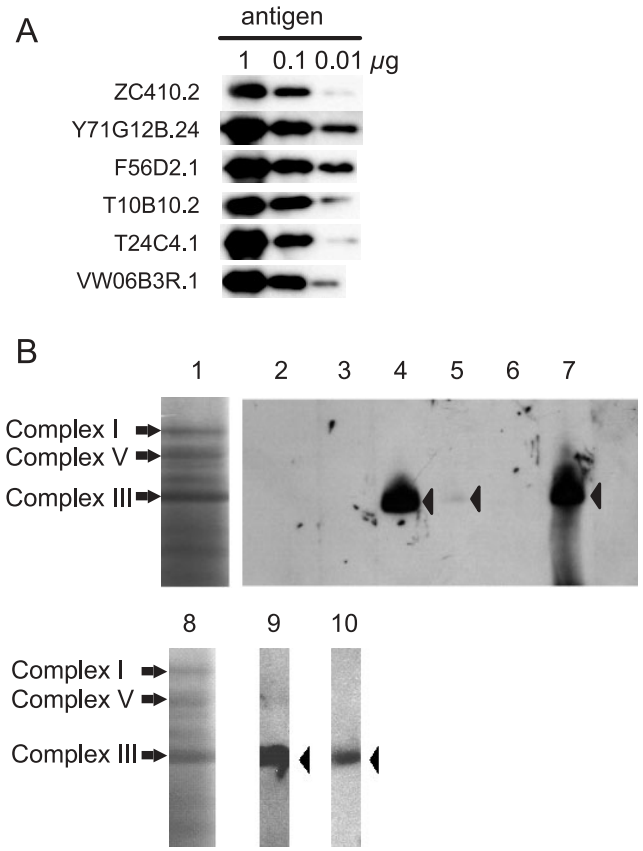

Fig. 4. **BN-PAGE and Western blotting of *C. elegans* mitochondria extracts.** The complexes in the mitochondrial membrane were separated by BN-PAGE under non-denaturing conditions and then transferred to a PVDF membrane. Immunostaining was performed with specific antibodies raised against each gene product and purified on a column of rProteinA Sepharose Fast Flow. The band of complex III (UCR) was presumed based on the result reported by Sanz *et al.* (*35*). (A) Western blotting after SDS-PAGE of recombinant proteins (0.01, 0.1, and 1 μg) with their specific antibodies (2 μg/ml). (B) Lane 1, BN-PAGE. Lanes 2–7, Western blotting after BN-PAGE using 2 μg/ml antibodies for the ZC410.2 protein (lane 2), the Y71G12B.24 protein (lane 3), the F56D2.1 protein (lane 4), the T10B10.2 protein (lane 5), the T24C4.1 protein (lane 6), and the VW06B3R.1 protein (lane 7). Lanes 8–10, twice the amount of protein was used for BN-PAGE. Lane 8, BN-PAGE. Lanes 9 and 10, Western blotting after BN-PAGE using a 10-times higher concentration of antibodies for the T10B10.2 protein (lane 9) and the T24C4.1 protein (lane 10).

MPP activity, although the metal-binding motif of the UCR-1 subunit, YFVEHX_{75}E, is different from that of β-MPP, HFLEHX_{75}E. Therefore, some mutations in the H*XX*EH motif of β-MPP seem to be acceptable without loss of proteolytic activity. In order to examine the structure/function relationship of the metal-binding motif, the HFLEH motif in the ZC410.2 protein was mutated to YFLEH (H100Y) and YFVEH (H100Y/L102V), which are found in human/mouse UCR-1 and bovine UCR-1, respectively. Additionally, we prepared mutants whose HFLEH motif is replaced with FFLEH (H100F), NFLEH (H100N), or HFLER (H104R), which are found in rainbow trout UCR-1, *Bm*-UCR-1, and the *C. elegans* F56D2.1 protein, respectively. The mutant proteins were expressed as fusion proteins with a $(His)_6$-tag, affinity-purified, and then mixed with the recombinant Y71G12B.24 protein. No MPP activity, however, was detected for any of the mutants. Thus, these mutations in the metal-binding motif are not acceptable for *C. elegans* β-MPP to retain its activity.

MPP and UCR are Essential for the Viability of C. elegans—RNAi was performed on the six genes by soaking *C. elegans* in a solution of dsRNA (Table 4). The interference of each gene led to embryonic lethality; the development was arrested in the late embryonic stage and various types of abnormal morphology were observed (Fig. 6). Among the six genes, the knockdown of F56D2.1 showed the highest lethality (>90%). Also the knockdown of ZC410.2 and Y71G12B.24 showed high lethality (>50%), and combination RNAi of the two genes led to >80% embryonic lethality. Although the lethal effect of single RNAi of T10B10.2, T24C4.1, or VW06B3R.1, or of combination RNAi of any two of the three genes was comparatively low (<30%), synthetic RNAi of all the three genes was 63% lethal. In addition, deletion mutants ZC410.2(tm1326) and F56D2.1(tm1181) were homozygously inviable. These results suggest the essentiality of MPP and the UCR core protein, and the functional redundancy among the UCR-2 subunits.

DISCUSSION

As described in the preceding section, primary structure comparison, and phylogenetic, immunochemical, and enzymological analyses led to the following assignment of the six *C. elegans* genes: Y71G12B.24, α-MPP; ZC410.2, β-MPP; F56D2.1, UCR-1; T10B10.2, T24C4.1; and VW06B3R.1, UCR-2. Immunochemical analysis of a *C. elegans* mitochondria extract showed that the VW06B3R.1 protein represents the major UCR-2, and the T24C4.1 protein the minor one. The expression patterns of VW06B3R.1 and T10B10.2 were examined by promoter-driven GFP expression analysis (data not shown), which demonstrated almost ubiquitous expression of both T10B10.2 and VW06B3R.1 and no significant difference between their expression patterns. Taken together with that synthetic RNAi of all the three UCR-2 genes caused a higher lethal effect than single RNAi or double RNAi (Table 4), the UCR-2 proteins are thought to be functionally redundant in *C. elegans*. It is unknown whether or not other nematodes such as *B. malayi* exhibit redundancy in the UCR-2 gene. The essentiality of F56D2.1 and ZC410.2 suggests the possibility that their orthologues in *B. malayi*, *Bm*-UCR-1 and *Bm*-MPP-β, are also indispensable for the viability of the filarial nematode. As mitochondria could be a good target for anti-parasitic drugs (*38*), specific inhibitors of MPP or the cytochrome bc_1 complex are expected to be potential anti-parasitic agents.

It is thought that β-MPP/UCR-1 and α-MPP/UCR-2 evolved from a common ancestral molecule, and then β-MPP and UCR-1 as well as α-MPP and UCR-2 became separated during the course of evolution (*12*, *40*). Figure 3 shows a phylogenetic tree including the nematode proteins, which suggests that divergence of UCR-1 and β-MPP occurred via a complicated process. The UCR-1 genes of mammals seem to have branched after the separation of nematodes and arthropods, while *C. elegans* F56D2.1 and *Bm*-UCR-1 as well as the UCR-1 genes of *S. cerevisiae* and

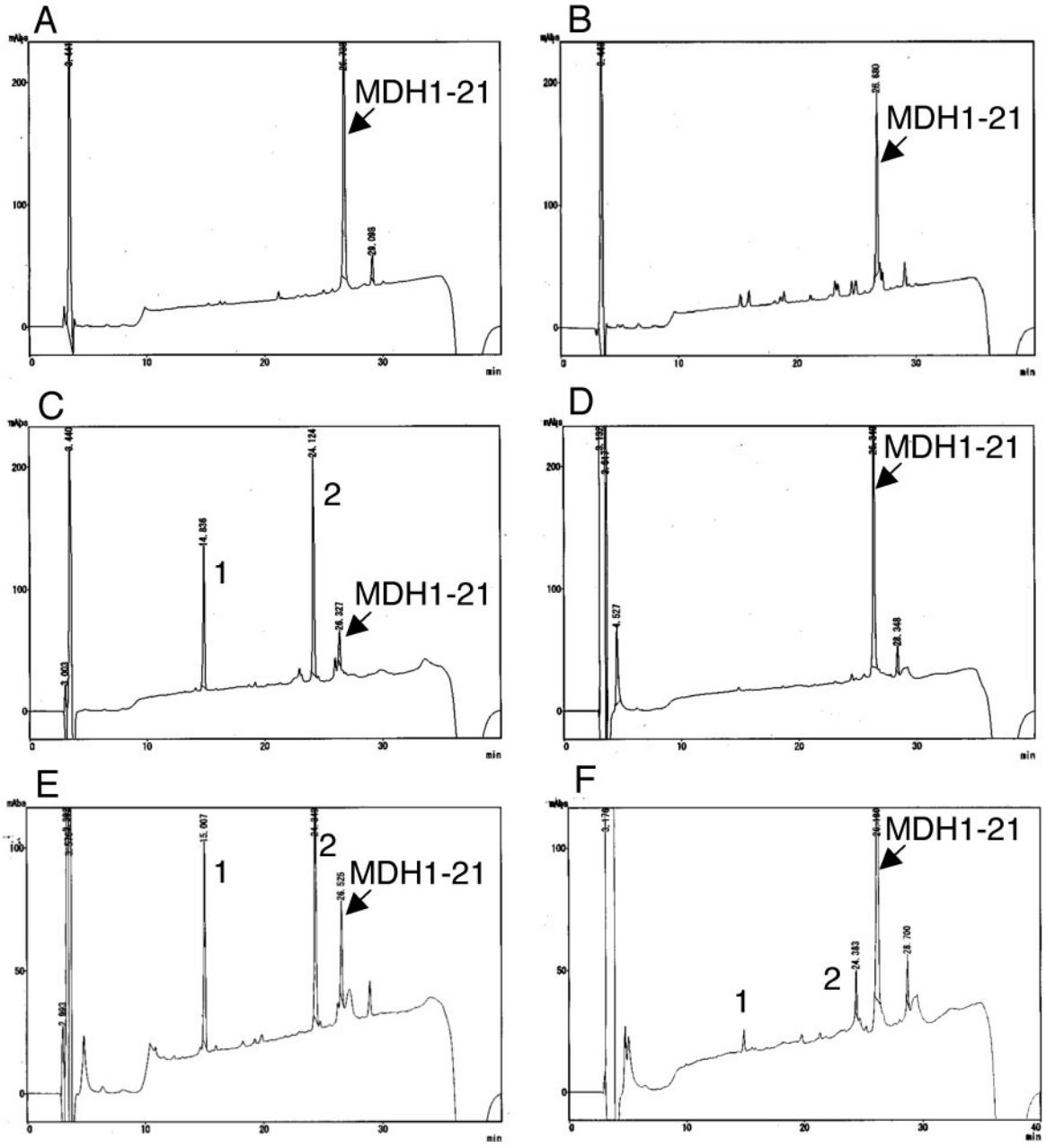

Fig. 5. **Reconstitution of MPP activity from the recombinant ZC410.2 and Y71G12B.24 proteins.** (A–D) The ZC410.2 and Y71G12B.24 proteins were expressed as soluble fusion proteins with a $(His)_6$-tag and a GST-tag, respectively. After each fusion protein had been affinity-purified, the MPP activity toward the synthetic peptides was assayed. (A, B) MDH1-21 was incubated with the recombinant ZC410.2 (A) or Y71G12B.24 (B) protein for 24 h. (C, D) MDH1-21 was incubated with a mixture of the ZC410.2 and Y71G12B.24 proteins for 2 h in the absence (C) and presence (D) of 12.5 mM EDTA. The amino acid sequences of fragments 1 and 2 in panel C were determined to be MLSALARPV-GAALRRS and FSTSA, respectively, on MS spectrometry. (E, F) The ZC410.2 and Y71G12B.24 proteins were co-expressed and incubated with MDH1-21 for 4 h in the absence (E) and presence (F) of 12.5 mM EDTA.

Euglena gracilis seem to have been derived from an ancestral gene before the separation of animals and fungi. Thus, on the assumption that the phylogenetic tree shows the course of molecular evolution, the divergence of UCR-1 and β-MPP is thought to have occurred at least twice during the evolution.

The core complex of bovine UCR is known to have MPP activity (*27*, *28*), although the biological significance of the activity is unclear. On the contrary, we could not detect MPP activity after the association of the UCR-1 and UCR-2 subunits of *C. elegans*. Although it cannot be ruled out that the *C. elegans* UCR-1/UCR-2 complex has protease activity, it is possible that *C. elegans* UCR-1 lost the potential protease activity that its ancestral molecule should have possessed, while bovine UCR-1, which diverged from β-MPP more

Table 4. **Embryonic lethality (%) with single RNAi (*) and double RNAi.**

	ZC410.2	Y71G12B.24	F56D2.1	VW06B3R.1	T10B10.2	T24C4.1
ZC410.2	63*	84	100	73	55	47
Y71G12B.24	84	54*	98	78	69	52
F56D2.1	100	98	91*	91	100	96
VW06B3R.1	73	78	91	27*	1	27
T10B10.2	55	69	100	1	14*	5
T24C4.1	47	52	96	27	5	9*

Triple RNAi of VW06B3R.1, T10B10.2, and T24C4.1 was 62% embryonic lethal.

Fig. 6. **RNAi of the family M16B genes.** (A) Wild-type embryo at the three-fold stage. (B, C) RNAi-treated embryos. RNAi of the family M16B genes caused various abnormalities in morphology. The pictures show embryos whose ZC410.2 gene was knocked down.

recently than *C. elegans* UCR-1, retains the potential activity.

The mammalian UCR-1 proteins are thought to have been derived from ancestral β-MPP or a bi-functional β-MPP/UCR-1 protein, and one of the active site His residues is thought to have become mutated during the molecular evolution. Since the bovine core protein has protease activity, some mutations may be acceptable without abolishing the protease activity. In this study, the HFLEH motif of *C. elegans* β-MPP was mutated into YFLEH, YFVEH, FFLEH, NFLEH, and HFLER, which are found in the UCR-1 proteins of various organisms, but the MPP activity was lost completely with all of these mutations. These results suggest that the His residues in the metal-binding motif are essential for the MPP activity. This is consistent with the report by Kitada *et al.* (*36*) that Arg mutations of the His residues of the H*XX*EH motif led to the complete loss of the activity of rat β-MPP. On the other hand, Striebel *et al.* (*17*) reported that Arg mutation of the first His residue in rat β-MPP abolished the activity, but that Gln mutation of the first His or Arg mutation of the second His residue led to a loss of 95% of the activity. Thus, some mutation of a His residue of the H*XX*EH motif might not abolish the activity completely, and a certain unknown structural factor(s) would be required for such a result.

In the case of *S. cerevisiae*, β-MPP is known to be essential for cell viability (*41*), while the deletion mutant of the UCR core protein-1 is viable (*42*). In the case of *C. elegans*, the lethality caused by RNAi of the family M16B genes and the inviability in homozygous animals with the deletion allele ZC410.2 (tm1326) or F56D2.1 (tm1181) demonstrated the essentiality of both MPP and the core protein of UCR for viability. The core protein is much more abundant than MPP and is thought to be present in the mitochondrial matrix before it is integrated into UCR (*37*). The essentiality of β-MPP subunit ZC410.2 indicates that the core protein present in the mitochondria matrix cannot compensate for the loss of ZC410.2. This fact suggests that *C. elegans* UCR-1 does not have enough activity to execute the role of MPP, consistent with the fact that no peptidase activity was detected with the recombinant core protein. Thus, it does not seem to be a common feature of the UCR core proteins that they possess peptidase activity.

The nucleotide sequence data for *Bm*-MPP-β and *Bm*-UCR-1 are available in the DDBJ/EMBL/GenBank databases under accession numbers AB163419 and AB163418, respectively. We would like to thank Prof. K. Kita (University of Tokyo) for the helpful discussions and suggestions. We also thank Prof. Y. Kohara (National Genetics Inst.) for providing us with the EST clones. We are grateful to Dr. S. Williams (Smith College) and the people involved in the Filarial Genome Project for the gift of the *Brugia malayi* and cDNA library, and EST clones, respectively. We also thank Dr. S. Mitani (Tokyo Women's Medical University) for the deletion mutants of *C. elegans*. This work was supported in part by Grants-in-Aid for Scientific Research, 11672218 and 13680725 (to HI) and 11694225 (to KT), from the Japan Society for the Promotion of Science.

REFERENCES

1. Williams, S.A., Lizotte-Waniewski, M.R., Guiliano, D., Daub, J., Scott, A.L., Slatko, B., and Blaxter, M.L. (2000) The filarial genome project: analysis of the nuclear, mitochondrial and endosymbiont genomes of *Brugia malayi*. *Int. J. Parasitol.* **30**, 411–419
2. Michel, E. and Bundy, D.A.P. (1997) Global mapping of lymphatic filariasis. *Parasitol. Today* **13**, 472–476
3. Tort, J., Brindley, P.J., Knox, D., Wolfe, K.H., and Dalton, J.P. (1999) Proteinases and associated genes of parasitic helminths. *Adv. Parasitol.* **43**, 161–266
4. Dame, J.B., Reddy, G.R., Yowell, C.A., Dunn, B.M., Kay J., and Berry C. (1994) Sequence, expression and modeled structure of an aspartic protease from the human malaria parasite

Plasmodium falciparum. *Mol. Biochem. Parasitol.* **64**, 177–190
5. Rosenthal P.J., Sijwali P.S., Singh A., and Shenai B.R. (2002) Cysteine proteases of malaria parasites: targets for chemotherapy. *Curr. Pharm. Des.* **8**, 1659–1672
6. Takahashi, M., Iwasaki, H., Inoue, H., and Takahashi, K. (2002) Reverse genetic analysis of the *Caenorhabditis elegans* 26S proteasome subunits by RNA interference. *Biol. Chem.* **383**, 1263–1266
7. Athauda, S.B.P., Nomura, H., Inoue, H., and Takahashi, K. (2003) Two distinct types of aspartic proteases in the filarial parasite *Brugia malayi*: Molecular cloning and tissue distribution. *Biomed. Res.* **24**, 269–276
8. Suzuki, M., Sagoh, N., Iwasaki, H., Inoue, H., and Takahashi, K. (2004) Metalloproteases with EGF, CUB, and thrombospondin-1 domains function in molting of *Caenorhabditis elegans*. *Biol. Chem.* **385**, 565–568
9. Nomura, H., Athauda, S.B.P., Takase, M., Ukai, A., Azuma, T., Hodotsuka, K., Inoue, H., and Takahashi, K. (2004) Cathepsin L-like cysteine proteases from *Brugia malayi*: cDNA cloning and comparison with *Caenorhabditis elegans*. *Biomed. Res.* **25**, 287–293
10. Rawlings N.D. and Barrett A.J. (1995) Evolutionary families of metallopeptidases. *Methods Enzymol.* **248**, 183–228
11. Voos, W., Martin, H., Krimmer, T., and Pfanner, N. (1999) Mechanisms of protein translocation into mitochondria. *Biochim. Biophys. Acta* **1422**, 235–254
12. Gakh, O., Cavadini, P., and Isaya, G. (2002) Mitochondrial processing peptidases. *Biochim. Biophys. Acta* **1592**, 63–77
13. Géli, V. (1993) Functional reconstitution in *Escherichia coli* of the yeast mitochondrial matrix peptidase from its two inactive subunits. *Proc. Natl. Acad. Sci. USA* **90**, 6247–6251
14. Arretz, M., Schneider, H., Guiard, B., Brunner, M., and Neupert, W. (1994) Characterization of the mitochondrial processing peptidase of *Neurospora crassa*. *J. Biol. Chem.* **269**, 4959–4967
15. Saavedra-Alanis, V.M., Rysavy, P., Rosenberg, L.E., and Kalousek, F. (1994) Rat liver mitochondrial processing peptidase. Both alpha- and beta-subunits are required for activity. *J. Biol. Chem.* **269**, 9284–9288.
16. Kitada, S., Kojima, K., Shimokata, K., Ogishima, T., and Ito, A. (1998) Glutamate residues required for substrate binding and cleavage activity in mitochondrial processing peptidase. *J. Biol. Chem.* **273**, 32547–32553
17. Striebel, H.M., Rysavy, P., Adamec, J., Spizek, J., and Kalousek, F. (1996) Mutational analysis of both subunits from rat mitochondrial processing peptidase. *Arch. Biochem. Biophys.* **335**, 211–218
18. Shimokata, K., Kitada, S., Ogishima, T., and Ito, A. (1998) Role of α-subunit of mitochondrial processing peptidase in substrate recognition. *J. Biol. Chem.* **273**, 25158–25163
19. Nagao, Y., Kitada, S., Kojima, K., Toh, H., Kuhara, S., Ogishima, T., and Ito, K. (2000) Glycine-rich region of mitochondrial processing peptidase α-subunit is essential for binding and cleavage of the precursor proteins. *J. Biol. Chem.* **275**, 34552–34556
20. Taylor, A.B., Smith, B.S., Kitada, S., Kojima, K., Miyaura, H., Otwinowski, Z., Ito, A., and Deisenhofer, J. (2001) Crystal structures of mitochondrial processing peptidase reveal the mode for specific cleavage of import signal sequences. *Structure (Camb.)* **9**, 615–625
21. Kitada, S., Yakasaki, E., Kojima, K., and Ito, A. (2003) Determination of the cleavage site of the presequence by mitochondrial processing peptidase on the substrate binding scaffold and the multiple subsite inside a molecular cavity. *J. Biol. Chem.* **278**, 1879–1885
22. Schulte, U., Arretz, M., Schneider, H., Tropschug, M., Wachter, E., Neupert, W., and Weiss, H. (1989) A family of mitochondrial proteins involved in bioenergetics and biogenesis. *Nature* **339**, 147–149
23. Braun, H.P., Emmermann, M., Kruft, V., and Schmitz, U.K. (1992) The general mitochondrial processing peptidase from potato is an integral part of cytochrome c reductase of the respiratory chain. *EMBO J.* **11**, 3219–3227
24. Braun, H.P., Emmermann, M., Kruft, V., Bodicker, M., and Schmitz, U.K. (1995) The general mitochondrial processing peptidase from wheat is integrated into the cytochrome bc_1-complex of the respiratory chain. *Planta* **195**, 396–402
25. Emmermann, M., Braun, H.P., Arretz, M., and Schmitz, U.K. (1993) Characterization of the bifunctional cytochrome c reductase-processing peptidase complex from potato mitochondria. *J. Biol. Chem.* **268**, 18936–18942
26. Eriksson, A.C., Sjoling, S., and Glaser, E. (1996) Characterization of the bifunctional mitochondrial processing peptidase (MPP)/bc1 complex in *Spinacia oleracea*. *J. Bioenerg. Biomembr.* **28**, 285–292
27. Deng, K., Zhang, L., Kachurin, A.M., Yu, L., Xia, D., Kim, H., Deisenhofer, J., and Yu, C.-A. (1998) Activation of a matrix processing peptidase from the crystalline cytochrome bc_1 complex of bovine heart mitochondria. *J. Biol. Chem.* **273**, 20752–20757
28. Deng, K., Shenoy, S.K., Tso, S.-C., Yu, L., and Yu, C.-A. (2001) Reconstitution of mitochondrial processing peptidase from the core proteins (subunits I and II) of bovine heart mitochondrial cytochrome bc_1 complex. *J. Biol. Chem.* **276**, 6499–6505
29. Walker, J. and Tait, A. (1997) *Ostertagia circumcincta*: Isolation of a partial cDNA encoding an unusual member of the mitochondrial processing peptidase subfamily of M16 metallopeptidases. *Int. J. Parasitol.* **27**, 1389–1394
30. Altshul, S.F., Gisg, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J.Mol. Biol.* **215**, 403–410
31. Miyadera, H., Amino, H., Hiraishi, A., Taka, H., Murayama, K., Miyoshi, H., Sakamoto, K., Ishii, N., Hekimi, S., and Kita, K. (2001) Altered quinone biosynthesis in the long-lived clk-1 mutants of *Caenorhabditis elegans*. *J. Biol. Chem.* **276**, 7713–7716
32. Schagger, H. (2001) Blue-native gels to isolate protein complexes from mitochondria. *Methods Cell Biol.* **65**, 231–244
33. Kitada, S., Kojima, K., and Ito, A. (2001) Glu(191) and Asp(195) in rat mitochondrial processing peptidase beta subunit are involved in effective cleavage of precursor protein through interaction with the proximal arginine. *Biochem. Biophys. Res. Commun.* **287**, 594–599
34. Tabara, H., Grishok, A., and Mello, C.C. (1998) RNAi in *C. elegans*: soaking in the genome sequence. *Science* **282**, 430–431
35. Sanz, M.A., Tsang, W.Y., Willems, E.M., Grivell, L.A., Lemire, B.D., van der Spek, H., and Nijtmans, L.G.J. (2003) The mitochondrial prohibitin complex is essential for embryonic viability and germline function in *Caenorhabditis elegans*. *J. Biol. Chem.* **278**, 32091–32099
36. Kitada, S., Shimokata, K., Niidome, T., Ogishima, T., and Ito, A. (1995) A putative metal-binding site in the beta subunit of rat mitochondrial processing peptidase is essential for its catalytic activity. *J. Biochem.* **117**, 1148–1150
37. Zara, V., Palmisano, I, Conte, L., and Trumpower, B.L., (2004) Further insights into the assembly of the yeast cytochrome bc_1 complex based on analysis of single and double deletion mutants lacking supernumerary subunits and cytochrome *b*. *Eur. J. Biochem.* **271**, 1209–1218
38. Kita, K., Nihei, C., and Tomitsuka, E. (2003) Parasite mitochondria as drug target: diversity and dynamic changes during the life cycle. *Curr. Med. Chem.* **10**, 2535–2548
39. Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic. Acids Res.* **22**, 4673–4680

40. Braun H.-P. and Schmitz, U.K. (1995) Are the 'core' proteins of the mitochondrial bc_1 complex evolutionary relics of a processing protease? *Trends Biochem. Sci.* **20**, 171–175
41. Yaffe, M.P., Ohta, S., and Schatz, G. (1985) A yeast mutant temperature-sensitive for mitochondrial assembly is deficient in a mitochondrial protease activity that cleaves imported precursor polypeptides. *EMBO J.* **4**, 2069–2074
42. Hsu, A.Y., Do, T.Q., Lee, P.T., and Clarke, C.F. (2000) Genetic evidence for a multi-subunit complex in the O-methyltransferase steps of coenzyme Q biosynthesis. *Biochim. Biophys. Acta* **1484**, 287–297