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Caenorhabditis elegans cDNA for a Menkes/Wilson Disease Gene Homologue and Its Function in a Yeast *CCC2* Gene Deletion Mutant[1]

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The full-length cDNA coding for a putative copper transporting P-type ATPase (Cu^{2+}-ATPase) was cloned from *Caenorhabditis elegans*. The putative Cu^{2+}-ATPase is a 1,238-amino acid protein, and highly homologous to the Menkes and Wilson disease gene products mutations of which are responsible for human defects of copper metabolism. The *Saccharomyces cerevisiae* mutant with a disrupted *CCC2* gene (yeast Menkes/Wilson disease gene homologue) was rescued by the cDNA for the *C. elegans* Cu^{2+}-ATPase but not by the cDNA with an Asp-786 (an invariant phosphorylation site) to Asn mutation, suggesting that the *C. elegans* Cu^{2+}-ATPase functions as a copper transporter in yeast. The expressed *C. elegans* protein was detected in yeast vacuolar membranes by immunofluorescence microscopy. The yeast expression system may facilitate further studies on copper transporting P-type ATPases.

Key words: *Caenorhabditis elegans*, copper transporting ATPase, immunofluorescence microscopy, Menkes/Wilson disease gene homologue, yeast *CCC2* gene.

Copper is required by most organisms as a trace element, because many enzymes for oxidative metabolism are dependent on copper together with iron. However, excess accumulation of the copper ion is toxic because of its ability to oxidize proteins and lipids. Menkes and Wilson diseases are human inherited disorders caused by genetic defects in copper metabolism (*1, 2*). Menkes disease is characterized by abnormal hair and facies, hypopigmentation, cerebral degeneration, connective tissue and vascular defects, and death during infancy. Most of these features are explained by a deficiency of copper-dependent enzymes. Defective intestinal absorption of copper results in a deficiency of copper in a patient (*1*). On the other hand, Wilson disease patient shows a reduction in the ceruloplasmin (multi-copper oxidase) concentration in plasma (*3*) and also defective biliary copper excretion (*4*), resulting in toxic copper accumulation in the liver.

The cDNAs responsible for Menkes and Wilson diseases (*5-11*) have been cloned and identified as putative copper transporting P-type ATPases (Cu^{2+}-ATPase). The two ATPases exhibit high sequence identity (56%) and contain characteristic motifs, such as Gly-Met-Thr-Cys-X-X-Cys, Cys-Pro-Cys, and Ser-Glu-His-Pro, probably involved in copper transport across membranes. Thus, the two putative Cu^{2+}-ATPase proteins may have similar functions. However, the Menkes gene is expressed in various tissues other than liver (*5, 6*), while the Wilson gene is mainly expressed in liver and kidney (*8, 9*). The tissue specific expression of these genes may partly explain the different diagnostic findings.

There is no convincing evidence of copper transport by eukaryotic putative Cu^{2+}-ATPases except that of *Saccharomyces cerevisiae*. The yeast *CCC2* gene (Menkes/Wilson disease gene homologue) has been analyzed by genetic studies. Disruption of the *CCC2* gene resulted in defective maturation of the Fet3 protein (multi-copper oxidase), which is essential for high affinity iron uptake (*12*) together with the Ftr1 protein (*13*). Phenotypically, the *Δccc2* (*CCC2* gene deletion) mutant could not grow under iron limited conditions because the Fet3 protein remained as an apo-form and there was no high affinity iron uptake (*12*). The requirement of the Ccc2 protein (*CCC2* gene product) for the delivery of copper to the Fet3 protein in yeast is analogous to that of the Wilson disease gene product for ceruloplasmin in human liver. Thus, the *Δccc2* mutant may constitute an excellent model system for investigating unknown roles of putative Cu^{2+}-ATPases from other eukaryotes.

At present, little is known about the tissue specific gene regulation and function of Menkes/Wilson disease-related proteins in mammals and other multicellular organisms. In order to investigate these problems, we chose *Caenorhabditis elegans* as a model animal. This nematode is one of the simplest animals having nervous, muscular, hypodermal, digestive and reproductive systems (*14*). The nematode requires copper for its aerobic life form, but a high concentration of the metal may be toxic because of its high oxidation capacity. Thus, the nematode should have mechanisms for recognizing copper and maintaining its

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Abbreviations: CUA-1 cDNA, *C. elegans* cDNA for putative copper ATPase; *cua-1*, *C. elegans* gene for putative copper ATPase; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; bp, base pairs; kb, kilobase pairs.

cellular homeostasis similar to that in mammals. In the present study, we cloned a *C. elegans* cDNA coding for a putative Cu^{2+}-ATPase (CUA-1) closely similar to those for Menkes and Wilson diseases, and showed that the cDNA could rescue the yeast *Δccc2* mutant. This is the first example of functional expression of a heterologous putative Cu^{2+}-ATPase in yeast.

EXPERIMENTAL PROCEDURES

General Methods—Standard methods were used for the handling and culturing of the *C. elegans* Bristol N2 strain (*15*). The molecular cloning methods used were as described previously (*16*). DNA sequencing was carried out with an ABI 373A Sequencer, and Western blot analysis was carried out by the published method (*17*) with minor modification. The primary and secondary antibodies used were monoclonal anti-hemagglutinin (HA) and sheep anti-mouse IgG conjugated with horse radish peroxidase, respectively. Immunochemical signals were detected using Amersham ECL kit. Protein concentrations were determined using bovine serum albumin as a standard (*18*).

Isolation of a Full-Length cDNA Coding for a Putative C. elegans Cu^{2+}-ATPase—The cDNA clone, yk29a9 (kindly supplied by Dr. Y. Kohara), was from a cDNA library of a mixed *C. elegans* population. Partial sequencing of the cDNA from its 5′-end (GenBank accession number D36097) revealed an amino acid sequence (residues 571 through 689, Fig. 1) related to those of Menkes and Wilson disease gene products.

The 5′-half of the entire cDNA was isolated from the total RNA of a mixed *C. elegans* population using TRIzol LS reagent (Gibco BRL). Synthesis of the first strand cDNA with a SuperScript Preamplification System (Gibco BRL) and reverse transcription polymerase chain reaction (RT-PCR) (Cetus Perkins-Elmer) were performed according to the manufacturers' instructions. The primers for RT-PCR were (Fig. 2): SL2, equivalent to the 22 nucleotide *C. elegans* spliced leader 2 sequence (*19*); and RTrv, complementary sequence corresponding to the amino acid residues between positions 708 and 715 of the ATPase (Fig. 1). ExTaq polymerase treated with TaqStart Antibody was used for 30 PCR cycles.

Full-length cDNA (CUA-1-cDNA, *C. elegans* Cu^{2+}-ATPase cDNA) was constructed and introduced into pBluescript II SK(+) by ligating the restriction fragment of yk29a9 (nucleotides 1886-4001) and the cDNA for the 5′-end of the transcript (nucleotides 1-1885) at the *Sal*I restriction site (Fig. 2).

Introduction of an Asp-786 to Asn Mutation into the Putative C. elegans ATPase—A *Hin*dIII-*Hin*dIII fragment (nucleotides 1733-2537, Fig. 2) from CUA-1 cDNA was subcloned into pBluescript II SK(+), and then used as a template for PCR to introduce an Asp-786 to Asn (GAC→AAT) mutation (Fig. 2). The combinations of primers D786Nfw and A, and D786Nrv and B were used for the first PCR, and primers A and B were used for the second PCR (Fig. 2). The mutated *Hin*dIII-*Hin*dIII fragment was replaced with the corresponding wild type fragment in the full-length cDNA.

Insertion of a Hemagglutinin (HA) Epitope into the Putative C. elegans ATPase—The HA epitope sequence from the human influenza virus was inserted into CUA-1 cDNA. A *Bam*HI-*Sal*I fragment (nucleotides 1-583) coding for the amino terminus of the ATPase was subcloned into pBluescript II SK(+) to generate a unique *Pma*CI restriction site (GTGCAC, Fig. 2) by PCR mutagenesis. The combinations of primers Pcmfw (Fig. 2) and B, and Pcmrv and A were used for the first PCR, and primers A and B were used for the second PCR (Fig. 2). The mutated *Bam*HI-*Sal*I fragment was obtained and replaced with that of the wild type. The oligonucleotide coding for three repeats of the HA epitope [5′-ATC(TACCCATACGATGT-TCCGGATTACGCT)$_3$GAT-3′] was inserted in-frame into the *Pma*CI site of the engineered cDNA. The same method was used to introduce the HA epitope into CUA-1 cDNA having Asp-786 to Asn mutation.

Disruption of the CCC2 Gene in Yeast and Transformation—A part of the yeast *CCC2* gene (*20*) was amplified from chromosomal DNA of *S. cerevisiae* YPH499 (*MAT*a, *ura3-52, leu2-Δ1, trp1-Δ63, his3-Δ200, ade2-101, lys2-801*) by PCR using two oligonucleotide primers (5′ primer, 5′-GGTTCAGGAACCATGGATAC-3′; 3′ primer, 5′-TTG-GCCAGTCCTCTCAAACT-3′). A *Bam*HI-*Sal*I fragment (0.6 kb, corresponding to amino acid residues 532-741 of the *CCC2*-coded protein) of the PCR product was replaced by the *HIS3* gene. This fragment was used to replace the *CCC2* gene using a one-step replacement strategy (*21*). The gene disruption (*Δccc2*) was confirmed by PCR and negative growth by respiration.

Full-length CUA-1 cDNA and derivatives of it were inserted into yeast multicopy vector pSY114 (YEp13-based plasmid with the *LEU2* marker) under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter and phosphoglucokinase terminator. The resulting recombinant plasmids were introduced into the *Δccc2* mutant and transformants were selected on solid synthetic dextrose (SD) medium (0.67% yeast nitrogen-base without amino acids, 2% glucose, 2% agar) supplemented with adenine, uracil, tryptophan, and lysine (*22*). The transformed cells were grown at 30°C on agar medium (2% bactopeptone, 1% yeast extract, 50 μM EDTA) containing 2% glucose (YPD) or 3% ethanol (YE), or the same medium with 1 mM $Fe(NH_4)(SO_4)_2$. Yeast cell lysate (*23*) was subjected to polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate for immunochemical detection.

Indirect Immunofluorescence Staining—Yeast cells grown in liquid SD medium to the middle of the logarithmic phase were fixed with 5% formaldehyde, treated with Zymolyase (100T) for 30 min, and then placed on a polylysine-coated slide glass. The slide glass was treated with 0.1% Triton X-100, and then incubated with the first antibody (mouse anti-HA diluted to 0.5 μg/ml), washed with phosphate-buffered saline, and then incubated with anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (diluted to 2.5 μg/ml).

Materials—Anti-HA monoclonal antibody and anti-mouse IgG conjugated with FITC were obtained from Boehringer Mannheim and Cappel, respectively. Anti-mouse IgG horse radish peroxidase conjugate and protein standards (Rainbow markers) for gel electrophoresis were from Amersham. Zymolyase (100T) was obtained from Seikagaku. The reagents for protein determination (Pierce Chem.) and TaqStart Antibody (Clonetech) were from commercial sources. Restriction enzymes, ExTaq polymerase, T4 DNA ligase and other reagents for DNA handling

were from Takara Shuzou Co. pBluescript II SK(+) was from Stratagene. All other chemicals used were of the highest grade commercially available.

RESULTS

Full-Length cDNA Clone Coding for a Putative Cu^{2+}-ATPase from C. elegans—Clone yk29a9 was isolated from a *C. elegans* cDNA library, and the result of partial sequencing (Val-571-Phe-689) suggested that it coded for a Menkes/Wilson disease protein homologue (Dr. Y. Kohara, personal communication). As judged from our sequencing study, the clone carried the carboxyl half of the protein (Fig. 1). We obtained the cDNA coding for the amino terminal half of the protein by RT-PCR, a primer having the yk29a9-specific sequence and that of a spliced leader 2 (SL2) being used. The full-length cDNA was constructed utilizing the *Sal*I restriction site at 1885 in the nucleotide sequence (Fig. 2), and then used for further studies.

Both strands of the full-length cDNA (4,001 bp) were sequenced repeatedly. The sequence of the RT-PCR product was also determined directly using primers derived from the cDNA sequences, and was consistent with that of the full-length cDNA. The cDNA codes for a 1,238-amino acid protein (Fig. 1) having a calculated molecular weight of 133,468. An initiation codon (nucleotides 70-72, Fig. 2) was within the consensus sequence for the *C. elegans* translation start site (ANN^A/C^A/GAAATGN, Ref. *24*).

Predicted Amino Acid Sequence of the Putative C. elegans Cu^{2+}-ATPase—The amino acid sequence of the putative ATPase (Fig. 1) shares all the features reported for the cation transporting P-type ATPases which form aspartyl phosphate intermediates during the catalytic cycle (*25*): Thr-Gly-Glu-Ser in the flexible loop, Asp-Lys-Thr-Gly-Thr in the phosphorylation domain, where the aspartate residue (Asp-786 in the *C. elegans* protein) is phosphorylated (*26*), Thr-Gly-Asp-Asn in the ATP-binding domain, and Gly-Asp-Gly-Val-Asn-Asp-Ser connecting the ATP-binding domain and the transmembrane helix are conserved. Moreover, the specific motifs for putative heavy metal ATPases (*27*), Gly-Met-Thr-Cys-X-X-Cys, Cys-Pro-Cys, and Ser-Glu-His-Pro, are also found (Fig. 1). Similar to the cases of other heavy metal ATPases, the presence of eight transmembrane helices (*27, 28*) in *C. elegans* ATPase was suggested from the hydropathy plot (*29*) (Fig. 1). The amino acid sequence of the *C. elegans* ATPase closely resembled those of other eukaryotic or bacterial enzymes. The identities between ATPases of *C. elegans* and other sources are: Menkes disease protein (*5*), 46.1% identical over 1,135 residues; Wilson disease protein (*11*), 42.6% over 1,220 residues; yeast Ccc2 protein (*20*), 29.6% over 928 residues; and *Enterococcus hirae* CopA (*30*), 30.7% over 680 residues. These high sequence similarities strongly suggest that the *C. elegans* ATPase has essentially the same functions as other ATPases.

Rescue of the Yeast Δccc2 Mutant with C. elegans cDNA (CUA-1 cDNA) for the Putative ATPase—The high sequence homology between the *C. elegans* ATPase and the *CCC2* gene product prompted us to examine whether or not CUA-1 cDNA could rescue the yeast *Δccc2* (*CCC2* gene

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MSENVSLLDG SPLPSRPSTS SIPRPSPSKN IQLLVDFGAP KTDGNVQETM LEIKGMTCNS   60
CVKNIQDVIG AKPGIHSIQV NLKEENAKCS FDTTKWTAEK VAEAVDDMGF DCKVLKKEPP  120
TQMAEKPKIR RAIVSIEGMT CHACVNNIQD TVGSKDGIVK IVVSLEQKQG TVDYNSEKWN  180
GESVAESIDD MGFDCKLITD QEIAAVEPQK ASTTKLSISP LKTVDLSDGK VELQLNGVKY  240
SKEGSSDHLE KCTFAVEGMT CASCVQYIER NISKIEGVHS IVVALIAAKA EVIYDGRVTS  300
SDAIREHMTG ELGYKATLLD SMGANPNYSK IRLIIGNLST ESDANRIESH VLSKSGIDSC  360
NVSIATSMAL VEFSPQVIGP RDIINVVESL GFTADLATRD DQMKRLDHSD DVKKWRNTFF  420
IALIFGVFVM IIMIIFHWIL RTPMHPDKQT PIFTPALSLD NFLLLCLCTP VQIFGGRYFY  480
VASWKAIKHG NANMDVLIML STTIAYTYSI VVLLLAIIFK WPSSPMTFFD VPPMLIVFIA  540
LGRMLEHKAK GKTSEALSKL MSLQAKEATL VTMDSEGRLT SEKGINIELV QRNDLIKVVP  600
GAKVPVDGVV VDGKSSVDES FITGESMPVV KKPGSTVIGG SVNQKGVLIV KATHVGNDST  660
LSQIVRLVEE AQTNRAPIQQ LADKIAGYFV PFVIVLSLFT LGVWIYIEYN SARNANLPPG  720
LRFEEALKIA FEAAITVLAI ACPCSLGLAT PTAVMVGTGV GAANGILIKG GEPLESVHKV  780
TTIVFDKTGT ITEGRPRVVQ IASFVNPSTM SLKLITFLSG ATEALSEHPI GNAVAAFAKQ  840
LLNEPTWPNT SRFHVSAGHG VTCRIDSIRQ SFSSLALSGS TCEIPRLPDG QTITIPGTEV  900
NLLQVSSKEV SQPNPDTANI VIGTERMMER HGIPVSEVVK MTLSEEQRKG HISVICAINA  960
EVVAVISIAD QVKKEASLAI YTLREMGLRV VLLTGDNSKT AESTAKQVGI DEVFAEVLPN 1020
QKQQKIKQLK GYKNKVAMVG DGVNDSPALA EANVGIAIAA GSDVAIESAG IVLVRNDLVD 1080
VVGAIKLSKM TTRRIRLNFL FAIIYNAIGI PIAAGVFRPF GFMLQPWMAA AAMALSSVSV 1140
VSSSLLLKNF RKPTIANLYT TSFKRHQKFL ESGSFQVQVH RGLDDSAVFR GAASSKLSIL 1200
SSKVGSLLGS TTSIVSSGSS KKQRLLDNVG SDLEDLIV                         1238
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Fig. 1. **Amino acid sequence of the putative *C. elegans* copper transporting ATPase.** The amino acid sequence was deduced from the nucleotide sequence of CUA-1 cDNA. Putative transmembrane helices deduced from the hydropathy plot (*29*) are underlined. Bold letters in boxes denote specific motifs found in heavy metal ATPases. Other boxed sequences are conserved in all P-type ATPases. The CUA-1 cDNA sequence has been deposited in GenBank (accession number D83665).

deletion) mutant. The yeast *Δccc2* mutant could not grow on iron limited ethanol-based medium (YE) by respiration because intracellular copper transport by the Ccc2 protein (*CCC2* gene product) is required for high affinity iron uptake (*12*), which is indispensable for growth under iron limited conditions. The *Δccc2* mutant could grow on the YE medium containing a large excess of the ferric ion (*12*) because iron is taken up by the low affinity transport system (Fig. 3). The *Δccc2* mutant carrying CUA-1 cDNA could grow on the YE medium similar to the mutant expressing the yeast *CCC2* gene (Fig. 3), suggesting that CUA-1 cDNA rescued the *Δccc2* mutant. On the other hand, the *Δccc2* mutant carrying CUA-1 cDNA with a mutation in a putative phosphorylation site (Asp-786 to Asn) or the vector without the cDNA insertion showed negative growth on the YE medium, although they could grow glycolytically on YPD medium (Fig. 3).

Synthesis of the Putative C. elegans ATPase in Yeast—To confirm the synthesis of the *C. elegans* ATPase in the yeast *Δccc2* mutant, an HA tag was introduced near the amino terminus of CUA-1 cDNA (between Asn-4 and Val-5). The wild type cDNA with the HA tag could rescue *Δccc2*, similar to that without the tag (Fig. 3). As shown by Western blot analysis, a single protein band with the HA tag was detected for the *Δccc2* cells carrying tagged CUA-1 cDNA but not for the control cells (Fig. 4). The approxi-

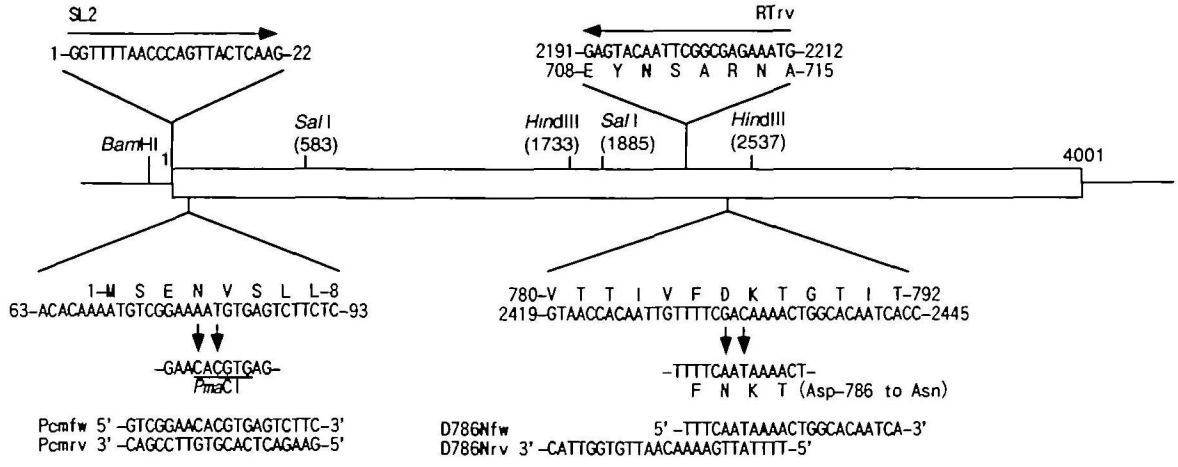

Fig. 2. **Construction of the full-length CUA-1 cDNA for the putative *C. elegans* ATPase, and introduction of a *Pma*CI restriction site and an Asp-786 to Asn mutation.** The entire CUA-1 cDNA (4,001 bp) is shown by an open bar with flanking regions derived from the pBluescript II SK(+) vector (plain lines). The 5′ half of the cDNA between nucleotides 1 and 1885 (*Sal*I site) was derived through PCR using primers SL2 and RTrv. No PCR product was obtained in the reaction with the SL1 (*33*) primer. The rest of the cDNA was derived from the yk29a9 clone. The primers used for mutagenesis, Pcmfw, Pcmrv, D786Nfw, and D786Nrv, are shown. Primers A and B (universal M13 reverse and forward primers, respectively) were also used for mutagenesis. The base changes for the *Pma*CI site introduction and Asp-786 to Asn mutation are shown by vertical arrows. The restriction sites used for subcloning are also shown.

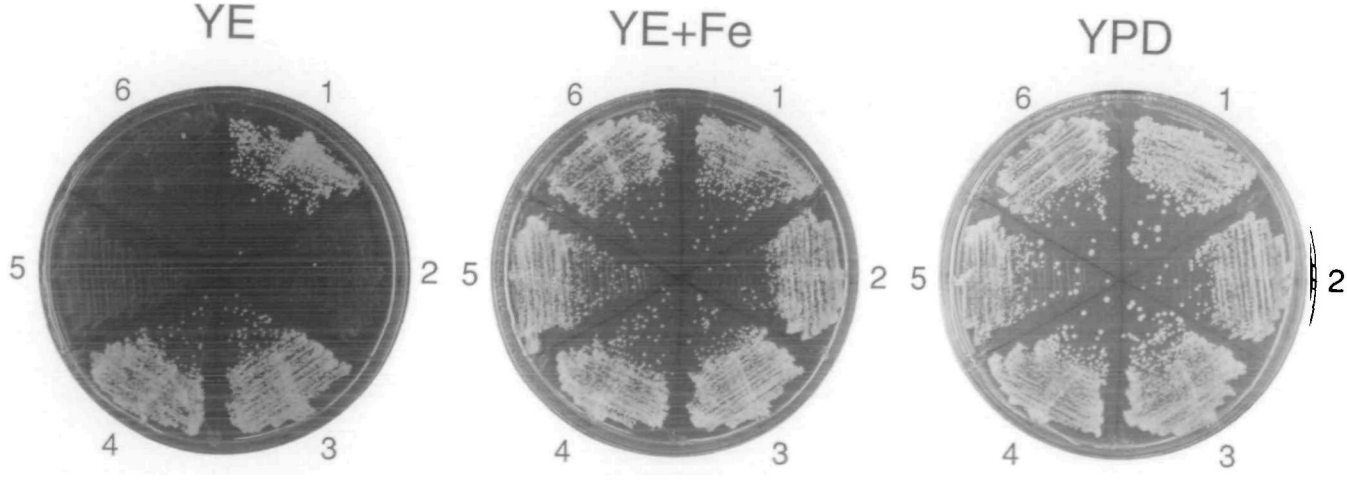

Fig. 3. **Rescue of the *Δccc2* mutant with the CUA-1 cDNA coding for the putative P-type ATPase.** Various plasmids were introduced into the yeast *Δccc2* mutant: *CCC2* gene, 1; vector with no insert, 2; wild type CUA-1 cDNA, 3; wild type CUA-1 cDNA with HA tag, 4; mutant CUA-1 cDNA (Asp-786 to Asn), 5; tagged mutant CUA-1 cDNA (Asp-786 to Asn), 6. A DNA fragment containing the entire yeast *CCC2* gene and its promoter region was inserted into a single copy vector, pRS314 (*42*), and the resulting recombinant plasmid was used in this study as a positive control. Single colonies of the transformants were suspended in sterile water, and then spread on YE medium, the same medium with 1 mM $Fe(NH_4)(SO_4)_2$ (YE+Fe), and YPD medium with 50 μM EDTA. The plates were incubated at 30°C and growth was recorded: YPD medium, after 2 days; YE-based media, after 3 days.

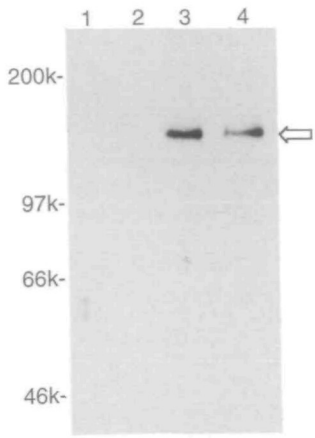

Fig. 4. **Presence of the putative *C. elegans* ATPase expressed in the *Δccc2* mutant.** Cell lysates (20 μg of protein) of yeast *Δccc2* harboring various plasmids were applied to a polyacrylamide (7.5%) gel in the presence of sodium dodecyl sulfate and blotted onto a nitrocellulose filter after the electrophoresis. The putative *C. elegans* ATPase with the HA tag was detected immunochemically in *Δccc2* cells carrying CUA-1 cDNA with the HA tag. Plasmids: lane 1, plasmid with no cDNA insert; lane 2, plasmid carrying CUA-1 cDNA; lane 3, plasmid carrying CUA-1 cDNA with the HA tag; lane 4, plasmid carrying mutant CUA-1 cDNA (Asp-786 to Asn) with the HA tag. The molecular weight of the putative *C. elegans* ATPase with the HA tag (arrow) was estimated to be 140,000 from the position of protein standards.

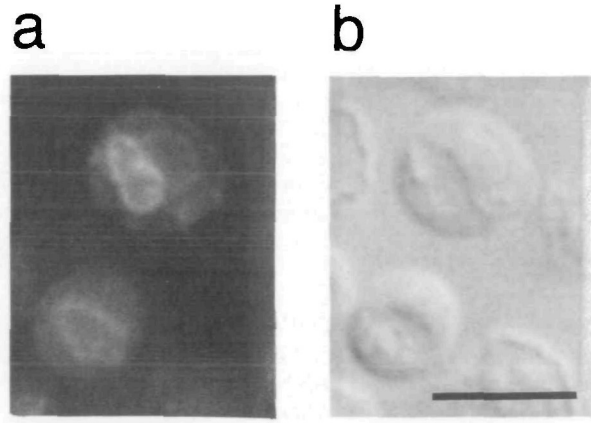

Fig. 5. **Localization of the putative *C. elegans* ATPase expressed in the *Δccc2* mutant.** The *Δccc2* mutant carrying CUA-1 cDNA with the HA tag was fixed in formaldehyde, and then probed with antibodies (anti-HA and anti-mouse IgG conjugated with fluorescein isothiocyanate, FITC) (a). The cells were visualized by fluorescence microscopy. Nomarski images are also shown (b). Bar, 5 μm.

mate molecular weight of the protein calculated from the migration distance on electrophoresis was 140,000, this being consistent with the value (136,950) calculated from the amino acid sequence of the ATPase with the HA tag. Essentially the same result was obtained with the Western blot analysis of tagged CUA-1 cDNA having Asp-786 to Asn mutation (Fig. 4), which could not rescue *Δccc2* (Fig. 3).

Localization of the Putative C. elegans ATPase in Yeast—It was of interest to identify the yeast organelles in which the *C. elegans* ATPase was located. As shown by immunofluorescence microscopy (Fig. 5), *C. elegans* ATPase with the HA tag was localized in a large intracellular membrane, possibly vacuolar membranes. Vacuolar structures were confirmed by the Nomarski images of the same cells (Fig. 5). Fluorescence staining of vacuolar membranes was not observed without the addition of the first antibody or in the control cells expressing *C. elegans* ATPase without the tag (data not shown).

DISCUSSION

The complete *C. elegans* cDNA (CUA-1 cDNA from the *cua-1* gene) of a Menkes/Wilson disease gene homologue was obtained in the present study by ligating amino and carboxyl terminal clones. The amino terminal cDNA was obtained by PCR using the primer for the *C. elegans* SL2 sequence. The nematode genes that acquire the SL2 through *trans*-splicing are suggested to lie immediately downstream of other gene(s) (*31*). Thus, the *cua-1* gene may be transcribed as a part of the polycistronic message and then spliced. It is of interest to identify other genes in the polycistronic unit, although no information is available at present. The *cua-1* gene was mapped on chromosome III (Dr. Y. Kohara, personal communication, and our unpublished result), and we found the 3′ part of the *cua-1* gene in the cosmid F45G2 sequence (Sanger Center, EMBL CEF45G2) in the *C. elegans* genome project. No other genes coding for similar proteins have been found in the *C. elegans* genome database.

Genetic studies indicated that the yeast Ccc2 protein is a copper transporter necessary for loading copper to the Fet3 protein, and that the copper-bound Fet3 protein is essential for high affinity iron uptake (*12*). In the present study, we showed that the *C. elegans* CUA-1 cDNA could rescue the yeast *Δccc2* (*CCC2* gene deletion) mutant, suggesting that the *C. elegans* ATPase substituted for the Ccc2 protein and transported copper to a compartment in which the Fet3 protein bound copper. This rescue was possibly due to complementation by the *C. elegans* ATPase functioning as a P-type ATPase, because the Asp-786 (phosphorylation site) to Asn mutant ATPase could not rescue the *Δccc2* mutant.

The *C. elegans* ATPase, when expressed in yeast, was observed in vacuolar membranes by immunofluorescence microscopy, implying that the ATPase functions in vacuoles. However, the yeast compartments in which the Ccc2 protein functions are unknown. Yuan *et al.* suggested, on analysis of mutants defective in post-Golgi sorting, that copper loading by the Ccc2 protein to Fet3 protein occurs in a post-Golgi compartment, possibly in the endocytotic pathway (*12*). Thus, it is also likely that part of the *C. elegans* ATPase was assembled and functioned in a yeast post-Golgi compartment, although most of the enzyme was assembled in vacuolar membranes. Vacuolar membranes are known to be default destinations for several yeast membrane proteins (*32-34*).

Biochemical characterization of putative heavy metal ATPases, such as the formation of a phosphorylated intermediate or ATP-driven metal ion transport activities, has been carried out only for bacterial enzyme (*35-37*). Our attempts to detect ATPase activity and a phosphorylated intermediate of the *C. elegans* ATPase expressed in yeast were not successful: we could not detect ATPase activity or an intermediate using vacuolar membranes (up to 15 μg

protein). The negative results may be due to the high protease sensitivity of the *C. elegans* enzyme. We noted degradation of the *C. elegans* enzyme in a vacuolar membrane fraction when yeast cells were fractionated without protease inhibitors (*38*). It is also noteworthy that the molecular activities of heavy metal transporting ATPases are very low compared with those of non-heavy metal ATPases. The cadmium transport rate for *Staphylococcus aureus* Cd^{2+}-ATPase (CadA) was estimated to be 0.13 μmol/min/mg of Cd^{2+}-ATPase protein, assuming that 10% of membrane proteins was Cd^{2+}-ATPase (*35*), whereas rabbit sarcoplasmic Ca^{2+}-ATPase exhibited Ca^{2+} transport activity of 6.2 μmol/min/mg of ATPase protein (*39*). The transport of copper by *E. hirae* ATPase (CopB) (*37*) also seemed to be much lower than that of Ca^{2+} by the Ca^{2+}-ATPase.

The structural features of putative heavy metal ATPases are clearly different from those of non-heavy metal transporting P-type ATPases (*27, 28*). The heavy metal ATPases have longer amino terminal sequences forming unique domains containing Gly-Met-Thr-Cys-X-X-Cys motifs. It has been assumed that these motifs are binding sites for heavy metal ions in the heavy metal ATPases (*40*), and similar motifs are found in other proteins capable of divalent cation binding such as mercury binding proteins (*41*). The number of the motifs in the heavy metal ATPases varies with the origin of the enzyme, although the significance of the differences is currently unknown: *C. elegans*, three; human (both Menkes and Wilson), six (*5, 11*); yeast (*CCC2* gene product), two (*20*); and bacterial enzymes, one (*27*). In this regard it is of interest that the *C. elegans* ATPase (three motifs) rescued the *Δccc2* mutant lacking the *CCC2* gene product (two motifs). The yeast expression system established in this study will allow further molecular characterization of the putative *C. elegans* Cu^{2+}-ATPase, and may be extended to human Menkes and Wilson disease gene products.

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