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<td><strong>Author(s)</strong></td>
<td>若林，篠光</td>
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Osaka University
Copper Trafficking in *Caenorhabditis elegans*: Copper Chaperone and Copper Transporting ATPase

Tokumitsu Wakabayashi

Division of Biological Sciences, Institute of Scientific and Industrial Research, Osaka University

1999

Ph. D. Thesis Submitted to Graduate School of Science, Osaka University
Acknowledgement

謝辞

本研究は、大阪大学産業科学研究所・生体応答科学部門・生体膜分子学研究部門（二井研究室）において行ったものです。私を二井研究室の学生として快く受け入れて下さり、本研究を行う機会を下さいました二井将光教授に深く感謝します。3年間にわたり、飽かず戦しく御指導を賜り、学問を志向するもののとしての姿勢を目の当たりにさせて頂けたことは得難い経験でした。

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**Abbreviations used**

Abbreviations used are:

- **ATP**: adenosine triphosphate
- **ATPase**: adenosine triphosphatase
- **bp**: base pair(s)
- **cDNA**: complementary DNA
- **EST**: expressed sequence tag
- **GFP**: green fluorescence protein
- **Kb**: kilo base(s)
- **Kbp**: kilo base pair(s)
- **kDa**: kilo dalton
- **LA-PCR**: long and accurate polymerase chain reaction
- **NGM**: nematode growth medium
- **ORF**: open reading frame
- **PCR**: polymerase chain reaction
- **RACE**: rapid amplification of cDNA end
- **RT-PCR**: polymerase chain reaction combined with reverse transcription
- **SDS-PAGE**: polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate

**Amino acids and their symbols used are:**

<table>
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<tr>
<th>Amino Acid</th>
<th>Symbol</th>
<th>Abbreviation</th>
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<tbody>
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<td>G</td>
</tr>
<tr>
<td>alanine</td>
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<td>A</td>
</tr>
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<td>valine</td>
<td>Val</td>
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</tr>
<tr>
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<td>Leu</td>
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</tr>
<tr>
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<td>Ile</td>
<td>I</td>
</tr>
<tr>
<td>serine</td>
<td>Ser</td>
<td>S</td>
</tr>
<tr>
<td>threonine</td>
<td>Thr</td>
<td>T</td>
</tr>
<tr>
<td>aspartic acid</td>
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<td>D</td>
</tr>
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<td>asparagine</td>
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<tr>
<td>proline</td>
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</table>
CHAPTER 1  INTRODUCTION

1.1 Dual features of copper ion in biological systems

Copper functions as a co-factor for variety of enzymes required for essential cellular metabolism (Table. 1). Copper proteins are located in all cellular compartment: Cu/Zn superoxide dismutase in the cytosol [1]; cytochrome c oxidase in mitochondria [2]; lysyl oxidase in the post-Golgi to secretory pathway [3]; and metallothionein in the nucleus, cytosol and lysosome [4,5]. However, the mechanisms underlying the intracellular delivery and compartmentalization of the copper are poorly understood.

Table 1.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceruloplasmin</td>
<td>Fe(II) -&gt;Fe(III) oxidation</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>Electron transfer, terminal oxidase</td>
</tr>
<tr>
<td>Dopamine-β-hydroxylase</td>
<td>Dopamine -&gt; norepinephrine</td>
</tr>
<tr>
<td>Lysyl oxidase</td>
<td>Collagen and elastin cross-linking</td>
</tr>
<tr>
<td>Metallothionein</td>
<td>Copper scavenging</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>Superoxide decomposition</td>
</tr>
</tbody>
</table>

Examples of cuproproteins are shown together with their function. More than 30 cuproproteins are known in mammals. Adapted from ref. [6].

On the other hand, an excess amount of copper is toxic because it generates extremely reactive hydroxyl radicals [7] causing cellular damage such as the oxidation of proteins and lipids, and cleavage of DNA and RNA [8,9]. Its improper incorporations into metalloprotein further cause toxicity. Copper has been shown in vitro to replace zinc
in the zinc finger domain of the human estrogen receptor and inhibits its binding to the promoter region [10,11], suggesting that copper may affect a series of transcriptional factors having zinc finger domain.

Thus, strict regulation of cellular copper concentration is required for metabolism and avoiding toxicity. Maintenance of copper homeostasis requires mechanism(s) such as transporters for uptake and efflux as well as proteins to sense copper ion concentrations.

1.2 Human inherited disorders of defective copper metabolism

The importance of copper homeostasis has been illustrated by two human inherited disorders, Menkes and Wilson diseases having defective copper metabolism (Table 2). Menkes disease is an X-linked recessive disorder characterized by diverse clinical manifestations including growth retardation, hypopigmentation, kinky hair, loose skin, and progressive neurological degenerations [12]. Since these manifestations resemble to those observed in nutritional copper deficiency, most of them are explained by a systemic copper deficiency and thus, defective activities of copper requiring enzymes [13]. In Menkes patients, copper is accumulated in intestine and kidney but low in other organs, suggesting that defective copper export from intestine cause the systemic copper deficiency [14]. Moreover, copper was accumulated in all cultured cell lines except hepatocyte established from patients [15-19].

In contrast to Menkes disease, autosomal recessive Wilson disease appears to be caused by toxic copper accumulation [20]. The major clinical manifestations of the Wilson disease are cirrhosis of liver with neurologic abnormalities and pigmented corneal rings (Kayser-Fleischer rings)[21]. The copper content is increased in liver, brain, and kidney of patients, suggesting that the disease is caused by defective cellular copper
Table 2.

Two human inherited disorders of copper metabolism.

<table>
<thead>
<tr>
<th></th>
<th><strong>Menkes disease</strong></th>
<th><strong>Wilson disease</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical observation</strong></td>
<td>Onset at birth</td>
<td>Onset at late childhood</td>
</tr>
<tr>
<td></td>
<td>Mental retardation</td>
<td>Liver cirrhosis</td>
</tr>
<tr>
<td></td>
<td>Neurological degeneration</td>
<td>Behavior disturbance</td>
</tr>
<tr>
<td></td>
<td>Kinky hair</td>
<td>Dysarthria</td>
</tr>
<tr>
<td></td>
<td>Hypopigmentation</td>
<td>Pigmented corneal rings</td>
</tr>
<tr>
<td></td>
<td>Skeletal defect (cutis laxa)</td>
<td>(Kayser-Fleischer ring)</td>
</tr>
<tr>
<td><strong>Laboratory findings</strong></td>
<td>Decreased serum, brain and liver Cu</td>
<td>Increased liver Cu</td>
</tr>
<tr>
<td></td>
<td>Increased intestinal and kidney Cu</td>
<td>Decreased serum Cu</td>
</tr>
<tr>
<td><strong>Basic defect</strong></td>
<td>Defect of intestinal Cu absorption</td>
<td>Defect of Cu excretion into bile</td>
</tr>
<tr>
<td></td>
<td>Deficiency of Cu dependent enzyme activity</td>
<td></td>
</tr>
<tr>
<td><strong>Locus</strong></td>
<td>Xq 13.3 / recessive</td>
<td>13q 14.3 / recessive</td>
</tr>
<tr>
<td><strong>Gene product</strong></td>
<td>Putative Cu transporting</td>
<td>Putative Cu transporting</td>
</tr>
<tr>
<td></td>
<td>P-type ATPase (ATP7A)</td>
<td>P-type ATPase (ATP7B)</td>
</tr>
<tr>
<td><strong>Expression</strong></td>
<td>All tissues except liver</td>
<td>Liver, kidney and placenta</td>
</tr>
</tbody>
</table>

Adopted from ref. [22]
export from these tissues [23,24].

In 1993, the genes responsible for the both diseases were cloned and identified to encode putative cation transporting P-type ATPase [25-30] closely related to bacterial enzymes conferring heavy metal resistance [31-33]. The P-type ATPase represents one of the three major families of ion translocating ATPases (F-, V-, and P-type). The P-type ATPase family includes more than 50 proteins responsible for the active transport of a variety of cations (H+, K+, Na+, Ca2+, Cd2+, Cu+) across membranes. Hallmark of this family is phosphorylation of the aspartic acid in the invariant sequence Asp-Lys-Thr-Gly during catalytic cycle [34]. P-type ATPases are classified structurally into two subfamilies, heavy metal type and non-heavy metal type [35-38]. Consistent with the clinical manifestations, both Menkes disease and Wilson disease gene products belong to the heavy metal P-type ATPase.

Menkes disease gene is expressed in all tissues tested except liver [25-27], whereas Wilson disease gene is expressed in liver and kidney [28,29]. These results are consistent with clinical observations. Payne et al. [39] demonstrated recently that the expression of Wilson disease protein in fibroblast derived from mottled mouse, an animal model of Menkes disease, restored the phenotypes. This observation supports the notion that these two gene products have similar function in the cellular copper metabolism. Immunohistochemical studies revealed that both Menkes and Wilson disease gene products are located in trans-Golgi membrane and exhibit copper-dependent translocation to plasma membranes, suggesting their direct roles in cellular copper export [40-42].

1.3 Cellular copper transport

Regardless of the importance of copper homeostasis, mechanisms underlying the
regulation of cellular copper contents are poorly understood. Although metallothionein plays a central role on detoxification of excess copper [43], it cannot participate in the uptake or export copper ions [44]. The insight into cellular copper transport came from the studies in *Saccharomyces cerevisiae* (Fig. 1) [45].

In aerobic conditions, copper ions are predominantly in the form of Cu(II). Cu(II) ions are reduced to Cu(I) at the yeast cell surface by the Fre1p and Fre2p, ferric and cupric reductase [46]. Cu(I) is then transported across the plasma membrane by Ctr1p [47,48]. Consistent with the key transport role, *CTR1* deletion mutation (Δ*ctr1*) causes decrease of activities of copper requiring enzymes Sod1p (Cu/Zn superoxide dismutase) and Fet3p (multi-copper oxidase)[48].

Once copper ion enters into the cells, it must be transferred to various cellular organelles. Three small cytoplasmic copper binding proteins are identified recently in yeast. Genetic evidences indicated that they have a common role in capturing copper, and deliver to the distinct destinations, and thus they are named copper chaperone. These chaperons are Cox17p, delivering copper to mitochondria [49,50], Lys7p to cytosol [51], and Atx1p to post-Golgi compartment [52,53]. Atx1p is suggested to transfer copper to Ccc2p which is involved in copper export pathway in yeast [52].

Ccc2p is a yeast homologue for human Menkes and Wilson disease gene products [54,55]. Similar to the mammalian counterparts, Ccc2p is located in the membranes of post-Golgi to secretory pathway [56]. Although, Ccc2p is not characterized biochemically, it appears to export copper from the cytosol to post-Golgi compartment. *CCC2* deletion mutant (Δ*ccc2*) cells fail to deliver copper to the Fet3p, a membrane-bound protein required for high affinity iron uptake, which has copper-binding domains facing organeller lumens [54,56].
Fig. 1  Model of cellular copper trafficking in yeast

Schematic model of cellular copper trafficking in yeast *Saccharomyces cerevisiae* (see text for detail). Ctr1p, high affinity copper transporter; Fre1p/Fre2p, ferric and cupric reductase; Atx1p, copper chaperone specific for Ccc2p; Lys7p, copper chaperone specific for Sod1p; Cox17p, copper chaperone specific for cytochrome c oxidase; Ccc2p, putative copper transporting P-type ATPase; Fet3p, multi copper oxidase required for iron uptake; Ftr1p, high affinity iron transprter.
In addition to the presence of homologous copper ATPase (Cu-ATPase) in yeast and human, homologues for all of the components described in yeast are identified in human [57,58], suggesting that the mechanism for cellular copper trafficking pathway is conserved evolutionarily.

1.4 Objectives of this study

In contrast to the growing knowledge on the cellular copper transport in yeast, mechanisms underlying the copper homeostasis in multicellular organisms are poorly understood. Characterization of genes involved in the copper trafficking is essential for understanding intracellular and whole-body copper homeostasis. In this study, I chose Caenorhabditis elegans as a model organism. The nematode is a simple animal and perhaps most extensively characterized metazoan in terms of anatomy, genetics, and behavior. Moreover, it shares many features that are central problems of mammalian biology.

I have isolated two C. elegans cDNAs cu-a-1 and cu-c-1 which have remarkable sequence similarities to human and yeast Cu-ATPase and copper chaperone protein, respectively, and assessed their functions by introduction of these cDNAs into yeast. The expression of CUA-1 and CUC-1 in the corresponding yeast null mutants (Δccv2 and Δatxl) restored the phenotype, suggested their conserved functions [59,60]. Expressions of the genes responsible for the cDNAs were examined in C. elegans using transgenic animals. Consistent with the notion that CUA-1 and CUC-1 constitute a copper trafficking pathway, these two genes were expressed in the same cell during worm development [60].
CHAPTER 2  ROLES OF COPPER ATPASE (MENKES/WILSON DISEASE GENE HOMOLOGUE) FROM CAENORHABDITIS ELEGANS

2.1 INTRODUCTION

There is no convincing evidence for copper transport by eukaryotic putative Cu-ATPases except that of Saccharomyces cerevisiae. The yeast CCC2 gene (Menkes/Wilson disease gene homologue) has been analyzed by genetic studies (Fig. 1). Disruption of the CCC2 gene resulted in defective maturation of the Fet3p (multi-copper oxidase) which is essential for high affinity iron uptake [54] together with the Ftr1p [61]. Phenotypically, the Δccc2 (CCC2 gene deletion) mutant could not grow under iron limited conditions because the Fet3p remained as an apo-form with no iron uptake activity [54]. The requirement of the Ccc2p for the delivery of copper to the Fet3p 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 roles of putative Cu-ATPases from other eukaryotes.

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, I chose Caenorhabditis elegans as a model animal. In this chapter, I cloned a C. elegans cDNA coding for a putative Cu-ATPase (CUA-1) closely similar to those for Menkes and Wilson diseases gene product, and showed that the cDNA could rescue the yeast Δccc2 mutant. This was the first example of functional expression of a heterologous putative Cu-ATPase in yeast.
2.2 MATERIALS AND METHODS

2.2.1 General methods

Wild-type (var. Bristol, strain N2) and transgenic *C. elegans* were grown at 20 °C on NGM agarose plates (0.3 % NaCl, 0.25% peptone, 5 μg/ml cholesterol, 1 mM CaCl₂, 1 mM MgSO₄, 25 mM potassium phosphate, pH 6.0, and 2 % agarose) seeded with *Escherichia coli* DH5α or with OP50-1 [62]. Standard methods [63] were used for the molecular cloning and the genomic DNA preparation from *C. elegans*. All chemicals used are highest grade commercially available.

2.2.2 Primers used

Oligonucleotide primers used in this study are listed below.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<td>C744Afw</td>
<td>5'-CCTGCAAGTCTGGTCTCGCAA-3'</td>
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<tr>
<td>C744Arv</td>
<td>5'-GACTTGCAGGACATGCAATGCAA-3'</td>
</tr>
<tr>
<td>D786Nfw</td>
<td>5'-TTTCATAAAAACTGCAACAACTCA-3'</td>
</tr>
<tr>
<td>D786Nrv</td>
<td>5'-TTTTATTGAAAAACAATTGTGGTTAC-3'</td>
</tr>
<tr>
<td>D-ScCCC23</td>
<td>5'-TGGGCCAGTCCTCTCAAACT-3'</td>
</tr>
<tr>
<td>D-ScCCC25</td>
<td>5'-GTTTCAGGAACCATGGGATAC-3'</td>
</tr>
<tr>
<td>HA fw</td>
<td>5'-ATC(TACCCATACGATGGTCCGGATTACGCT)₃GAT-3'</td>
</tr>
<tr>
<td>HA rv</td>
<td>5'-ATC(AGCGTAATCCCGGAACATCGTATGGGTA)₃GAT-3'</td>
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<tr>
<td>Pmafw</td>
<td>5'-GTCGAACACGTGAGTCTTC-3'</td>
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<tr>
<td>Pmarv</td>
<td>5'-GAAGACTCAGTGTCCGAC-3'</td>
</tr>
<tr>
<td>RTrv</td>
<td>5'-CATTTTCGCCGAATTGTACTC-3'</td>
</tr>
<tr>
<td>SL2</td>
<td>5'-GGTTTTAACCCAGTTACTCAAG-3'</td>
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</table>
universal M13 fw  
5'-GTAAACGACGGCCAGT-3'

universal M13 rv  
5'-GGAAACAGCTATGACCATG-3'

2.2.3 Isolation of putative copper ATPase (cua-1) cDNA from *C. elegans*

The EST clone, yk29a9 (kindly supplied by Dr. Y. Kohara), was from a cDNA library of a mixed *C. elegans* population (eggs, larvae, and adult worm). Partial sequencing of the cDNA from its 5'-end (GenBank™ accession number D36097) revealed an amino acid sequence (residues 571 through 689, Fig. 2) related to those of the 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 RT-PCR was performed according to the manufacturers' instructions. The primers for RT-PCR were (Fig. 2): SL2, equivalent to the 22-nucleotide *C. elegans* trans-spliced leader 2 sequence [64]; and RTrv, complementary sequence corresponding to the amino acid residues between positions 708 and 715 of the ATPase (Fig. 2). ExTaq polymerase (Takara) treated with TaqStart Antibody (CLONETECH) was used for 30 PCR cycles.

Full-length cDNA (*cua-1* cDNA, *C. elegans* Cu-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 (GenBank™ accession number D83665) (Fig. 3).
2.2.4 Introduction of Asp-786 to Asn, Cys-744 to Ala mutation and hemagglutinin (HA) epitope tag into CUA-1

An *HindIII-HindIII* fragment (nucleotides 1733 - 2537, Fig. 3) from CUA-1 cDNA was subcloned into pBluescript II SK(+), and used as a template for PCR to introduce an Asp-786 to Asn (GAC to AAT) mutation (Fig. 3). The combinations of primers D786Nfw and universal M13 rv, and D786Nrv and universal M13 fw were used for the first PCR, and primers universal M13 rv and M13 fw were used for the second PCR (Fig. 3). The mutant *HindIII-HindIII* fragment was replaced with that of the wild type in the full-length cDNA. Primers C744Afw and C744Arv were used for first round PCR to introduce a Cys-744 to Ala (TGT to GCA) mutation.

The HA epitope sequence from the human influenza virus was inserted into CUA-1 cDNA. A *BamHI-SalI* fragment (nucleotides 1 - 583) coding for the amino terminus of the ATPase was subcloned into pBluescript II SK(+) to generate a unique *PmaCl* restriction site (GTGCAC, Fig. 3) by PCR mediated mutagenesis. The combinations of primers Pmafw (Fig. 3) and universal M13 fw, and Pmarv and universal M13 rv were used for the first PCR, and primers universal M13 fw and rv were used for the second PCR. The mutated *BamHI-SalI* fragment was obtained and replaced with that of the wild type. The oligonucleotide coding for three repeats of the HA epitope was inserted in-frame into the *PmaCl* site of the engineered cDNA. These manipulations caused a single amino acid substitution of the CUA-1 reading frame and 29 amino acids insertion at the N-terminal portion of the protein. The same method was used to introduce the HA epitope into CUA-1 cDNA having Asp-786 to Asn and Cys-744 to Ala mutations.
2.2.5 Expression of CUA-1 in yeast CCC2 gene deletion mutant

A part of the yeast CCC2 gene [55] was amplified from chromosomal DNA of S. cerevisiae YPH499 (MATa, ura3-52, leu2-Δl, trp1-Δ63, his3-Δ200, ade2-101, lys2-801) by PCR using two oligonucleotide primers D-ScCCC25 and D-ScCCC23. A BamHI-Sal I fragment (0.6 kbp, 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 [65]. The gene disruption (Δccc2) was confirmed by PCR and negative growth by respiration.

Full-length cua-1 cDNA and its derivatives were inserted into yeast multicopy vector pSY114 (YEp13-based plasmid with the LEU2 marker, kindly provided by Drs. Sone and Yoshida) under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter and phosphoglucomokinase terminator. The resulting recombinant plasmids were introduced into the Δccc2 mutant and transformants were selected on a solid synthetic dextrose (SD) medium (0.67 % yeast nitrogen-base without amino acids, 2 % glucose, and 2 % agar) supplemented with adenine, uracil, tryptophan and lysine [66]. The transformed cells were grown at 30 °C on an agar medium (2 % bactopeptone, 1 % yeast extract, 50 μM EDTA) containing 2 % glucose (YPD) or 3 % ethanol (YPE), or the same medium with 1 mM Fe(NH₄)(SO₄)₂.

2.2.6 Immunological detection of CUA-1 in yeast

Yeast crude cell lysate was prepared as described in [67]. The lysate was applied to a 7.5 % (w/v) SDS-PAGE using standard protocol [63]. Protein immobilization and immunological detection were performed using standard procedure [68] with minor modification: the 20 % (w/v) skim milk was used for blocking. The HA epitope was probed by 12CA5 anti-HA monoclonal antibody (Boeringer Mannheim) followed by
visualization using ECL detection kit (Amersham). Protein concentrations of the lysate was determined with bicinchoninic acid (Pierce Chemical Co.) using bovine serum albumin as a standard.

Immunohistochemical staining was carried out as follows. Yeast cells grown exponentially in liquid SD medium were fixed with 5% formaldehyde, treated with Zymolyase 100T for 30 min, and placed on a polylysine-coated slide glass. The slide glass was treated with 0.1% Triton X-100, incubated with the first antibody (mouse anti-HA, clone 12CA5 diluted to 0.5 μg/ml), washed with phosphate-buffered saline, and then incubated with fluorescein isothiocyanate (FITC) conjugated goat Fab fragment to mouse IgG (Organon Teknika Corp.) (diluted to 2.5 μg/ml).

2.3 RESULTS

2.3.1 Isolation of cDNA coding for a putative Cu-ATPase (CUA-1) from C. elegans

An EST clone yk29a9 was isolated from a C. elegans cDNA library, and the result of partial sequencing suggested that it coded for a Menkes/Wilson disease protein homologue (Dr. Y. Kohara, personal communication). DNA sequencing revealed that the EST clone apparently lacks its 5' half, because the clone did not contained trans-spliced leader sequence, which is attached to the 5' end of C. elegans transcript. The missing part of the cDNA was obtained by RT-PCR, using a primer designed from the yk29a9 specific sequence and that of a trans-spliced leader 2 (SL2) (Fig. 2).
Fig. 2  Sequence of the cDNA of C. elegans putative copper transporting ATPase (cua-1) and the derived amino acid sequence.

Arrows above the DNA sequence indicate the annealing sites for the primers used in RT-PCR experiments. Putative transmembrane helices derived from the hydrophobic plot analysis [73] are underlined. Blue boxes denote specific motifs found in heavy metal ATPases. Red boxes are conserved residues in all P-type ATPases. Possible polyadenylation signal is double underlined. The cua-1 cDNA sequence has been deposited in GenBank (Accession Number D83665).
Fig. 3  Introduction of a hemagglutinin(HA) epitope tag and Cys-744 to Ala, Asp-786 to Asn mutations.

The entire cua-1 cDNA (4001 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 from PCR. The rest of the cDNA was derived from EST clone yk29a9. The primers used for mutagenesis, Pmafw, Pmarv (lower left), C744Afw, C744Arv (upper right), D786Nfw and D786Nrv (lower right), are shown. Universal primers (M13 reverse and forward primers) were also used for mutagenesis. The base changes for these mutations are shown by vertical arrows. The sequence encoding HA epitope tag (upper left) was inserted into the PmaCI site. The restriction sites used for subcloning are also shown.
**Fig. 4  Chromosomal location of *cua-1* gene**

Most of the *C. elegans* genome is represented by a grid of 958 YAC clones on yeast artificial chromosome (YAC) polytene membrane. These YAC clones were selected to give twofold coverage of the genome. The *cua-1* cDNA was labelled and hybridized to the membrane, and revealed that *cua-1* gene was located near the right end of the chromosome III (in the cosmid gap between F45G2 and T27E9).
The putative full-length cDNA was constructed utilizing the SalI restriction site at 1885 in the nucleotide sequence (Fig. 3). Both strands of the constructed cDNA (4001 bp) were sequenced repeatedly. The presence of a trans-spliced leader sequence and a poly A tail in the cDNA strongly suggested that the cDNA covers the full length. The BLAST search of C. elegans genomic sequence by the cDNA revealed a cua-1 gene located on chromosome III, being consistent with the result of Southern blotting (Fig. 4). The sequence of the cDNA completely identical with that obtained from genomic sequencing project (YAC clone Y76A2 of chromosome III) [69]. Thus, I concluded that the cDNA is the same as the one derived from the full-length mRNA, and used for further studies.

2.3.2 Amino acid sequence of the putative C. elegans Cu-ATPase

The cDNA coded for a 1,238-amino acid protein (Fig. 2) having a calculated molecular weight of 133,468. Nucleotide sequence immediately upstream the initiation codon (nucleotides 63 - 73, ACACAAAAATGT) (Fig. 2) was within the consensus for the C. elegans translation start site (ANN^N/C^N/G^NAAATGN) [70].

The amino acid sequence of the putative ATPase (Fig. 2) shares all the features reported for the cation transporting P-type ATPases which form aspartyl phosphate intermediates during the catalytic cycle [71]: Thr-Gly-Glu-Ser in the flexible loop (Fig. 5C), Asp-Lys-Thr-Gly-Thr in the phosphorylation domain (Fig. 5B), where the aspartate residue (Asp-786 in the C. elegans protein) is phosphorylated [72], 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 (Fig. 5D) are conserved. Moreover, the specific motifs for the putative heavy metal ATPases [35], Gly-Met-Thr-Cys-X-X-
A

Copper binding motif

**\* **** \* **

CUA-1 Cu1  IKGMTNSCVKNIQDVIGAKPGIHISIQVNL
CUA-1 Cu2  IEGMTCHAVCNNIQDVTGSDKGIVKIVSL
CUA-1 Cu3  VEGTCASCVQYIERNSKIEGVHISIVAL
Human MNK Cu1  VEGTCNSCVWTIEQIQGKVNGVHIIKVSL
Human MNK Cu2  VEGTCCHCTSTIEGKGLQGVQRKIVSL
Human MNK Cu3  IDGMHCKSCVSNIESLTLALQYVSVSIVSL
Human MNK Cu4  IDGMCTSCVQSIEGVISKKPGKSVRISVSL
Human MNK Cu5  VTGMCASCVANIERNLRGBGIYISSLVAL
Human MNK Cu6  VRGTCASCVHKIESSLTKHRGILYCSVAL
Human WND Cu1  ILGTMQSCVKSIEDRSLNKIGTSMKVSL
Human WND Cu2  VEGTCQSCVSSIESGKVRKLQGVVRKVLAS
Human WND Cu3  IDGMHCKSVLNEENIGQLGVLQISIQVSL
Human WND Cu4  IAGMTCASCVHSIEGMRQLEQVQISVSL
Human WND Cu5  IAGMTCASCVSNIERNLQKEAGVLSVLVAL
Human WND Cu6  ITGMCASCVHNIESLKLTRNGITYASVAL
Yeast Ccc2p Cu1VHGMCSCACTNTINTQLRALKGVTKCDISL
Yeast Ccc2p Cu2VQGMCSCVSTVTQKEEGEVESVVSL
Yeast Pca1p  VSGMCTGCESCKLKKSFGALKVHGLTLS
E. hirae  ITGMCASCARIKELNQPGVMSATVNL
E. coli o732  VSGMDCAACARKVENAVRQLAGVNVQVLF

B

CPC, DKTG, SEHP motifs

**\* **** \* **

CUA-1 740 IAIPCSLGLATPTAVMGTGVGAANGILIKGGEPTSCSVHKVTVTTFDKTG
Human MNK 999 IAIPCSLGLATPTAVMGTGVGAQNGILIKGGEPLEMAHKVKVVFDKTG
Human WND 951 IAIPCSLGLATPTAVMGTGVQAQNGILIKGGEPLMAMHKKTMDKFGT
Yeast Ccc2p 581 VASPCALSLATPTAIVMTGVGVGAQNGILIKGGEVLEFKNSITTFVDKTG
Yeast Pca1p 857 VSPCIGLAVLPVFVIASGVAAAKRGVIFRKSAESIEEVSNHVTVDVTKTG
E. hirae CopA 379 IACPSCALATPTAIVMTGVGHNGILIKGGEALEGGAHLNISLLDTTG
Rabbit Ca 305 AAIPEQLAPAVITTCLALGTRNMAKKNIARSLSPVETLGCTSVICSDDKTG
Yeast Pma1p 332 IGVPVLAPAVVTITMVAAGYALKQIAVQKLSATIESLAVGLCDDKTG

CUA-1  ITEG-RPRVQIASVFNPSTMKLITFLSGATEALSEHPIGNA 833
Human Mcl  ITHG-TPVNVQVKLTESRISHHKILAVGTAESNSEHPLGA 1092
Human Wcl  IIHG-VPRVMRLLGDVTLLPLRVKLAVGTAESSEHPLGA 1044
Yeast Ccc2p  LTTGFMVKKFLDSNWGQNEDEVLACIKATSIDSHPVS 675
Yeast Pca1p  LTEG-KLTQVH----ETVRGDRHSQSSLGLLTEGEEHPVS 945
E. hirae CopA  ITQG-RPEVTDVIGPKD-------TISLFYSLHASEHPLGA 464
C

TGES/A motif

CUA-1  GAKVPVDGVVVDGKSSVDESFITGESMPV
Human MNK GGKFVFDGRVIEGHSMVDESITGEAMPV
Human WND GGKFVFDGKVLEGNTMADESITGEAMPV
Yeast Ccc2p GMKIPADGIIITGSESEIDESLMTGESILV
Yeast Pcalp DSRIPTDGTVISGSSEVDEALITGESMPV
_E. hirae_ CopA GEQVPTDGRIAGTSALDESMLTGESVPV
_E. coli o732_ GGRLPADGKLSPFASFDSEALTGESIPV

D

GDGXND motif

CUA-1  VAMVGDGVNDSPALAEANVGIA
Human MNK VAMVGDGDPSALAMANVGIA
Human WND VAMVGDGVNDSPALAQADMGVA
Yeast Ccc2p VAVVGDGINPALALSVDGLIA
Yeast Pcalp VVFCGDGNDAILTGATIGVH
_E. hirae_ CopA VGMVGDGINPALRLADVAGIA
_E. coli o732_ LAMVGDGINDAPAMKAAAIGIA

Fig. 5  Amino acid sequence alignment of CUA-1

Sequences of heavy metal ATPases from various species are aligned. Rabbit sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, yeast plasma membrane H\(^+\)-ATPase are also shown in B).

A) copper binding motif, conserved residues were boxed. GMTCCXXC, found in many copper binding proteins were indicated by asterisk.

B) CPC, DKTG, and SEHP motifs, residues conserved among heavy metal ATPase were boxed. Residues conserved in all P-type ATPase are boxed. Residues analyzed in this chapter are indicated by asterisk.

C) and D) TGES/A and GDGXND motifs. Conserved residues are boxed.
Fig. 6 Schematic alignment of heavy metal ATPase

Open and closed boxes represent the cytoplasmic and transmembrane domains of these ATPases, respectively. Amino acid sequence motifs typical for heavy metal ATPase are indicated above.
Cys (Fig. 5A), Cys-Pro-Cys and Ser-Glu-His-Pro (Fig. 5B), are also found (Fig. 1). Similar to other heavy metal ATPases, the presence of eight transmembrane helices [35,38] in CUA-1 was suggested from the hydropathy plot (Figs. 2 and 6) [73]. The amino acid sequence of the CUA-1 closely resembled to those of other eukaryotic or bacterial enzymes (Fig. 6). The identities between ATPases of *C. elegans* and other sources are: Menkes disease protein [27], 46.1% identical over 1135 residues; Wilson disease protein[28], 42.6% over 1220 residues; yeast Ccc2p [55], 29.6% over 928 residues; and *Enterococcus hirae* CopA [33], 30.7% over 680 residues. These high sequence similarities strongly suggest that the CUA-1 has essentially the same functions as other heavy metal ATPases.

**2.3.3 Function of CUA-1 in Δccc2 mutant**

The high sequence homology between the CUA-1 and the yeast Ccc2p prompted me to examine whether expression of CUA-1 could rescue the yeast Δccc2 (*CCC2* gene deletion) mutant. The Δccc2 mutant could not grow on iron limited ethanol-based medium (YPE) by respiration because intracellular copper transport by the Ccc2p is required for high affinity iron uptake [54], which is indispensable for growth under iron limited conditions (Fig. 7B). The Δccc2 mutant could grow on the YPE medium containing a large excess of the ferric ion [54] because iron is taken up by the low affinity transport system. The Δccc2 mutant carrying *C. elegans cua-1* cDNA could grow on the YPE medium similar to the mutant expressing the yeast *CCC2* gene (Fig. 7C), suggesting that CUA-1 could complement the Δccc2 mutation. On the other hand, the Δccc2 mutant carrying cua-1 cDNA with a mutation in a putative phosphorylation site (Asp-786 to Asn), the conserved 6th transmembrane Cys-Pro-Cys motif (Cys-744 to Ala) (Fig. 7A) and the
Fig. 7 Rescue of Δccc2 mutant by CUA-1

A) Structural model for CUA-1 based on hydropathy plot and homology to other P-type ATPases. Characteristic motifs are indicated. Residues analyzed in this chapter are boxed.

B) Function of Ccc2p in yeast. Ccc2p plays an essential role for the function of Fet3p, which is required for high affinity iron uptake in yeast. Because of the reason, Δccc2 cell cannot grow by respiration.

C) Rescue of yeast Δccc2 mutant by CUA-1. Yeast Δccc2 mutant cannot grow on YPE medium without addition of excess iron to the medium (vector). The Δccc2 mutant carrying wild type cua-1 cDNA (WT, WT (tagged)) could grow on iron limited YPE medium similar to the mutant harboring the yeast CCC2 gene (CCC2). On the other hand, the Δccc2 mutant carrying the mutant cua-1 cDNA (C744A; Cys-744 to Ala, D786N; Asp-786 to Asn) could not restore the growth defect, suggesting that CUA-1 functioned as P-ATPase.
vector without the cDNA insert showed negative growth on the YPE medium, although they could grow glycolytically on YPD medium (Fig. 7C).

To confirm the synthesis of the CUA-1 in the yeast Δccc2 mutant, an HA tag was introduced near the amino terminus of *cuc-I* 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. 7C). As shown by Western blot analysis (Fig. 8A), a single protein band with the HA tag was detected for the Δccc2 cells carrying tagged *cua-I* cDNA but not for the control cells. The approximate 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-I* cDNA having Asp-786 to Asn and Cys-744 to Ala mutations (Fig. 8A).

### 2.3.4 Subcellular localization of the CUA-1 expressed in yeast

It was of interest to identify the yeast organelles in which the CUA-1 was located.

As shown by immunofluorescence microscopy (Fig. 8B, left), CUA-1 with the HA tag was localized in membranes of a large intracellular membrane, possibly vacuoles. Vacuolar structures were confirmed by the Nomarski images of the same cells (Fig. 8B, right). Fluorescence staining of vacuolar membranes was not observed without the addition of the anti-HA antibody or in the control cells expressing CUA-1 without the tag (data not shown).
Fig. 8  Immunological detection of CUA-1 expressed in yeast

A)  Presence of CUA-1 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 CUA-1 with the HA tag was detected immunochemically in Δccc2 cells carrying cua-1 cDNA with the HA tag. The molecular weight of the CUA-1 with the HA tag (arrow) was estimated to be 140,000 from the position of protein standards.

B)  Localization of the CUA-1 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) (left). The cells were visualized by fluorescence microscopy. Nomarski images are also shown (right).
2.3.5 Functional analysis of human Wilson disease gene product in yeast Δccc2 mutant

Yeast Δccc2 mutant has been useful for the functional analysis of CUA-1 as described above. It became of interest to know whether human Wilson or Menkes disease gene can be functionally expressed in yeast. If the disease gene could rescue the yeast Δccc2 mutant, we can analyze function of the gene product. Studies in this direction was carried out with Wilson disease gene product in collaboration with Dr. Sugiyama and his colleagues in Akita University Medical School [74].

As shown in Fig. 10, the wild type human Wilson disease gene product could be expressed in the yeast, and the expression restored the growth defect of Δccc2 mutant on iron limited medium (Fig. 11). Although Wilson disease patient's genes were sequenced [75], the relationships between mutations and functional defects are poorly understood. The Wilson ATPase (ATP7B product) has six copper binding motifs (Gly-Met-Thr-Cys-X-X-Cys) in its amino terminus (Fig. 9), whereas Ccc2p has two motifs (Fig. 6). However, functional differences among these motifs are unknown. To address these questions, I started collaboration. Various plasmids carrying cDNA for mutant proteins found in the disease patients were constructed (Fig. 9) and introduced into the yeast Δccc2 mutant.

Amounts of mutant proteins except Cu 1-4 and Cu 1-5 were comparable to wild type as shown by Western blot (Fig. 10). Then I tested whether mutant protein could support growth of Δccc2 mutant on iron limited medium. Unexpectedly, His-1069 to Gln mutant ATPase could rescue the growth defect of Δccc2 mutant, while other mutants (Asn-1027 to Ala, Thr-1029 to Ala and Asp-1270 to Ser mutants) could not (Fig. 11). This observation suggests that the Wilson disease gene product complement the growth defect by its function as the P-type ATPase like C. elegans CUA-1, since the mutants
failed to reverse the phenotype.

Analysis of a series of deletion of amino terminus copper binding motifs (Cu 0, Cu 1-2, Cu 1-3, Cu 1-4, Cu 1-5 and Cu 6) showed that only 6th motif is sufficient to complement the Δcsc2 phenotype on the iron limited medium. This observation is consistent with the severe clinical observation of the Wilson disease patients having the 6th motif deleted [75].

![Diagram of Wilson wild type and various mutants](image)

**Fig. 9 Mutant cDNAs analyzed**

Dr. Iida of Akita University Medical School constructed various mutant cDNAs shown above. All cDNAs contain the sequence encoding three repeat of HA epitope tag at their C-terminus. Thin line represents portion deleted.
Fig. 10  Presence of Wilson disease gene product (WND) in yeast Δccc2 mutant cells

Total lysate from \(2 \times 10^7\) cells of each transformant was electrophoresed on polyacrylamide gel (7.5 %) in the presence of 0.1% sodium dodecyl sulfate, and transferred to a nitrocellulose membrane. All other procedures were as in Fig. 8A.

A) WND proteins having various point mutations found in the disease patients.

B) WND proteins lacking copper binding domains.

Mutations are as shown in the Fig. 9.
Fig. 11 Complementation of yeast Δcc2 mutant by wild-type or mutant human Wilson disease gene products.

The yeast Δcc2 mutant cells were transformed with the WND cDNA having various point mutations found in the disease patients (upper) or cDNA lacking copper binding motifs (lower). Transformed cells were spread onto an SD medium with (SD+Fe) or without (SD) 500 μM ferric ammonium sulfate and incubated at 30 °C for 4 days. Mutants are as shown in Fig. 9.
2.4 DISCUSSION

Genetic studies indicated that the yeast Ccc2p is a copper transporter necessary for loading copper to the Fet3p, and that the copper-bound Fet3p is essential for high affinity iron uptake [54]. Present study clearly showed that the C. elegans cua-1 cDNA could rescue the yeast Δccc2 (CCC2 gene deletion) mutant, suggesting that the CUA-1 substituted for the Ccc2p and transported copper to a compartment in which the Fet3p accepts copper. This rescue was possibly due to complementation by the CUA-1 functioning as a P-type ATPase, because the Asp-786 (phosphorylation site) to Asn mutant ATPase could not rescue the Δccc2 mutant.

The Cys-Pro-Cys motif is conserved in heavy metal ATPase and located in the middle of a predicted transmembrane domain. A few exceptions could be found in prokaryotic heavy metal ATPases: Enterococcus hirae CopB (Cys-Pro-His) [33]; Helicobacter pylori hpCopA (Cys-Pro-Ser-Cys) [76]. The proline residue in the Cys-Pro-Cys motif of heavy metal ATPase is also found in non-heavy metal ATPases. However the invariant proline is not flanked by cysteines: the corresponding sequence in the Ca²⁺-ATPase is Ile-Pro-Glu [77]. This region has been proposed to form a domain essential for calcium ion transport [78]. I found that the alteration of Cys-Pro-Cys to Cys-Pro-Ala in CUA-1 abolished its function in the yeast, suggesting that the thiol group of the carboxyl terminal cysteine residue in the motif may serve as a copper ion transport domain.

The CUA-1, when expressed in yeast, was observed in vacuolar membranes by immunofluorescence microscopy, implying that the ATPase functions in vacuoles. However, Yuan et al. suggested, on analysis of mutants defective in post-Golgi sorting,
that copper loading by the Ccc2p to Fet3p occurs in a post-Golgi compartment, possibly in the secretory pathway [56]. Thus, it is likely that a part of the CUA-1 was assembled and functioned in a yeast post-Golgi compartment, although most of the enzymes were assembled in vacuolar membranes. Yeast vacuolar membranes are known to be default destinations for several proteins [79-81].

Biochemical characterization of putative heavy metal ATPases, such as the detection of a phosphorylated intermediate or ATP-driven metal ion transport, has been carried out only for bacterial enzyme [82-84]. My attempts to detect ATPase activity and a phosphorylated intermediate of the CUA-1 expressed in yeast were not successful: I could not detect ATPase activity (assayed with 20 mM MOPS-NaOH pH 7.0, 200 mM KCl, 5 mM MgCl₂, 5 mM DTT, 10 μM Bafilomycine A₁, 2 mM ATP, 10 μM CuCl₂) or an intermediate (assayed with the same buffer using 40 μM γ³²P-ATP instead of 2 mM ATP) using vacuolar membranes (up to 15 μg protein). The negative results may be due to the high protease sensitivity of CUA-1. I noted degradation of the CUA-1 in a vacuolar membrane upon fractionation of yeast cells. It is also noteworthy that the transport activities of heavy metal ATPases detected in bacteria are very low compared with those of non-heavy metal ATPases: the rate for Staphylococcus aureus Cd²⁺-ATPase (CadA) was estimated to be 0.13 μmoles/min/mg of Cd²⁺-ATPase, assuming that 10 % of membrane proteins was Cd²⁺-ATPase [82], whereas rabbit sarcoplasmic Ca²⁺-ATPase exhibited Ca²⁺ transport activity of 6.2 μmoles/min/mg of ATPase protein [85]. The transport of copper by E. hirae ATPase (CopB) [84] was much lower than that of Ca²⁺ by the Ca²⁺-ATPase.

The structural features of putative heavy metal ATPases are clearly different from those of non-heavy metal transporting P-type ATPases [35,38]. The heavy metal
ATPases have longer amino terminal sequences forming unique domains containing Gly-Met-Thr-Cys-X-X-Cys motifs. It has been demonstrated that these motifs are binding sites for heavy metal ions in Menkes and Wilson ATPases [86]. The similar motifs are found in other proteins capable of heavy metal binding such as mercury binding proteins [87]. The number of the motifs in the heavy metal ATPases varies with the origin of the enzymes, although the significance of the differences is currently unknown: *C. elegans*, three motifs; human (both Menkes and Wilson), six [27,28]; yeast (Ccc2p), two [55]; and CopA, one [33]. In this regard, it is of interest that the CUA-1 (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-ATPase, and may be extended to human Menkes and Wilson disease gene products [41,74,88].

As described in section 2.3.5, the human Wilson disease gene product restored the growth defect of Δccc2 mutant, demonstrating that the human cDNA was expressed functionally in the yeast. Furthermore, expression of mutant cDNA (His-1069 to Gln), which causes relatively mild clinical effect in patients [89], complemented the yeast defect, whereas mutations giving severe effects (Asn-1027 to Ala, Thr-1029 to Ala and Asp-1270 to Ser) [89] could not complement Δccc2. Thus clinical effects qualitatively correspond to the analysis using Δccc2. Payne et al. recently demonstrated that the His-1069 to Gln mutant ATPase showed mislocalization, misfolding and subsequent degradation in mammalian cell line at 37 °C but not at 28 °C [39]. The restoration of yeast Δccc2 by this mutant protein may be due to the proper localization of the mutant protein at 30 °C (the yeast growth temperature).

The six Met-Thr-Cys-X-X-Cys motif of human Menkes and Wilson disease gene product has been shown *in vitro* to bind copper selectively with stoichiometry of one
copper per one motif [86], demonstrating the equivalence among the six motifs in terms of copper binding. However, the result described in this study showed that only the 6th motif (most proximal one to the C-terminus) is sufficient to rescue the Δcc2 mutant. Consistent with the result, the patients with the deletion of the 6th copper binding motif caused by an exon skipping mutation showed severe clinical observation [75], and thus indicating that the six motifs are not equivalent functionally with respect to copper transport.
CHAPTER 3 ROLES OF COPPER CHAPERONE IN C. ELEGANS

3.1 INTRODUCTION

Three independent pathways for intracellular copper ion trafficking have been shown in yeast (Fig. 12) [49,51,52,90]. A pathway including Atx1p and Ccc2p carries copper to post-Golgi compartment: Cu(I) is transported into cytosol through plasma membrane by Ctr1p, transferred to copper chaperone Atx1p and carried to Cu-ATPase Ccc2p in the post-Golgi membranes [52,90].

Ccc2p is a homologue of human Menkes [25-27] and Wilson [28-30] diseases and C. elegans CUA-1 copper ATPases [59,91]. Expressions of complementary DNAs coding for these ATPases could complement a null mutation of yeast CCC2 gene [41,59,74,88, 91]. Potential ATX1 homologues were noted in the GenBank™dbest database of A. thaliana, O. sativa and human and C. elegans from genome sequence [92], suggesting that Atx1p / Ccc2p pathway is present in higher eukaryotes. Consistent with this prediction, the human homologue HAH1 could complement yeast Δatx1 mutation [93]. RNA blot analysis identified corresponding mRNA in all human tissues and cell lines examined [93]. On the other hand, Menkes and Wilson copper ATPases showed more restricted tissue distribution [25,27-29].

It became of interest to know whether the ATX1 homologue of C. elegans is expressed in all worm cells similar to HAH1 or in restricted cellular distribution. The homologue may be expressed in the same cells, if it functions with copper ATPase. In this chapter, I cloned a cDNA for C. elegans Atx1p homologue CUC-1 which could complement yeast Δatx1 mutation. The cuc-1 expression is developmental stage-specific:
External copper is imported into yeast by plasma membrane transporter, Ctr1p. Genetic studies revealed that the internalized copper is delivered to different cellular locations by distinct copper chaperone proteins: Lys7p targets Cu to cytosolic Cu Zn SOD (Sod1p), Cox17p guides Cu to mitochondria for insertion into cytochrome c oxidase complex, and Atx1p directs Cu to a Cu-ATPase of post-Golgi compartment (Ccc2p).
expressed in intestinal and hypodermal cells of adult and larvae, respectively. The expression pattern was similar to *cua-1* except that pharyngeal muscle cells preferentially expressed CUA-1 ATPase.

### 3.2 MATERIALS AND METHODS

#### 3.2.1 Primers used

Oligonucleotide primers used in this study were listed below.

- **CeATX-1 AAAXho** 5'-CCGCTCGAGTTTTTTTTTTTTAAGTTTAGAAAAAT-3'
- **CeATX-1 fw1** 5'-TACGTTTTCGAAATCGGACATGACAT-3'
- **CeATX-1 fw2** 5'-GCAATGACATGCAATGGGCTGT-3'
- **CeATX-1 Nhe** 5'-CGGAATTCATGCTAGCACACAGTACCGTTTTCGAAA-TGGGCATG-3'
- **CeATX-1 rv1** 5'-GTAATTGCTTATCTCTTTTCTCCTG-3'
- **CeATX-1 rv2** 5'-CAAGTACATCAGATGCTGGT-3'
- **cuaLA10** 5'-ACCGGTGACTCCCTCAAATTGTGGTCCGGTTCCTGT-TTCT-3'
- **cuaex2.0** 5'-GTAATTGGAGCGAAGCCAGAATTCATAGGGATCCC-G-3'
- **cucE3** 5'-CGGGATCCGAGTTGCTTGATCTCTTTTCTTG-3'
- **cucUP** 5'-CACATGCATGCTTGGTTCACTGGTTGAACACTGCGGA-3'
- **D-ScATX13** 5'-AGTAGCTACAAATACAAAATAGTAAAAACTATTTTC-
3.2.2 Isolation of copper chaperone, ATX1 homologue cDNA from C. elegans

The Marathon™ cDNA amplification Kit (CLONTECH) and C. elegans total RNA were used to isolate a yeast ATX1 homologue, cuc-1 cDNA. Primers CeATX-1 Fw1 and CeATX-1 Rv1 combined with adaptor primer 1 (CLONTECH) were used for the first-round PCR to amplify 3' and 5' fragment of the cDNA, respectively. For nested second-round PCR, primers CeATX-1 Fw2 and CeATX-1 Rv2 combined with adaptor primer 2 (CLONTECH) were used (Fig. 13). The resulting PCR fragments were ligated to construct the full length cDNA for cuc-1. The resulting 0.5 kbp fragment was digested by BamHI and inserted into the corresponding site of pBluescript II SK(+) (STRATAGENE) to generate pSKcuc-1. Nucleotide sequence of the cDNA was determined (GeneBank™ Accession Number AB017201).
3.2.3 Northern blot hybridization

The $^{32}$P-labelled CUC-1 cDNA was prepared using Random Primed DNA Labelling kit (Boehringer Mannheim) and used as a probe. Twenty-five µg of *C. elegans* total RNA was electrophoresed, transferred to a Hybond-N+ nylon membrane (Amersham Life Science Inc.), and hybridized with the probe by using standard methods [63]. The membrane was washed to a final stringency of 0.1 x SSC (1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1 % SDS at 65 °C after the hybridization.

3.2.4 Plasmids used for yeast transformation

Plasmid pKTcuc-1 was constructed by mobilizing *cuc-1* ORF into a multicopy yeast expression plasmid pKT10 [94]. A 0.4 kbp fragment carrying a synthetic *NheI* site after the initiation codon of *cuc-1* cDNA was amplified from pSKcuc-1 by PCR using a pair of primers CeATX-1 Nhe and CeATX-1 AAAXho. The fragment was digested by *EcoRI* and *XhoI*, then introduced into the corresponding restriction sites of pKT10. Two oligonucleotides encoding a partial sequence of vesicular stomatitis virus glycoprotein (VSV-G) (corresponding to T500 - K511) [95,96] were annealed, digested by *NheI*, and inserted into the *NheI* site of the plasmid to generate pKTcuc-1 VSV encoding CUC-1 with VSV-G epitope tag. The direction and in-frame insertion were confirmed by sequencing. The 1.2 kbp fragment containing the yeast *ATX1* sequence was amplified from yeast genomic DNA by PCR using a pair of primers ScATXGNM5 and ScATXGNM3 [53] and inserted into a yeast centromeric shuttle vector pRS316 [97].
3.2.5 Expression of CUC-1 in yeast $\Delta atx1$

The *S. cerevisiae* $\Delta atx1$ strain was constructed as follows. A PCR fragment of $\Delta atx1::LEU2$ was amplified from pJJ283 [98] by using primers D-ScATX15 and D-ScATX13. The fragment was introduced into YPH499 to delete ATXI gene. Disruption of chromosomal $ATXI$ gene was confirmed by diagnostic PCR analysis (Fig. 16). The $\Delta atx1$ cells were transformed with the plasmid by using standard method [99]. Yeast cells harboring the plasmid were selected by SD medium with appropriate supplements. Iron-dependent growth was examined as described in [57,61], on SD-agarose plates containing 25 mM Na-MES (2-(N-morpholino)ethanesulfonic acid) pH 6.1. Where indicated, 1 mM ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine; Sigma), or 350 $\mu$M ferric ammonium sulfate (Sigma) was added to the medium.

3.2.6 Subcellular fractionation of CUC-1 in $\Delta atx1$ cells

Subcellular fractionation was carried out as described in [100] with minor modifications. Complete™ EDTA(-) proteinase inhibitor cocktail (Boeringer Mannheim) was added to all buffers. Exponentially growing cells ($2 \times 10^8$ cells) were converted to spheroplast by Zymolyase 100T (Seikagaku kougyou) and then osmotically lysed. The cell suspension was centrifuged for 5 min at $500 \times g$ to yield total cell lysate (lysate). The lysate was subjected to centrifugation at $10,000 \times g$ for 15 min to yield pellet ($P_{10}$) and supernatant ($S_{10}$) fractions. The $S_{10}$ fraction was further subjected to centrifugation at $100,000 \times g$ for 60 min to yield pellet ($P_{100}$) and supernatant ($S_{100}$) fractions. Proteins in
these fractions from $1.5 \times 10^7$ cells were resolved by SDS-PAGE. The CUC-1 tagged with VSV-G epitope was detected by Western blotting using a mouse anti VSV-G monoclonal antibody (clone P5D4, Boeringer Mannheim). Organelle marker proteins ADH (cytosol), ALP (vacuolar membrane), and Kex2p (late Golgi) were also detected by specific antibodies.

### 3.2.7 Construction of reporter fusion gene of *cua-1*

Upstream regions of *cua-1* gene were amplified by PCR. Genomic sequence of *cua-1* gene found in a YAC clone of Y76A2 is available from Sanger Centre, U.K. [69]. *C. elegans* genomic DNA (500 ng) and the combination of primers cuaLA10 and cuax2.0 were used for PCR by LA-PCR Kit ver.2 (TaKaRa) to amplify the upstream regions of *cua-1* gene. The PCR products containing about 2.5 kbp upstream sequence of *cua-1* gene and the first two exons were used for subsequent construction of reporter genes.

The amplified genomic DNA fragments were digested by *BamHI* and *SalI*, and ligated with the corresponding restriction sites of a promoterless NLS-GFP vector pPD95.67 (A. Fire *et al.*, personal communication). Both ends of inserted PCR fragments were sequenced to confirm in-frame fusion between the second exon of *cua-1* gene and the reporter genes.

### 3.2.8 Construction of reporter fusion gene *cuc-1*

A 5' flanking region of *cuc-1* gene was amplified from cosmid ZK652 by LA-PCR. A combination of primers cucUP and cucE3 was used. The amplified genomic DNA fragments were digested by *SphI* and *BamHI* and inserted into the corresponding
restriction sites of pPD95.70 to generate a *cuc-1::GFP* reporter fusion construct. The correct insertion was confirmed by sequencing.

### 3.2.9 Transformation of *C. elegans* by microinjection

Transformation of *C. elegans* was performed by microinjection of the plasmids into the distal arm of hermaphrodite gonad [101]. The *cuc-1::GFP* reporter constructs were injected at a final concentration of 100 µg/ml. Transmitting lines were established from cloned F1 scored by observing GFP expressions. In each experiment, I isolated at least three transmitting lines derived from independent injection and examined the expression. Expression patterns of fusion genes were indistinguishable among these lines.

### 3 RESULTS

#### 3.3.1 Cloning of cDNA for an ATX1 homologue from *C. elegans*

An ORF exhibiting strong homology to a yeast copper chaperone (Atx1p) was predicted in *C. elegans* genome by genome sequencing project [92]. The ORF was covered by cosmid ZK652 from chromosome III and named *cuc-1* gene in this study. I cloned a cDNA corresponding to the ORF by 5' and 3'-RACE based on the genomic sequence [92]. The isolated cDNA contained a trans-spliced leader sequence (SL1) (Fig. 13A). Spliced leaders are attached to 5'-end of *C. elegans* transcripts and SL1 is attached only to the most upstream genes of the operons [64,102], suggesting that *cuc-1* gene is located immediate downstream of the promoter. Consistent with this notion, *cuc-1* mRNA was amplified by reverse transcription PCR with SL1 primer but not with SL2 primer (Fig. 13B). A single transcript of about 0.5 kb was detected in *C. elegans* by
**Fig. 13.** cDNA structure (A) and SL-PCR (B) of *cuc-1*.

**A)** Structure of the *cuc-1* cDNA and its coding protein. The partial spliced leader sequence (SL1) and polyadenylation signal are underlined: +1, spliced leader acceptor site. Primers used for cloning and SL-PCR are indicated above the sequence.

**B)** RT-PCR using primer corresponding to spliced leader sequence (SL-PCR) was carried out. A single band having the size consistent with that of the cDNA was obtained by using SL1 primer but not by SL2 primer.
**Fig. 14** Detection of *cuc-1* transcript

Northern blot analysis of total RNA from mixed-stage *C. elegans* population using *cuc-1* cDNA as a probe (upper). The worms were cultured in NGM (no addition) or in NGM containing 1 μM CdCl₂ [Cd], 5 μM (CH₃COO)₂Cu [Cu], 1 μM (CH₃COO)₂Ni [Ni], 5 μM (CH₃COO)₂Zn [Zn], 500 μM ferrozine [-Fe], 500 μM bathocuproine sulfate [-Cu]. The same blot was also probed with ubiquitously expressed rp21 (*rp21*) and cadmium inducible metallothionein (*mtl-1*) cDNA. Open and filled arrow heads represent position of ribosomal RNA and each transcripts, respectively.
CUC-1  M---TQYVFEMGMCNAGARKVGLGKEDKIKIID
Atx1p  MAEIYQFNVMTCSGCSGAVNKVTLEPDVSKID-
HAH1  M---PKH-ESVDMTCGCASEAVSRVLNLG-GV-KYD-

CUC-1  INVEKKITVTTLPSADVLLEALKTGKEI----KQLQ
Atx1p  ISLEKSQDVYTLTLPYDFILEKIKKGKEVRSGKQL-
HAH1  IDLPNKKKCIESHSMDTLLATLKKTGKTV-SYLGLE

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<td>HAH1</td>
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Fig. 15  Alignment of Atx1p homologues

Deduced amino acid sequence of CUC-1 was aligned with those of yeast Atx1p and human HAH1. Conserved residues among the three proteins are boxed. Residues identical to CUC-1 are in bold letters. The characteristic copper binding motif, and the lysine rich sequence, KKTGK, are indicated by an asterisk. The sequence identities are shown below.
Northern blotting, the size of the transcript being consistent with that of the cDNA (Fig. 14).

The cDNA contained an open reading frame of 207 bp coding for a protein of 69 amino acid residues having a predicted molecular weight of 7,587 (Fig. 13A). The deduced amino acid sequence showed 49.3 and 39.1 % identities to yeast Atx1p [53] and human HAH1 [57], respectively (Fig. 15). The characteristic copper binding (Met-Thr-Cys-X-X-Cys) and lysine-rich (Lys-Lys-Thr-Gly-Lys) motifs are conserved among these sequences (Fig. 15).

3.3.2 Roles of CUC-1 in yeast Δatxl mutant

The cuc-1 cDNA was introduced into the yeast Δatxl mutant which could not grow on an iron-depleted medium [52]. As shown in Fig. 17, expression of cuc-1 could complement the null mutation, suggesting that the CUC-1 has the similar function to Atx1p. To enable immunochemical detection of CUC-1, a VSV-G epitope tag was introduced in-frame after initiation codon of cuc-1. The introduction of the epitope tag did not disrupt the function of CUC-1, since the tagged construct could complement Δatxl mutant (Fig. 17, -Fe). We detected a single distinct band with an apparent molecular mass of 7.0 kDa in the cell lysate carrying cuc-1 cDNA with the epitope (Fig. 18, lysate) but absent in the cell lysate carrying vector alone. The CUC-1 with the epitope migrated on gel electrophoresis slightly faster than a position expected from the sequence (8,908, mol. wt. with tag). The Atx1p behaved similarly on the electrophoresis [52]. The CUC-1 was co-fractionated with alcohol dehydrogenase (ADH) in the supernatant after centrifugation at 100,000 x g, indicating that the CUC-1 is localized mainly in the cytosol similar to Atx1p (Fig. 18) [52].

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Fig. 16  Disruption of the yeast ATX1 gene

A) Schematic diagram of yeast ATX1 gene disruption. Yeast ATX1 ORF was replaced by LEU2 gene. Primers used for diagnostic PCR (a, b) and PCR mediated gene replacement (c, d) are indicated by arrows.

B) Chromosomal gene disruption was confirmed by diagnostic PCR. A combination of primers a and b amplified 1.2 kbp and 3.1 kbp fragment containing an entire region of ATX1 locus from wild-type (W.T) and Δatx1 genomic DNA, respectively.
Fig. 17  Complementation of iron dependent growth of Δatx1 mutant by expression of CUC-1.

The Δatx1 cells harboring vector with no insert (vector), the CUC-1 expression plasmid (CUC-1), CUC-1-VSV tag expression plasmid (CUC-1-VSV), or the ATX1 on a single copy plasmid (ATX1) were grown for 5 days at 30 °C on minimal medium containing 1 mM ferrozine (-Fe) or 350 μM ferric ammonium sulfate (+Fe).
Fig. 18  Subcellular localization of CUC-1.

Cells (Δatx1) harboring vector with no insert and CUC-1-VSV tag expression plasmid were converted to spheroplasts and osmotically lysed. The total lysate of the spheroplasts (lysate) was centrifuged at 10,000 x g to obtain low speed pellet (P₁₀). Then, the supernatant was centrifuged at 100,000 x g to obtain pellet (P₁₀₀) and supernatant (S₁₀₀) fractions. Fractionation procedure is schematically shown (right). Each fraction from 1.5 x 10⁷ cells was loaded on a polyacrylamide gel in the presence of sodium dodecylsulfate. After electrophoresis, the proteins were electrotransferred to nitrocellulose and probed for VSV-tagged CUC-1 using the mouse monoclonal antibody P5D4 (1 µg/ml, Boehringer Mannheim) (left). Organelle marker proteins alcohol dehydrogenase (ADH, cytosol), alkaline phosphatase (ALP, vacuolar membrane), and Kex2p (late Golgi) were also detected by the specific antibodies. Blots were developed using the ECL detection kit (Amersham).
3.3.3 Expression patterns of cua-1 and cuc-1 genes

The yeast Atxl1p and Ccc2p constitute a cytosolic copper trafficking pathway to post-Golgi compartment [52,90]. To determine the expression patterns of CUC-1 and CUA-1 in C. elegans, fusion genes were constructed by inserting genomic fragments containing cuc-1 or cua-1 5' regulatory region into GFP expression vector (Fig. 19A, 20A).

In adult transgenic worms, cuc-1::GFP fusion genes were strongly expressed in intestine (Fig. 19B). On the other hand, in the larval stages, high level expressions were observed in the hypodermal cells of the head and body region (Fig. 19D). Dauer larva is a non-aging diapausal stage specialized for inadequate conditions to successful reproduction. In this stage, the cuc-1 fusion gene was almost repressed (data not shown).

The cuc-1::GFP showed essentially the same expression patterns as cuc-1::GFP in adult and larval stage (Fig. 20). In addition, signals were also prominent in the pharyngeal muscle cells in terminal bulb (pm6) (Fig. 20B; arrow, 20D; arrow head and Fig. 21) during all stages of development. In dauer larvae, the expressions of cua-1 were detectable only in the hypodermal cells (Fig. 20F).
Fig. 19  Expression of *cuc-1* in intestinal and hypodermal cells.

A) Structure of *cuc-1* gene and a fusion gene *cuc-1::GFP*. Filled and open boxes represent coding and untranslated regions, respectively. Hatched boxes represent exons corresponding to clone ykl40h8 identified in the Expressed Sequence Tag Database. Direction of transcription is indicated by arrows. A 1.7 kbp DNA fragment containing the upstream region and the *cuc-1* open reading frame was fused with a GFP reporter gene. Three transmitting lines carrying the *cuc-1::GFP* construct were established and had indistinguishable expression patterns.

B) Fluorescence micrograph of a young adult worm showing intestinal expression.

C) Nomarski micrograph of the same worm in B.

D) and E) Lateral view of an anterior half of L1 larvae. Nuclei in the head corresponding to the hyp-5, hyp-6, and hyp-7 cells are indicated by the arrows. Hypodermal cell nuclei of the body region are indicated by the arrow heads. Expressions were repressed in dauer larvae (not shown). Scale bars indicate 100 μm.
**Fig. 20** Expression of *cua-1* in intestinal and hypodermal cells.

A) Structure of *cua-1* gene and a fusion gene *cua-1::GFP*. Filled and open boxes represent coding and untranslated regions of *cua-1*, respectively. The hatched box indicates the last exon of the upstream gene identified in the genomic sequence. Direction of *cua-1* transcription is indicated by an arrow. A 3.0 kbp DNA fragment containing the upstream region and the first two exons of *cua-1* gene was fused with the reporter gene as indicated below. Three transmitting lines carrying the *cua-1::GFP* construct were established and had indistinguishable expression patterns.

B) and C) Fluorescence micrograph of an adult worm showing intestinal and pharyngeal expression. A pharyngeal muscle cell (pm6) is indicated by the arrow. Nomarski micrograph of the same worm is shown in C.

D and E) Lateral view of the anterior half of the L1 larvae. Nuclei of hypodermal cells in the head and body regions are indicated by the arrows. Nuclei of the pharynx and anterior intestinal cells are indicated by the arrow heads.

F and G) Ventral view of the dauer larvae. The expression of *cua-1::GFP* fusion gene is restricted to hypodermal cells in each lateral surface. Scale bars indicate 100 μm.
Fig. 21  Expression of *cua-1* gene in pharyngeal muscle cell, pm6

A) Fluorescent micrograph of adult transgenic worm. A medial plane of focus is shown.

B) Same field as in A), lateral plane of focus is shown.

C) Nomarski micrograph of the same worm in A) and B), medial plane of focus is shown.

D) Schematic diagram of the worm in the field. Pharyngeal muscle pm6 is located in anterior half of pharyngeal terminal bulb and consisted of three mononucleated cells: one in dorsal (pm6D), and two in subventral (pm6VL and pm6VR).

Three pm6 cells are indicated by arrow.
3.4 DISCUSSION

I have identified CUC-1, a copper chaperone protein expressed in C. elegans. CUC-1 showed remarkable sequence similarities with yeast Atx1p [53] and human HAH1 [57], and could complement yeast Δatx1 mutation. These findings strongly suggest that the CUC-1 has similar function as Atx1p and that the copper trafficking pathway similar to yeast is present in higher eukaryotes. In yeast, the ATX1 gene expression is regulated by the iron concentration [52,103]. However, the amount of the cuc-1 transcript did not change with the iron or copper in the growth medium. Similarly, the amount of HAH1 transcript in human cell lines is not altered by the copper ion concentration [57].

The expressions of cuc-1 in C. elegans were clearly tissue and developmental-stage specific: expressed in hypodermal cells of L1 larval stage, and intestinal cells of adult worm. Transgenic studies suggested that cua-1 gene is expressed similar to cuc-1 gene, consistent with a model that CUC-1 transfers copper to CUA-1 ATPase. The intestinal expression of both genes may be essential for copper ion intake into the worm. The both expressions were repressed in intestine when L1 worms became dauer larvae. This result may be reasonable because dauer worms are dormant and do not take food [104]. The hypodermal cells in larval stage may transport copper for copper containing enzymes required to cross-link collagens in extracellular space. Cell specific expression of cuc-1 is different from the human homologue HAH1 which is expressed in all tissues and cell lines tested [57].

It should be noted that expression patterns of the two genes were slightly but significantly different. The cua-1::GFP was expressed in pharyngeal muscle, whereas no cuc-1::GFP signals were detectable. Thus CUA-1 copper ATPase may function in the muscle differently from intestinal or hypodermal cells. The regulations of the expression
of the two genes are of interest to study: they may be regulated similarly in intestinal and hypodermal cells but differently in pharyngeal muscle cells. In this regard, it is noteworthy that no significant sequence homology was found between the 5' flanking sequences of *cuc-1* and *cua-1*. 
CHAPTER 4 CONCLUSION

Old but common question on copper metabolism is how organisms overcome its toxicity. Regardless of species, they may deal with excess copper in combination of the three ways: (a) copper binding to specific detoxification proteins such as metallothioneins; (b) shutting off its intracellular uptake; (c) secreting (or transporting) it to the outside of the cell or transport it into cellular compartment. This study has been focused on the constituent of cellular copper export machinery and I have identified two C. elegans cDNAs cua-1 and cuc-1 which have remarkable sequence similarities to Cu-ATPase and copper chaperone protein, respectively, found in human [25-30,57] and yeast [53,55].

Both CUA-1 and CUC-1 shared all the sequence motifs typical for Cu-ATPase (e.g. copper-binding, phosphorylation, and ATP-binding motifs etc.) [34-36] and copper chaperone protein (copper-binding and lysine-rich motifs) [53,57], respectively. Presence of these homologues in C. elegans supports the concept that the pathway for copper trafficking is conserved during evolution. Most importantly, the expression of these two cDNAs restored the growth defect of the corresponding yeast null mutant (Δccc2 and Δatxl) [59,60,91], indicating that these two cDNAs encode a functional copper transporter and a chaperone protein. Furthermore, two CUA-1 mutant cDNA (Cys-744 to Ala and Asp-786 to Asn) failed to rescue the defect, suggesting the function of CUA-1 as a copper ion specific P-type ATPase.

The expression of cuc-1 in C. elegans was examined by Northern blot. Many genes involved in iron and copper ion homeostasis including ATX1, are under the control of metal ion sensitive transcriptional regulator in yeast [43,103,105]. However, we could not detect obvious changes in the amount of the cuc-1 transcript in the presence of altered concentrations of heavy metals. Similarly, amount of HAHL transcript in human cell lines
is not altered by the copper ion concentrations [57], indicating that the transcription of the copper chaperone genes in multicellular organisms are not regulated by metal ion concentrations.

The expression pattern of cuc-1 showed striking specificity in tissue and worm developmental stages: its expression in hypodermal cells in larval stages, and intestinal cells of adult worm. On the other hand, HAHI is expressed ubiquitously [57]. Although no studies were carried out for the copper chaperone expression during development, it may be regulated in a tissue and developmental stage specific manner.

Transgenic studies suggested that the cua-1 is expressed similar to cuc-1, consistent with the model that CUC-1 transfers copper to CUA-1 for transport copper into post Golgi compartment [52,54,90]. It became of interest to know the physiological role(s) of these gene products in multicellular organism. The intestinal cells are responsible for dietary intake of many nutrients including copper ion into the animal body. It might be reasonable that the expressions of cuc-1 and cua-1 repressed in intestine of the dauer larvae, because in this stage, the worms do not take any food [104]. The hypodermal cells in larval stage may secrete copper-containing lysyl oxidase required for cross-linking of collagens which form worm's exoskeletal cuticles. Occipital horn syndrome (OHS) is the most mild allele of the Menkes disease [106]. The mutations in the OHS were identified to have effects for precise splicing. Although a small amount of normally spliced message is observed in the OHS patients, connective tissues of the patients are severely damaged, whereas nervous systems remain normal [106]. This suggests that the efficient copper transport into the post Golgi compartment by the Menkes disease gene product is required for lysyl oxidase. Further molecular analysis is required to support the hypothesis that CUC-1 and CUA-1 are required for the cellular function(s) of intestinal and hypodermal cells through copper ion transport.
I have attempted in this study to elucidate the mechanism for copper trafficking in multicellular organism to fill the gaps of knowledge between the yeast copper transport and human disease, using the simple model animal. Almost all the results support the idea that the copper transport system is conserved evolutionally. One exception which I want to point out here is the different expression of cua-1 and cuc-1 in pharyngeal muscle and in hypodermal cells of dauer larvae. Although no cuc-1 expression was observed in these cells, cua-1 was strongly expressed, suggesting the direct copper transport by Cu-ATPase without the function of copper chaperone protein. The yeast Δatx1 mutant cells still uptake iron to some extent, indicating the copper transport by Ccc2p independent of Atx1p [52]. It should be necessary to isolate the mutants in which cua-1 or cuc-1 gene was deleted for further understanding of the copper trafficking in C. elegans.
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CHAPTER 2

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*J. Biochem.* 121, 1169-1175 (1997)

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Essential Cys-Pro-Cys motif of *Caenorhabditis elegans* copper transport ATPase.

CHAPTER 3

Tokumitsu Wakabayashi, Norihiro Nakamura, Yoshihiro Sambongi, Yoh Wada,
Toshihiko Oka, and Masamitsu Futai

Identification of the copper chaperone, CUC-1, in Caenorhabditis elegans: tissue specific co-expression with the copper transporting ATPase, CUA-1.

FEBS lett. 440, 141-146 (1998)