Copper Trafficking in *Caenorhabditis elegans*: Copper Chaperone and Copper Transporting ATPase

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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將光教授に深く感謝します。３年間にわたり、飽かず厳しく御指導を賜り、学問を志向するも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。

本研究を行うにあたり、直接の御指導、そして多くの貴重な御助言を賜りました、三本木至宏、中村徳弘、両博士に心から感謝します。お二人の絶え間ない協力と励ましなくしては、こんにちを迎えることは適いませんでした。

本研究に終始、御指導、御協力を頂きました、和田　洋博士をはじめとする二井研究室のスタッフの皆様に感謝します。

唯一の先輩であった、西　毅博士（現 Tufts University School of Medicene）、長江隆司氏、吉水孝緒氏、武田健志氏らをはじめとする二井研究室の学生、卒業生の皆様との出会いは、この３年間を豊かなものとして下さいました。ここに厚く御礼申し上げます。

最後に、私の大学院進学に理解を示し、全ての面でそれを支えてくれた両親、家族同様に励ましてくれた山口尚稔夫妻、そして、私の全てを見守り、共に喜び、悲しみ、大きな理解と、深い愛情で励まし続けてくれた、最愛の妻、朱里に言葉にならない、あらん限りのの感謝をここに表します。

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:

glycine	Gly	G	glutamine	Gln	Q
alanine	Ala	A	lysine	Lys	K
valine	Val	V	arginine	Arg	R
leucine	Leu	L	cysteine	Cys	C
isoleucine	Ile	I	methionine	Met	M
serine	Ser	S	phenylalanine	Phe	F
threonine	Thr	T	tyrosine	Tyr	Y
aspartic acid	Asp	D	tryptophan	Trp	W
asparagine	Asn	N	histidine	His	H
glutamic acid	Glu	E	proline	Pro	P

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.

Examples of cuproproteins

Protein	Function
Ceruloplasmin	Fe(II) ->Fe(III) oxidation
Cytochrome *c* oxidase	Electron transfer, terminal oxidase
Dopamine-β-hydroxylase	Dopamine -> norepinephrine
Lysyl oxidase	Collagen and elastin cross-linking
Metallothionein	Copper scavenging
Superoxide dismutase	Superoxide decomposition

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.

	Menkes disease	Wilson disease
Discovery	John H. Menkes (1962) [12]	S. A. Kinner-Wilson (1912) [21]
Clinical observation	Onset at birth Mental retardation Neurological degeneration Kinky hair Hypopigmentation Skeletal defect (cutis laxa)	Onset at late childhood Liver cirrhosis Behavior disturbance Dysarthria Pigmented corneal rings (Kayser-Fleischer ring)
Laboratory findings	Decreased serum, brain and liver Cu Increased intestinal and kidney Cu	Increased liver Cu Decreased serum Cu
Basic defect	Defect of intestinal Cu absorption Deficiency of Cu dependent enzyme activity	Defect of Cu excretion into bile
Locus	Xq 13.3 / recessive	13q 14.3 / recessive
Gene product	Putative Cu transporting P-type ATPase (ATP7A)	Putative Cu transporting P-type ATPase (ATP7B)
Expression	All tissues except liver	Liver, kidney and placenta

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^+, Ca^{2+}, Cd^{2+}, 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 (*Δctr1*) 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 (*Δccc2*) 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].

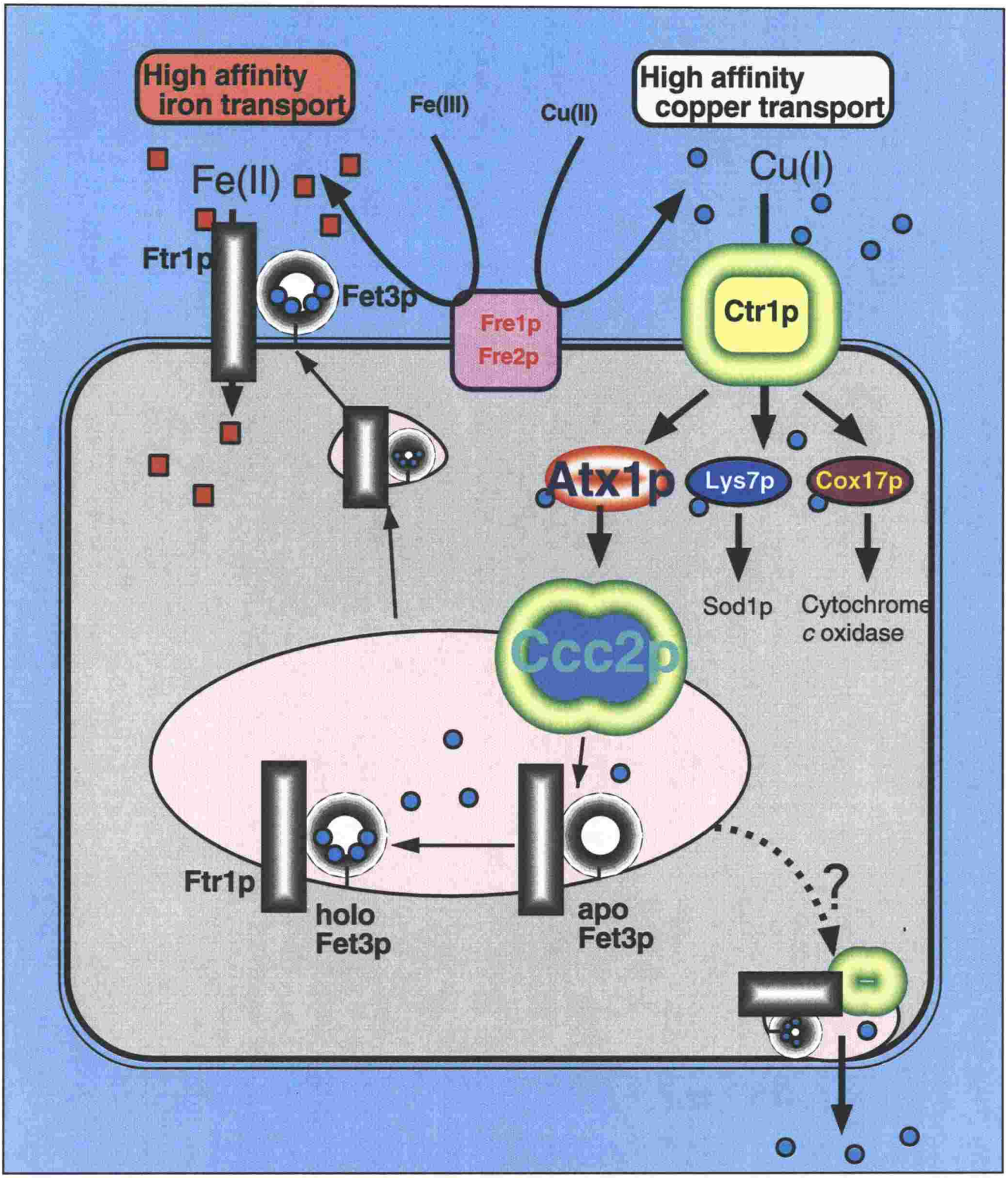

Fig. 1 Model of cellular copper trafficking in yeast

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 evolutionary.

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 *cua-1* and *cuc-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 (*Δccc2 and Δatx1*) 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 $CaCl_2$, 1 mM $MgSO_4$, 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.

C744Afw	5'-CCTGCAAGTCTGGGTCTCGCAA-3'
C744Arv	5'-GACTTGCAGGACATGCAATTGCAA-3'
D786Nfw	5'-TTTCAATAAAACTGCACAATCA-3'
D786Nrv	5'-TTTTATTGAAAACAATTGTGGTTAC-3'
D-ScCCC23	5'-TTGGCCAGTCCTCTCAAACT-3'
D-ScCCC25	5'-GGTTCAGGAACCATGGATAC-3'
HA fw	5'-ATC(TACCCATACGATGTTCCGGATTACGCT)$_3$GAT-3'
HA rv	5'-ATC(AGCGTAATCCGGAACATCGTATGGGTA)$_3$GAT-3'
Pmafw	5'-GTCGGAACACGTGAGTCTTC-3'
Pmarv	5'-GAAGACTCACGTGTTCCGAC-3'
RTrv	5'-CATTTCTCGCCGAATTGTACTC-3'
SL2	5'-GGTTTTAACCCAGTTACTCAAG-3'

universal M13 fw 5'-GTAAAACGACGGCCAGT-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 *Hin*dIII-*Hin*dIII 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 *Hin*dIII-*Hin*dIII 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 *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. 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 *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 was inserted in-frame into the *Pma*CI 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 (*MAT***a**, *ura3-52, leu2-Δ1, trp1-Δ63, his3-Δ200, ade2-101, lys2-801*) by PCR using two oligonucleotide primers D-ScCCC25 and D-ScCCC23. A *Bam*HI-*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 phosphoglucokinase 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 $Fe(NH_4)(SO_4)_2$.

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 (Amersharm). 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 deduced from the hydrophobicity 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).

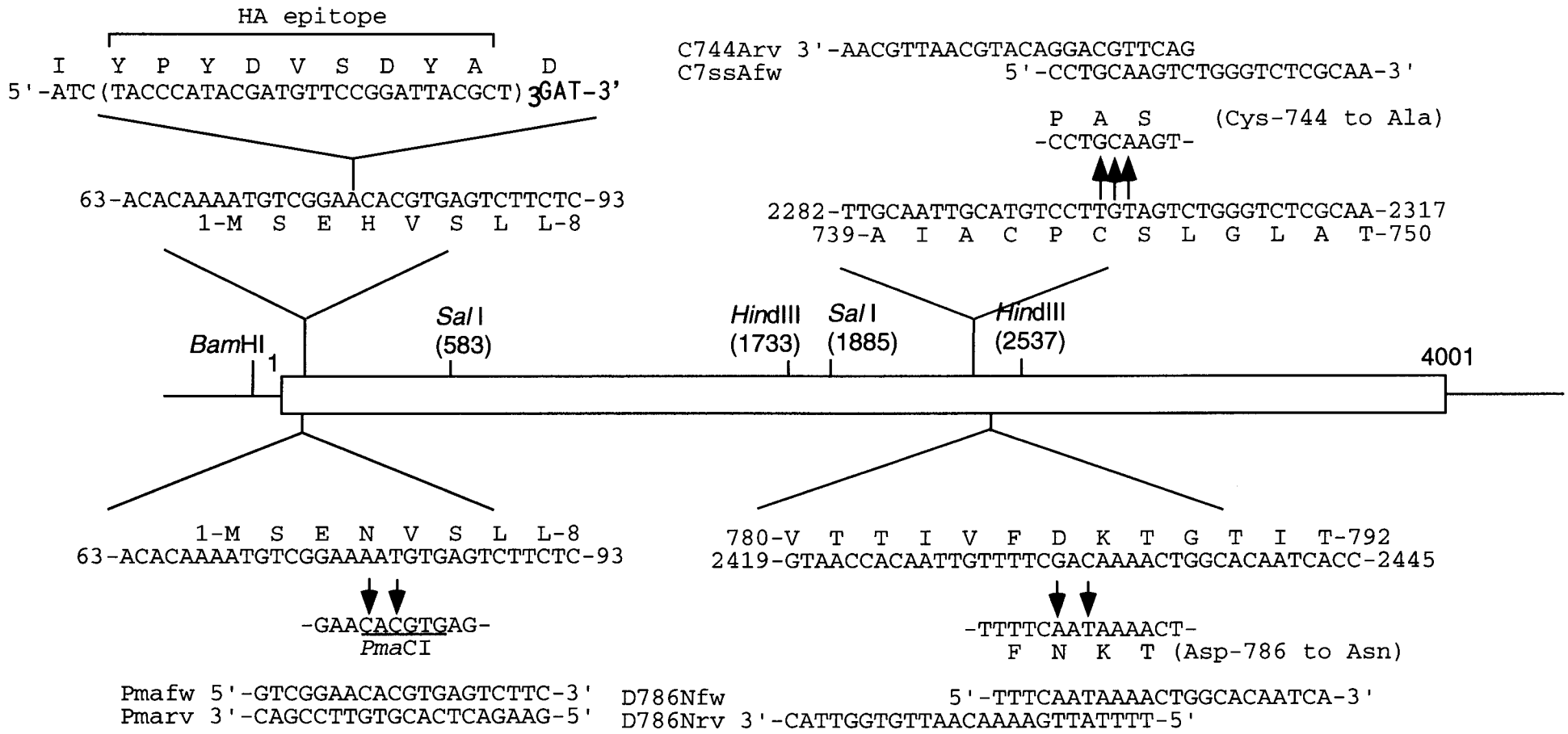

Fig. 3 Introduction of a hemaggulutinin(HA) epitope tag and Cys-744 to Ala, Asp-786 to Asn mutations.

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 *Pma*CI site. The restriction sites used for subcloning are also shown.

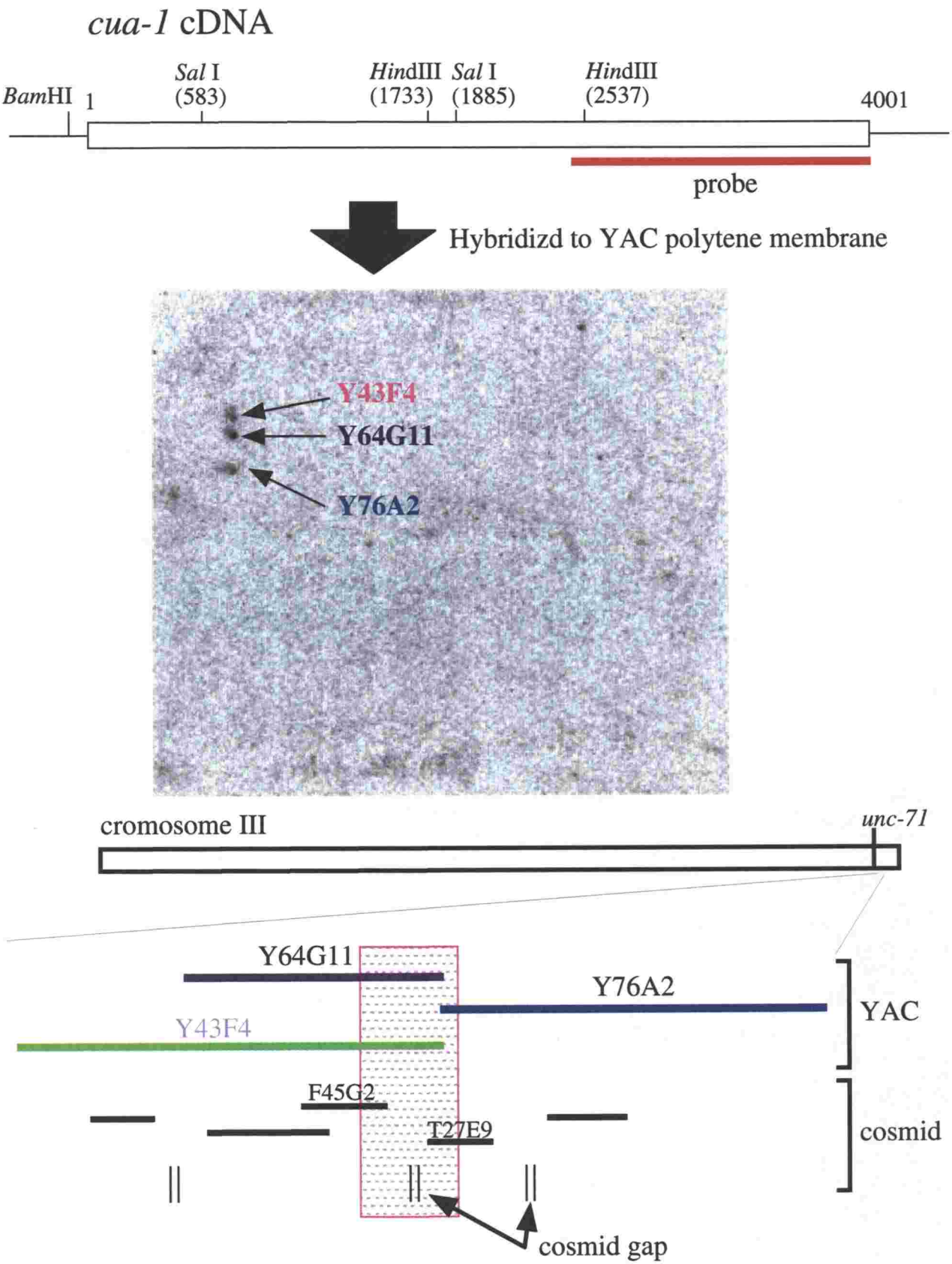

Fig. 4 Chromosomal location of *cua-1* gene

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, ACACAAAATGT) (Fig. 2) was within the consensus for the *C. elegans* translation start site ($ANN^{A}/_{C}{}^{A}/_{G}AAATGN$) [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

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                          ****  *
CUA-1 Cu1          IKGMTCNSCVKNIQDVIGAKPGIHSIQVNL
CUA-1 Cu2          IEGMTCHACVNNIQDTVGSKDGIVKIVVSL
CUA-1 Cu3          VEGMTCASCVQYIERNISKIEGVHSIVVAL
Human MNK Cu1      VEGMTCNSCVWTIEQQIGKVNGVHHIKVSL
Human MNK Cu2      VEGMTCHSCTSTIEGKIGKLQGVQRIKVSL
Human MNK Cu3      IDGMHCKSCVSNIESTLSALQYVSSIVVSL
Human MNK Cu4      IDGMTCNSCVQSIEGVISKKPGVKSIRVSL
Human MNK Cu5      VTGMTCASCVANIERNLRREEGIYSILVAL
Human MNK Cu6      VRGMTCASCVHKIESSLTKHRGILYCSVAL
Human WND Cu1      ILGMTCQSCVKSIEDRISNLKGIISMKVSL
Human WND Cu2      VEGMTCQSCVSSIEGKVRKLQGVVRVKVSL
Human WND Cu3      IDGMHCKSCVLNIEENIGQLLGVQSIQVSL
Human WND Cu4      IAGMTCASCVHSIEGMISQLEGVQQISVSL
Human WND Cu5      IKGMTCASCVSNIERNLQKEAGVLSVLVAL
Human WND Cu6      ITGMTCASCVHNIESKLTRTNGITYASVAL
Yeast Ccc2p Cu1VHGMTCSACTNTINTQLRALKGVTKCDISL
Yeast Ccc2p Cu2VQGMTCGSCVSTVTKQVEGIEGVESVVVSL
Yeast Pca1p        VSGMSCTGCESKLKKSFGALKCVHGLKTSL
E. hirae           ITGMTCANCSARIEKELNEQPGVMSATVNL
E. coli o732       VSGMDCAACARKVENAVRQLAGVNQVQVLF
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B

CPC, DKTG, SEHP motifs

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                        *                                                *
CUA-1          740 IACPCSLGLATPTAVMVGTGVGAANGILIKGGEPLESVHKVTTIVFDKTGT
Human MNK      999 IACPCSLGLATPTAVMVGTGVGAQNGILIKGGEPLEMAHKVKVVVFDKTGT
Human WND      951 IACPCSLGLATPTAVMVGTGVAAQNGILIKGGKPLEMAHKIKTVMFDKTGT
Yeast Ccc2p    581 VACPCALGLATPTAIMVGTGVGAQNGVLIKGGEVLEKFNSITTFVFDKTGT
Yeast Pca1p    857 VSCPCVIGLAVPIVFVIASGVAAKRGVIFKSAESIEVAHNTSHVVFDKTGT
E. hirae CopA379 IACPCALGLATPTAIMVGTGVGAHNGILIKGGEALEGAAHLNSIILDKTGT

Rabbit Ca      305 AAIPEGLPAVITTCLALGTRRMAKKNAIVRSLPSVETLGCTSVICSDKTGT
Yeast Pma1p    332 IGVPVGLPAVVTTTMAVGAAYLAKKQAIVQKLSAIESLAGVEILCSDKTGT


CUA-1              ITEG-RPRVVQIASFVNPSTMSLKLITFLSGATEALSEHPIGNA  833
Human Mc1          ITHG-TPVVNQVKVLTESNRISHHKILAIVGTAESNSEHPLGTA 1092
Human Wc1          IIHG-VPRVMRVLLLGDVATLPLRKVLAVVGTAEASSEHPLGVA 1044
Yeast Ccc2p        LTTGFMVVKKFLKDSNWVGNVDEDEVLACIKATESISDHPVSKA  675
Yeast Pca1p        LTEG-KLTVVH----ETVRGDRHNSQSLLLGLTEGI-KHPVSMA  945
E. hirae CopA      ITQG-RPEVTDVIGPKD--------IISLFYSLEHASEHPLGKA  464
```

C

TGES/A motif

CUA-1	GAKVPVDGVVVDGKSSVDESFITGESMPV
Human MNK	GGKFPVDGRVIEGHSMVDESLITGEAMPV
Human WND	GGKFPVDGKVLEGNTMADESLITGEAMPV
Yeast Ccc2p	GMKIPADGIITRGESEIDESLMTGESILV
Yeast Pca1p	DSRIPTDGTVISGSSEVDEALITGESMPV
E. hirae CopA	GEQVPTDGRIIAGTSALDESMLTGESVPV
E. coli o732	GGRLPADGKLLSPFASFDESALTGESIPV

D

GDGXND motif

CUA-1	VAMVGDGVNDSPALAEANVGIA
Human MNK	VAMVGDGINDSPALAMANVGIA
Human WND	VAMVGDGVNDSPALAQADMGVA
Yeast Ccc2p	VAVVGDGINDAPALALSDLGIA
Yeast Pca1p	VVFCGDGTNDAIGLTQATIGVH
E. hirae CopA	VGMVGDGINDAPALRLADVGIA
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. GMTCXXC, 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. Conserverd residues are boxed.

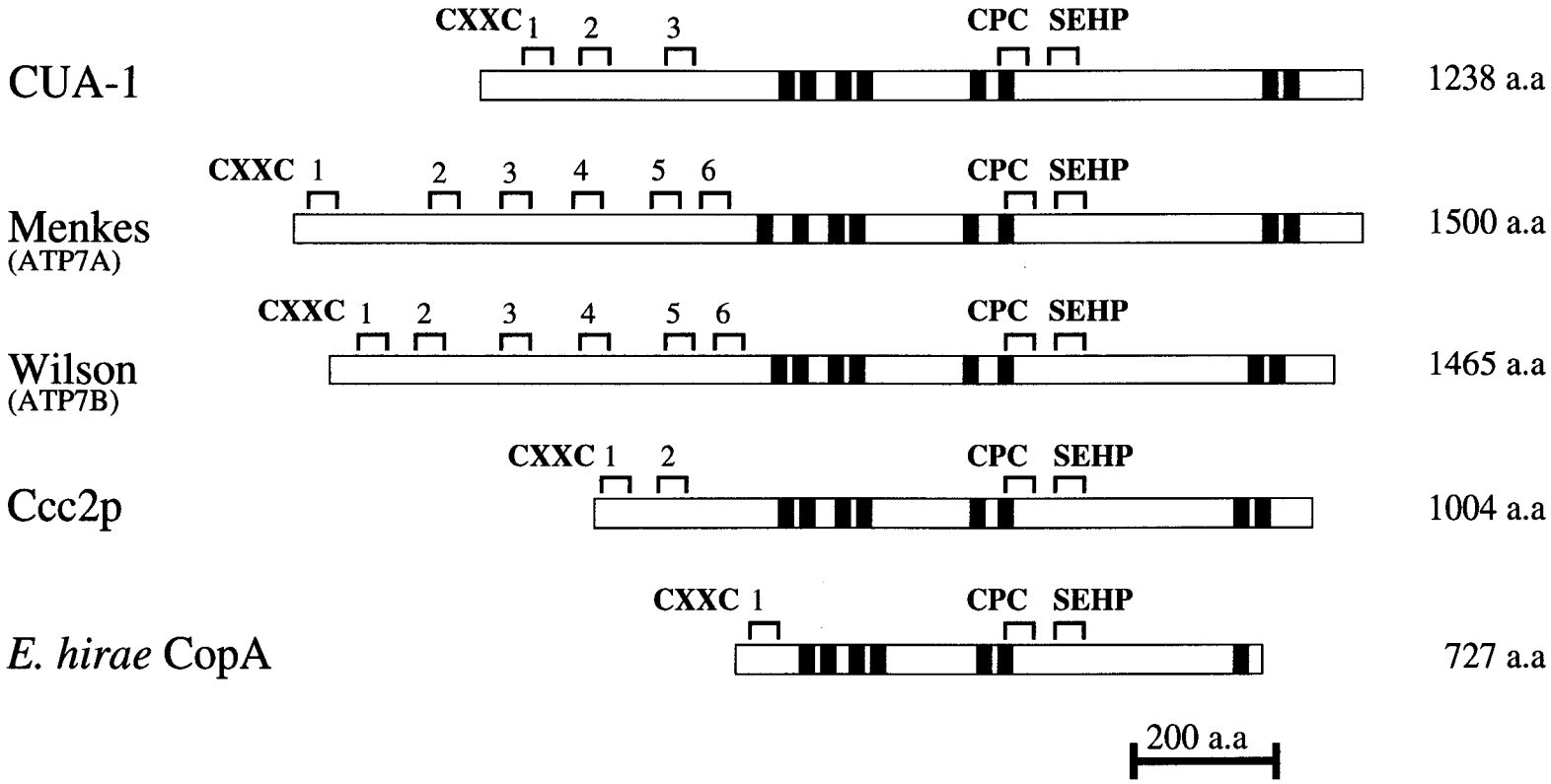


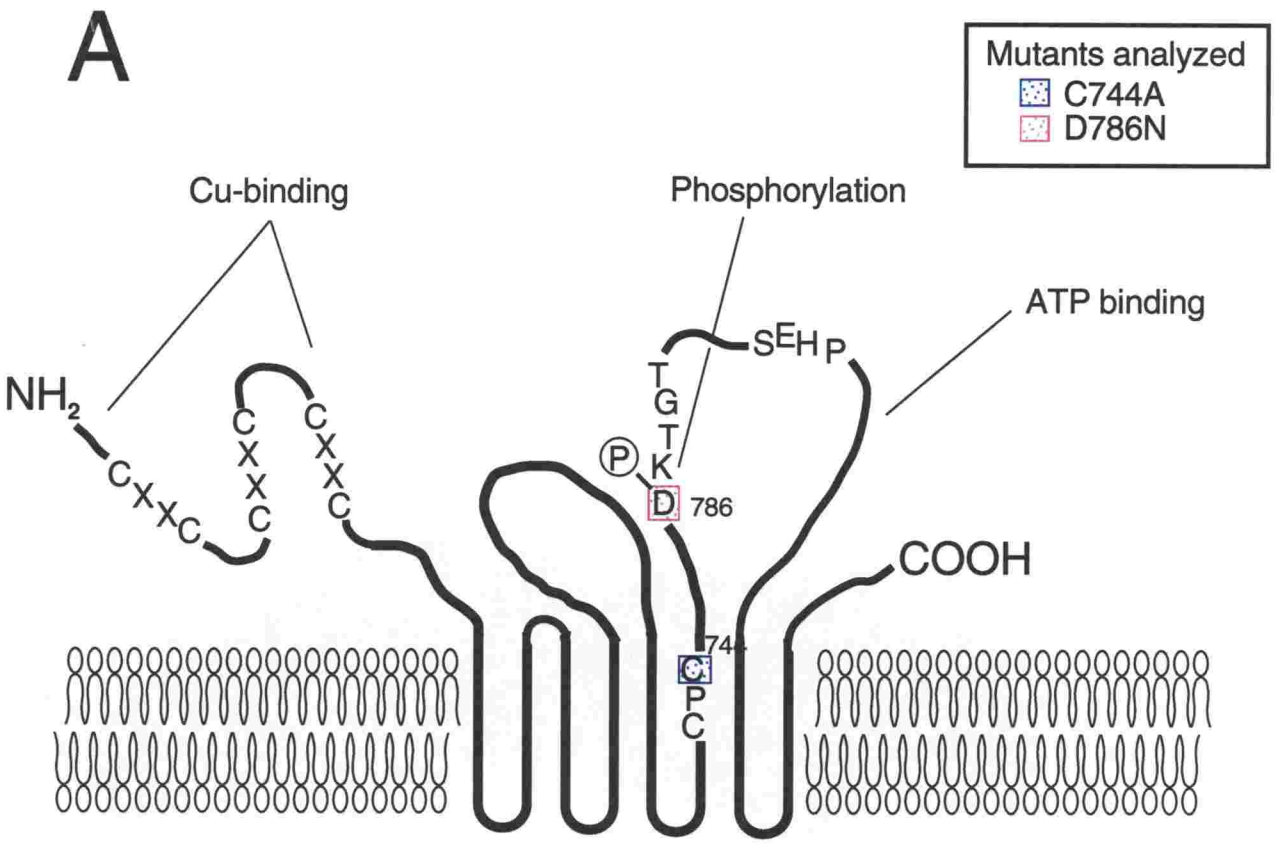
Fig. 6 Schematic alignment of heavy metal ATPase

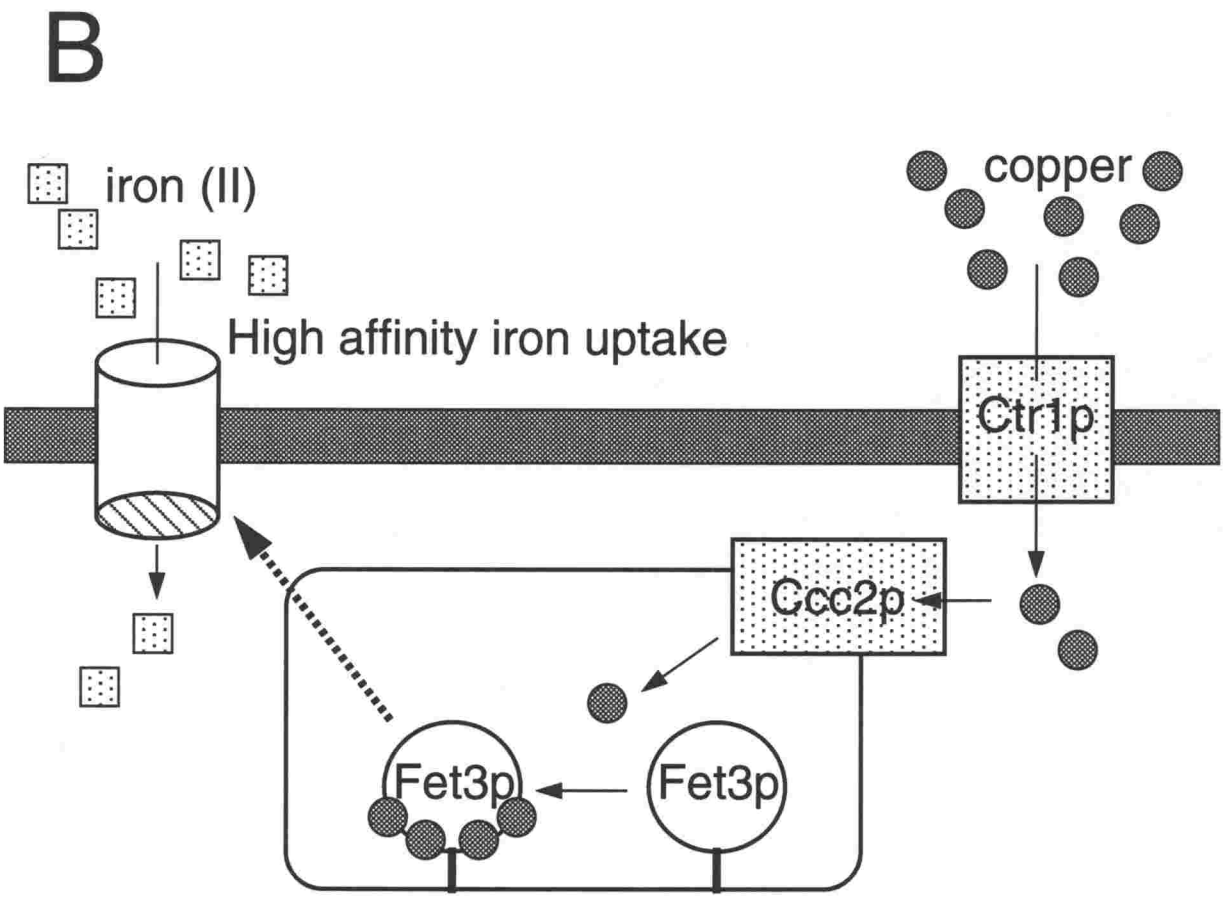
Open and closed boxes represent the cytoplasmic and transmembrane domains of these ATPases, respectivery. Amino acid sequence motifs typical for heavy metal ATPase are indicated above.

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


A
Mutants analyzed
C744A
D786N
Cu-binding
Phosphorylation
ATP binding
NH2
CXXC
CXXC
CXXC
SEHP
TGTK
P
D 786
COOH
744
PC


B
iron (II)
copper
High affinity iron uptake
Ctr1p
Ccc2p
Fet3p
Fet3p

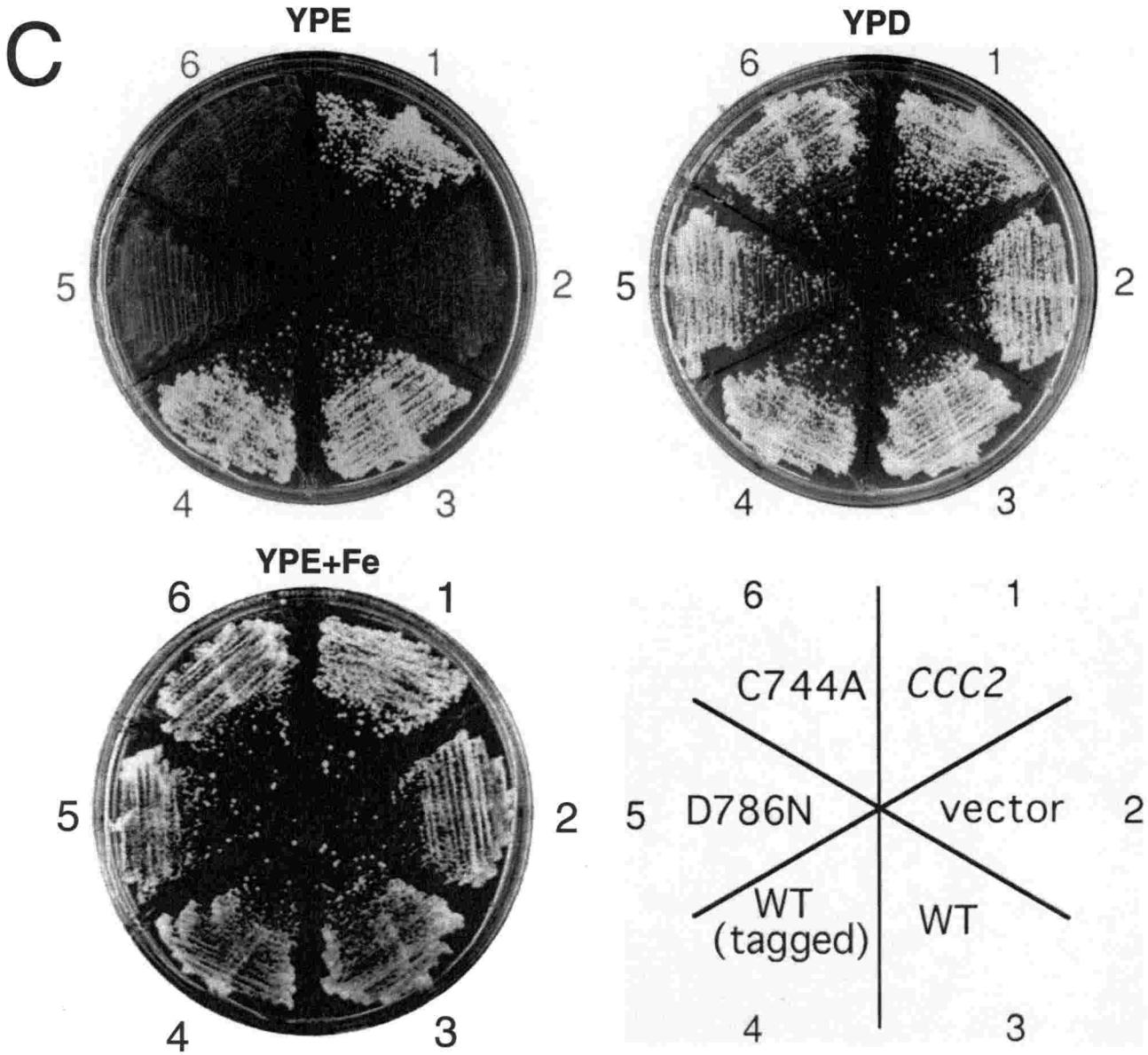


Fig. 7 Rescue of Δ*ccc2* mutant by CUA-1

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, Δ*ccc2* cell cannot grow by respiration.

C) Rescue of yeast Δ*ccc2* mutant by CUA-1. Yeast Δ*ccc2* mutant cannot grow on YPE medium without addition of excess iron to the medium (vector). The Δ*ccc2* mutant car rying wild type *cua-1* cDNA (WT, WT (tagged)) could grow on iron limited YPE medi um similar to the mutant horboring the yeast *CCC2* gene (*CCC2*). On the other hand, the Δ*ccc2* 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-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. 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-1* 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-1* 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).

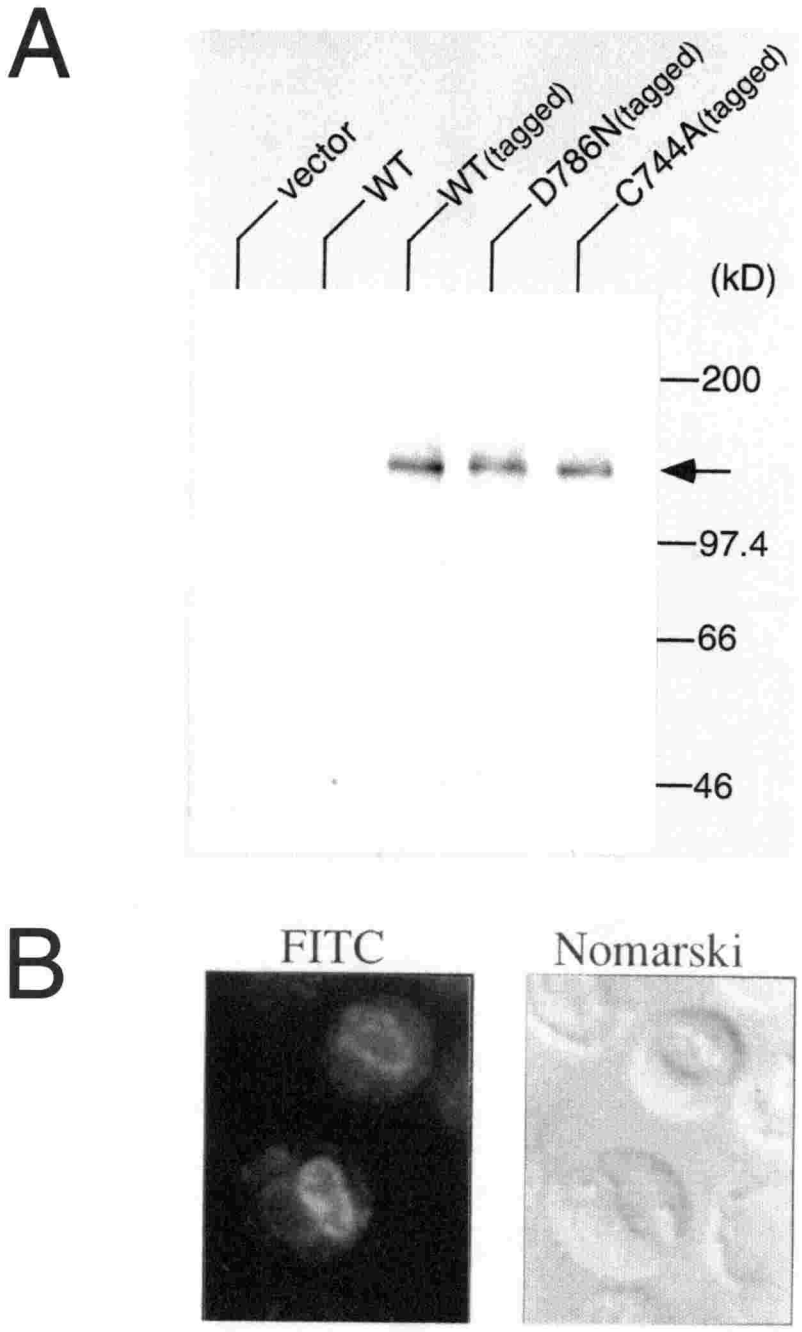


Fig. 8 Immunological detection of CUA-1 expressed in yeast

A) Presence of CUA-1 expressed in the Δ*ccc2* mutant. Cell lysates (20 μg of protein) of yeast Δ*ccc2* 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 Δ*ccc2* 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 Δ*ccc2* mutant. The Δ*ccc2* 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 *Δccc2* 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].

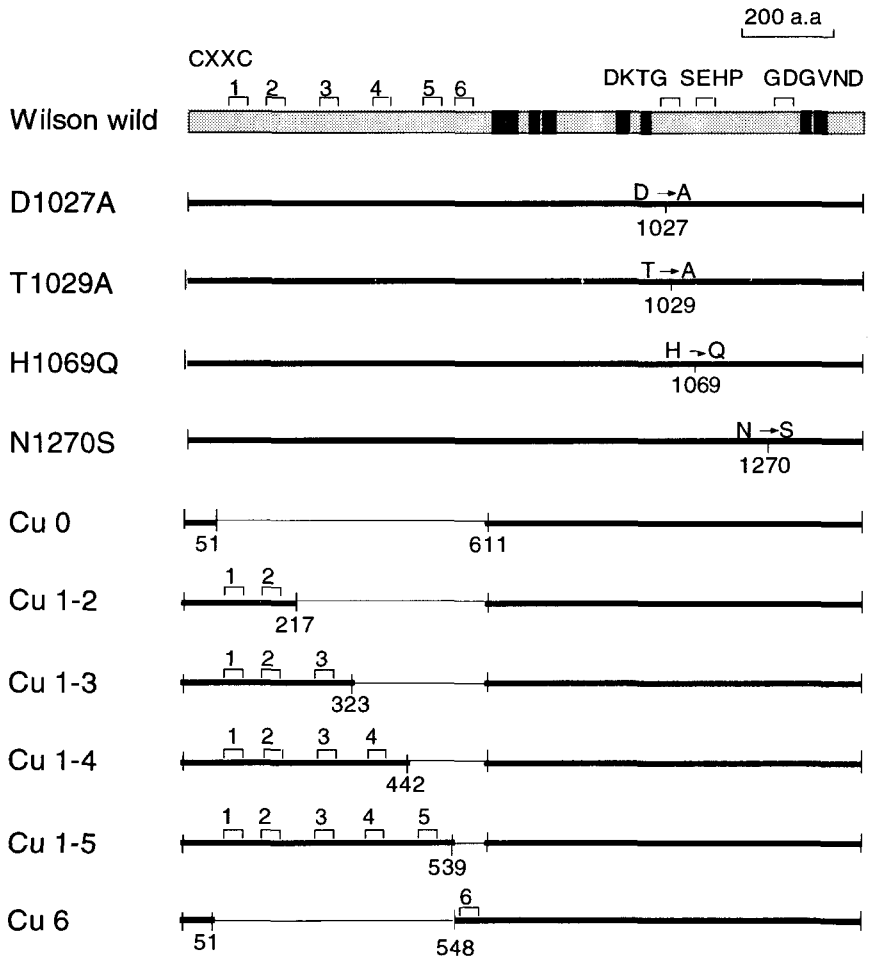


Fig. 9 Mutant cDNAs analyzed

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.

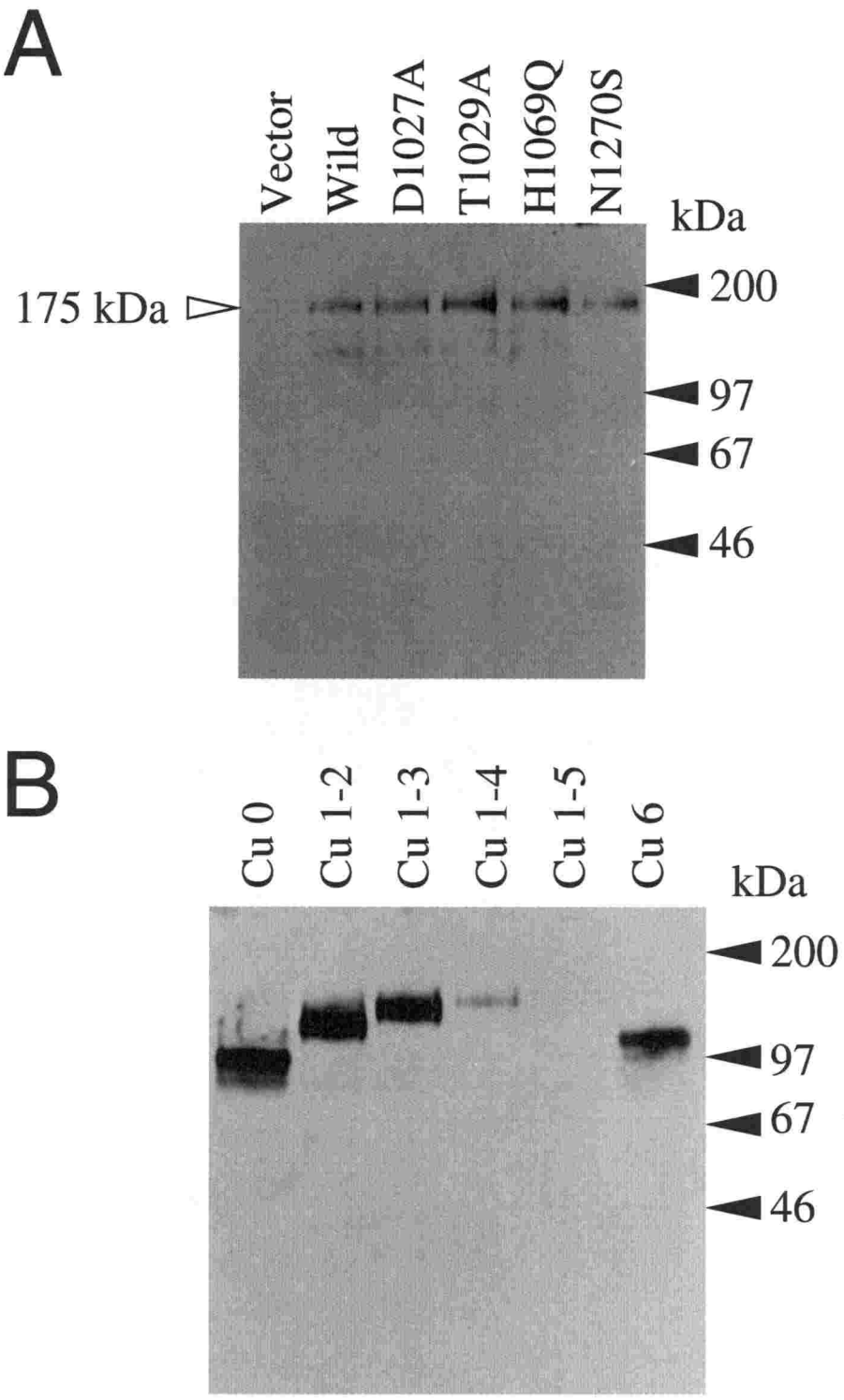


Fig. 10 Presence of Wilson disease gene product (WND) in yeast Δ*ccc2* mutant cells

Total lysate from 2×10^7 cells of each transformant was electrophoresed on polyacrylamide gel (7.5 %) in the presense 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.

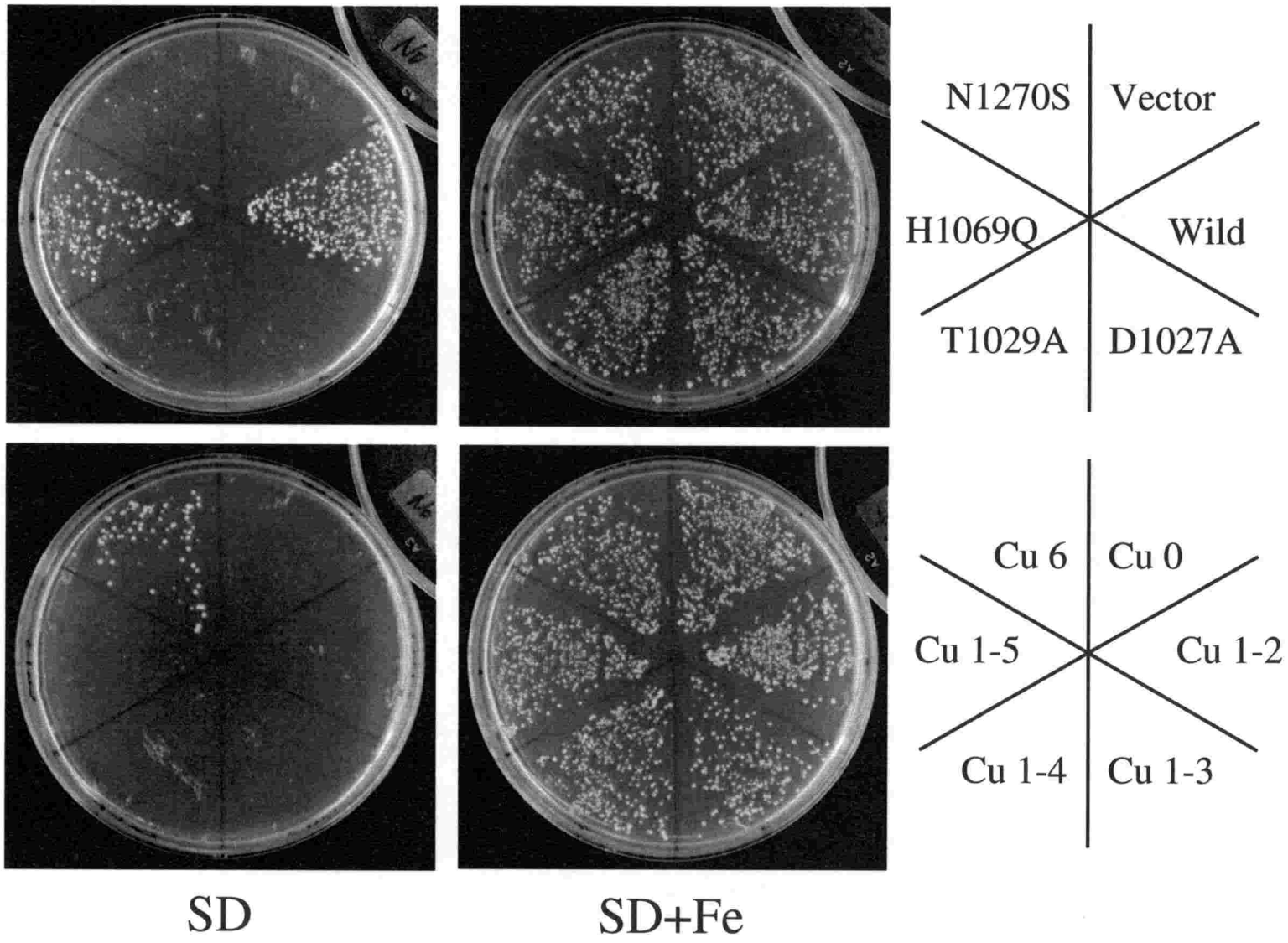


Fig. 11 Complementation of yeast Δ*ccc2* mutant by wild-type or mutant human Wilson disease gene products.

The yeast Δ*ccc2* 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 conseved 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 Ca^{2+}-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 $MgCl_2$, 5 mM DTT, 10 μM Bafilomycine A_1, 2 mM ATP, 10 μM $CuCl_2$) or an intermediate (assayed with the same buffer using 40 μM γ^{32}P-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* Cd^{2+}-ATPase (CadA) was estimated to be 0.13 μmoles/min/mg of Cd^{2+}-ATPase, assuming that 10 % of membrane proteins was Cd^{2+}-ATPase [82], whereas rabbit sarcoplasmic Ca^{2+}-ATPase exhibited Ca^{2+} 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 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 [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 *Δccc2* 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:

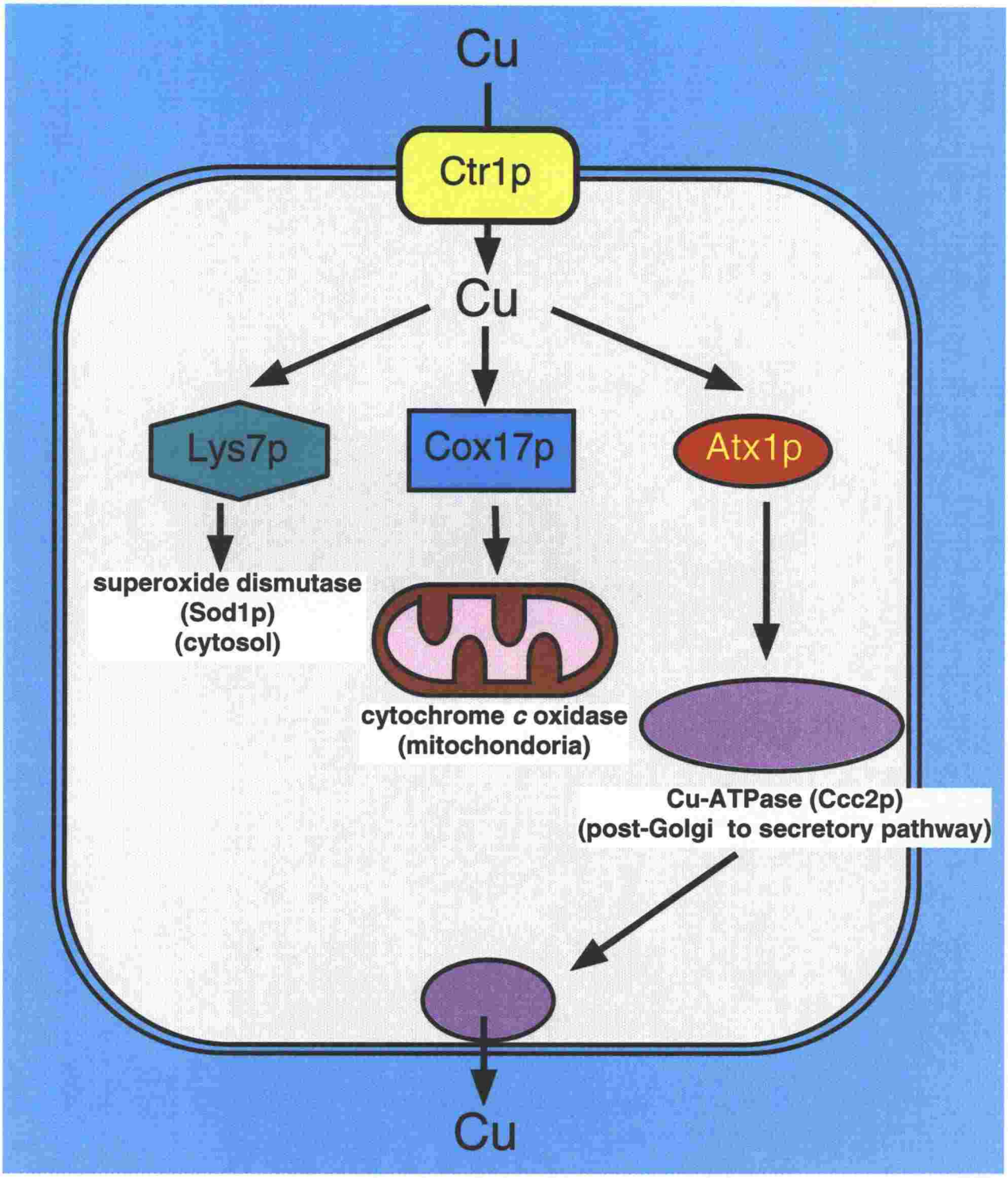


Fig. 12 Routes for intracellular delivery of copper in yeast

External copper is inported 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 mitochondoria 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'-CCGCTCGAGTTTTTTTTTTTAAGTTTAGAAAAAT-3'
CeATX-1 fw1	5'-TACGTTTTCGAAATCGGCATGACAT-3'
CeATX-1 fw2	5'-GCATGACATGCAATGGCTGT-3'
CeATX-1 Nhe	5'-CGGAATTCATGGCTAGCACACAGTACGTTTTCGAAA-TGGGCATG-3'
CeATX-1 rv1	5'-GTAGTTGCTTGATCTCTTTTCCTG-3'
CeATX-1 rv2	5'-CAAGTACATCAGATGCTGGT-3'
cuaLA10	5'-ACGCGTCGACTCCTCCAATTGTTGGTCCGGTTCCTGT-TTCT-3'
cuaex2.0	5'-GTAATTGGAGCGAAGCCAGGAATTCATAGGGATCCC-G-3'
cucE3	5'-CGGGATCCGTAGTTGCTTGATCTCTTTTCCTG-3'
cucUP	5'-CACATGCATGCTTGGGTTCACTGGTTGAACTGCGGA--3'
D-ScATX13	5'-AGTAGCTACAATACAAAATAGAGTAAAAAACTATTTC-

	TCTTCACTTTCATGGAAACAGCTATGACCATG-3'
D-ScATX15	5'-ACATTGCAGGATGAGATTTCCAACGACACAAGAGAG-
	AACTAGCGCAAAAGGTAAAACGACGGCCAGT-3'
ScATXGNM3	5'-AGACTCGAGCCATCACACTTTCATCATCGTT-3'
ScATXGNM5	5'-AGAGGATCCTTTAAAGGAGATTATGGAGATG-3'
VSV-G fw	5'-AGCTAGCTAGCTACACTGATATCGAAATGAACCGCCT-
	GGGTAAGGCTAGCTAGCT-3'
VSV-G rv	5'-AGCTAGCTAGCCTTACCCAGGCGGTTCATTTCGATAT-
	CAGTGTAGCTAGCTAGCT-3'

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 *Bam*HI 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 *Nhe*I 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 *Eco*RI and *Xho*I, 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 *Nhe*I, and inserted into the *Nhe*I 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 *Δatx1*

The *S. cerevisiae Δatx1* strain was constructed as follows. A PCR fragment of *Δatx1::LEU2* was amplified from pJJ283 [98] by using primers D-ScATX15 and D-ScATX13. The fragment was introduced into YPH499 to delete *ATX1* gene. Disruption of chromosomal *ATX1* gene was confirmed by diagnostic PCR analysis (Fig. 16). The *Δatx1* 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 μM ferric ammonium sulfate (Sigma) was added to the medium.

3.2.6 Subcellular fractionation of CUC-1 in *Δatx1* 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 (2×10^8 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 1.5×10^7 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 cuaex2.0 were used for PCR by LA-PCR Kit ver.2 (TaKaRa) to amplify the upstream regions of *cua-1* gene. The PCR products containing abcut 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 *Bam*HI and *Sal*I, 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 *Sph*I and *Bam*HI 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

A

```
          +1
GTTTGAGGCAAGCAGAAGCAGAGTTTCCAGATTTCTGACTCAACAAATATCATG    47
SL1                                                    M
                          CeATX-1 fw2
ACACAGTACGTTTTCGAAATGGGCATGACATGCAATGGCTGTGCAAATGCTGCG    101
 T  Q  Y  V  F  E  M  G  M  T  C  N  G  C  A  N  A  A

CGAAAAGTTCTAGGAAAGCTTGGAGAAGACAAAATCAAAATTGACGATATTAAC    155
 R  K  V  L  G  K  L  G  E  D  K  I  K  I  D  D  I  N
                                          CeATX-1 rv2
GTGGAAACCAAGAAAATCACAGTTACAACTGATTTACCAGCATCTGATGTACTT    209
 V  E  T  K  K  I  T  V  T  T  D  L  P  A  S  D  V  L
                CeATX-1 rv1
GAAGCTCTGAAGAAAACAGGAAAAGAGATCAAGCAACTACAATAAATGACCCTT    263
 E  A  L  K  K  T  G  K  E  I  K  Q  L  Q  *

TTGATTGTCTCTTTAATTTCTATCCGAATGGTTGTGAATATCGTTATTTTTTGA    317

CATTTCTGTAATTTTTGTATTAATTTATGACTCTTGCTTGTAAAATTGTCCTAT    371

TCTAAAAATAAACGCATTTTCTAAACTTAAAAAAAAAAAAAAAAAAAAAAAAA
    polyadenylation signal
```

B

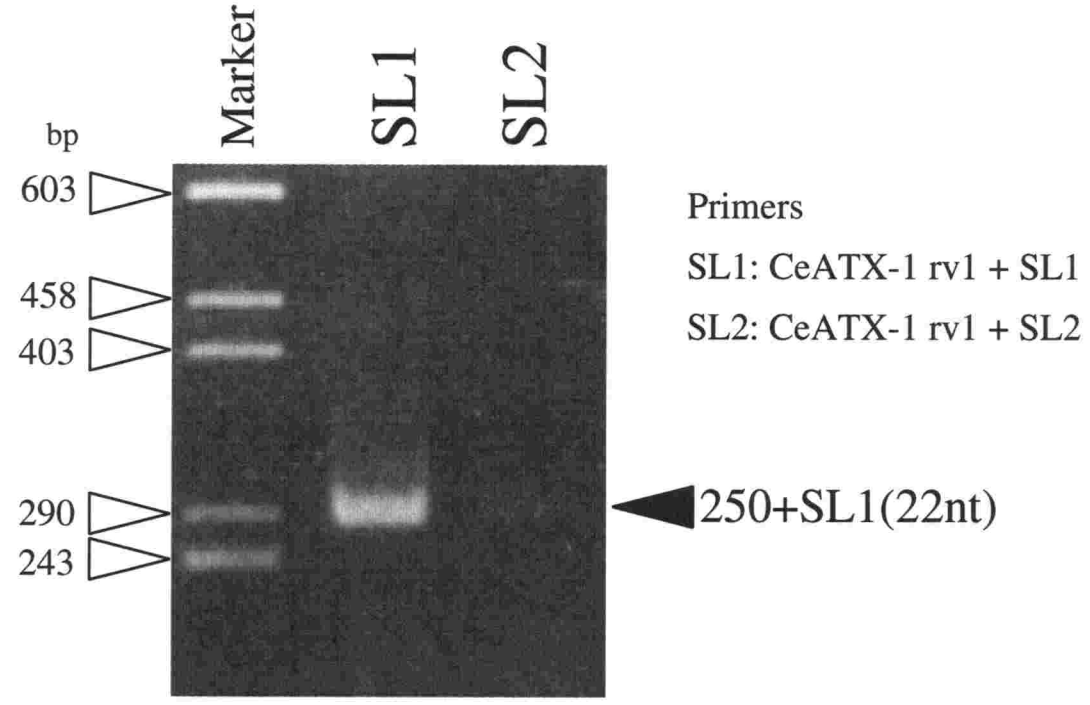


Fig. 13. cDNA structure (A) and SL-PCR (B) of *cuc-1*.

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.

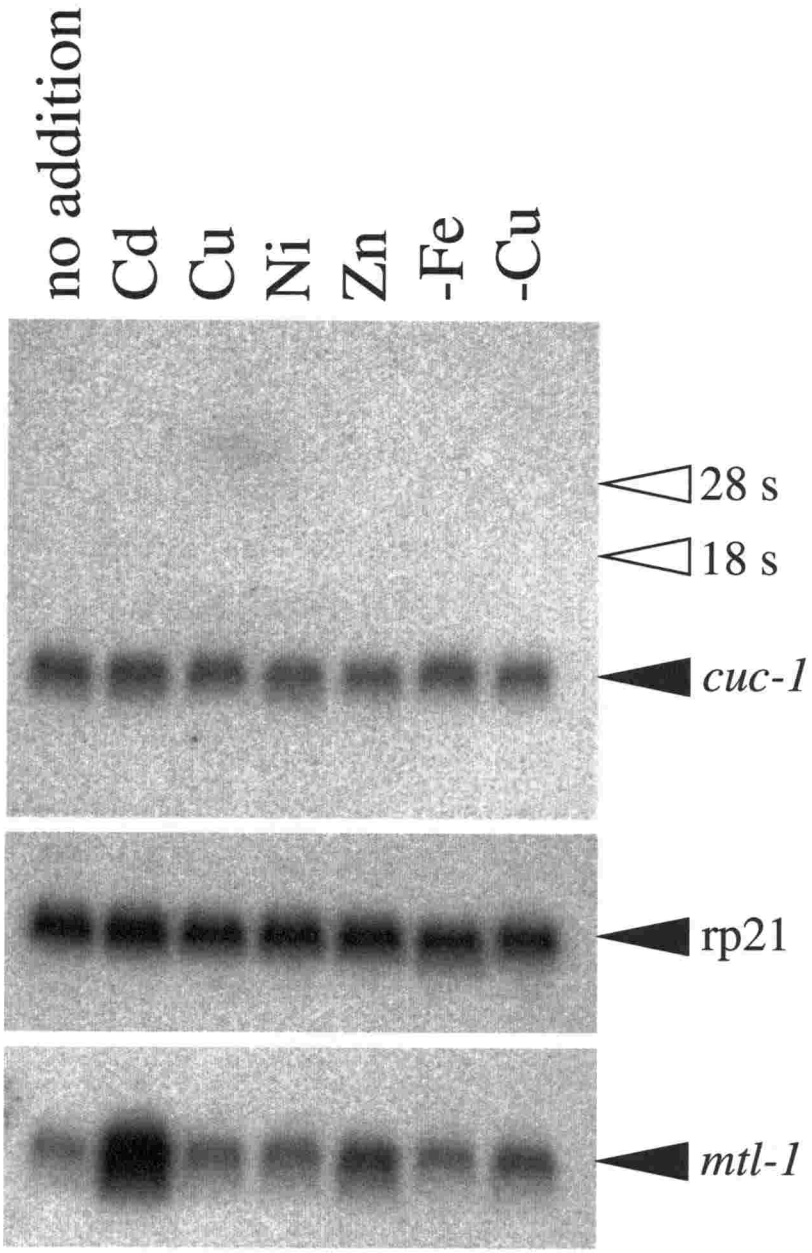
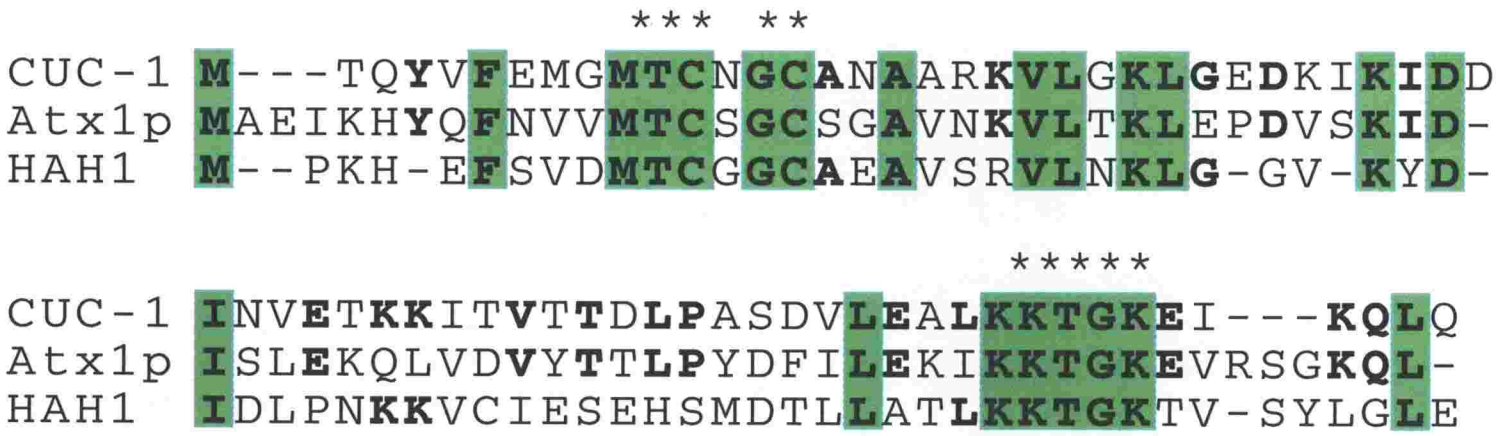


Fig. 14 Detection of *cuc-1* transcript

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 $CdCl_2$ [Cd], 5 μM $(CH_3COO)_2Cu$ [Cu], 1 μM $(CH_3COO)_2Ni$ [Ni], 5 μM $(CH_3COO)_2Zn$ [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	Atx1p	HAH1
CUC-1	——	49.3	39.1
Atx1p		——	42.5
HAH1			——

(%)

Fig. 15 Alignment of Atx1p homologues

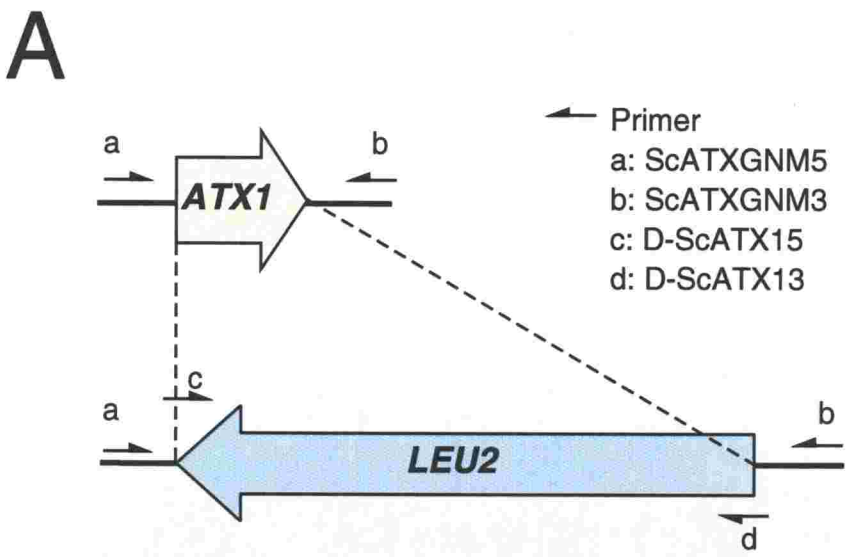
Deduced amino acid sequence of CUC-1 was aligned with those of yeast Atx1p and human HAH1. Conserved residues among the three proteins are boxed. Residues identical to CUC-1 are in bold letters. The characteristic copper binding motif, and the lysine rich sequence, KKTGK, are indicated by asterisk. The 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 *Δatx1* mutant

The *cuc-1* cDNA was introduced into the yeast *Δatx1* 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 *Δatx1* 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].



B

	a+b	c+b
W.T.	1.2 kbp	(0.8 kbp)
Δatx1	3.1 kbp	2.7 kbp

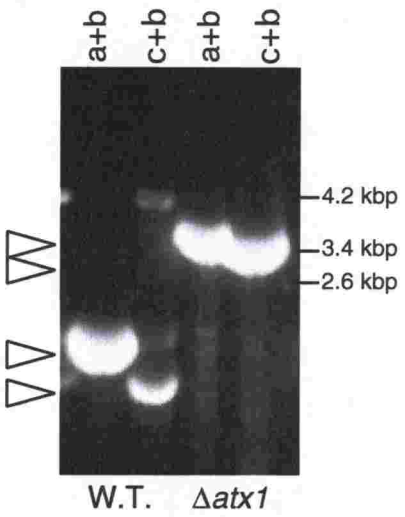


Fig. 16 Disruption of the yeast *ATX1* gene

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.

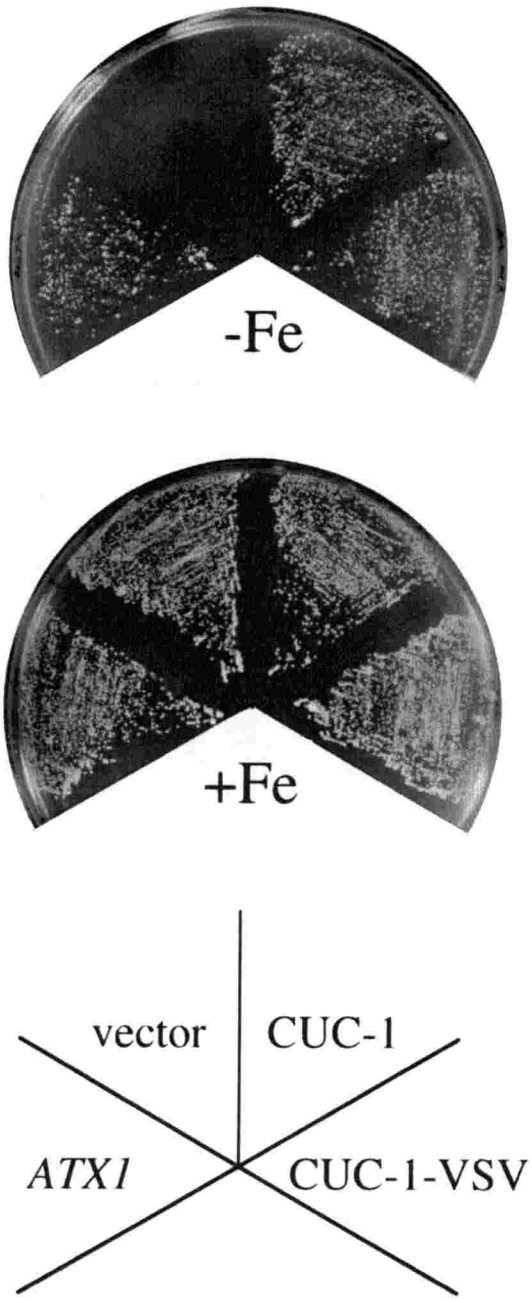


Fig. 17 Complementation of iron dependent growth of Δ*atx1* mutant by expression of CUC-1.

The Δ*atx1* 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).

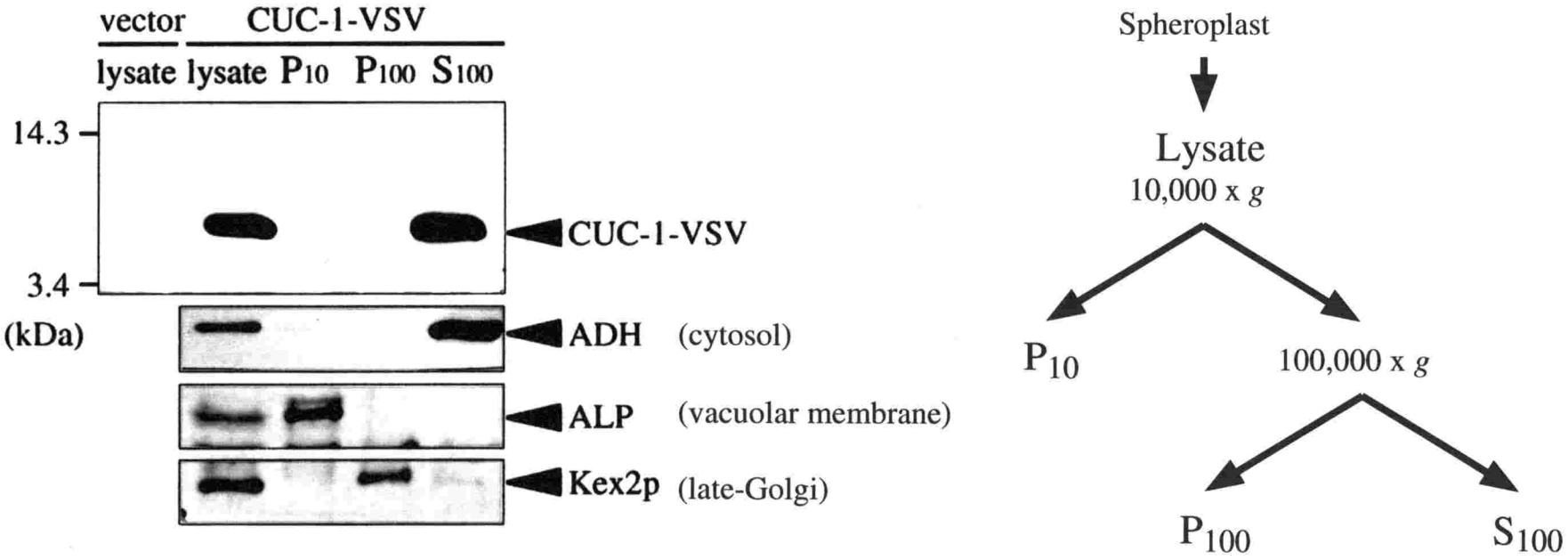


Fig. 18 Subcellular localization of CUC-1.

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 (P_{10}). Then, the supernatant was centrifuged at 100,000 x *g* to obtain pellet (P_{100}) and supernatant (S_{100}) fractions. Fractionation procedure is schematically shown (right). Each fraction from 1.5 x 10^7 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 Atx1p 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 tansgenic 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 *cua-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).

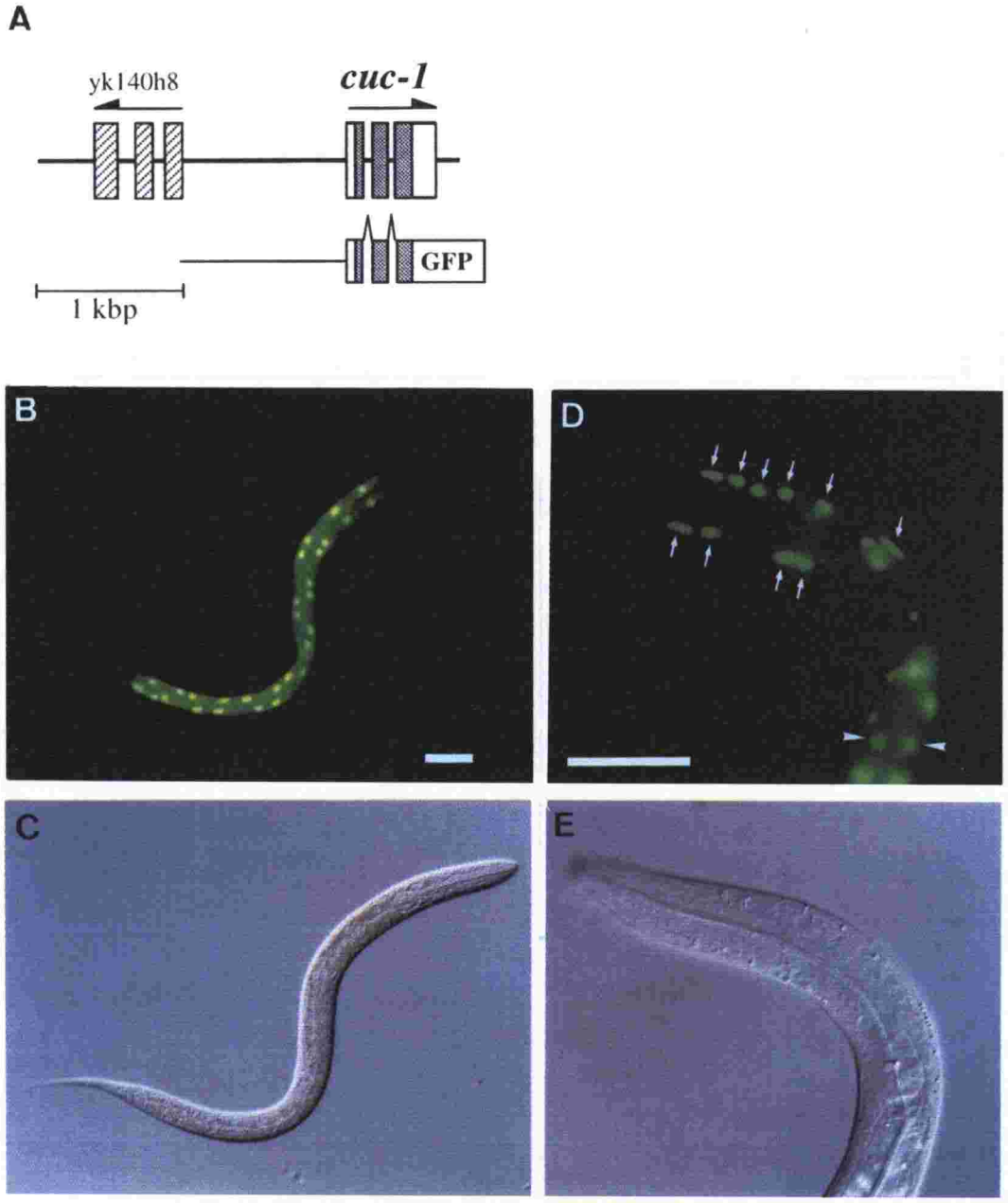


Fig. 19 Expression of *cuc-1* in intestinal and hypodermal cells.

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 yk140h8 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* con struct 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.

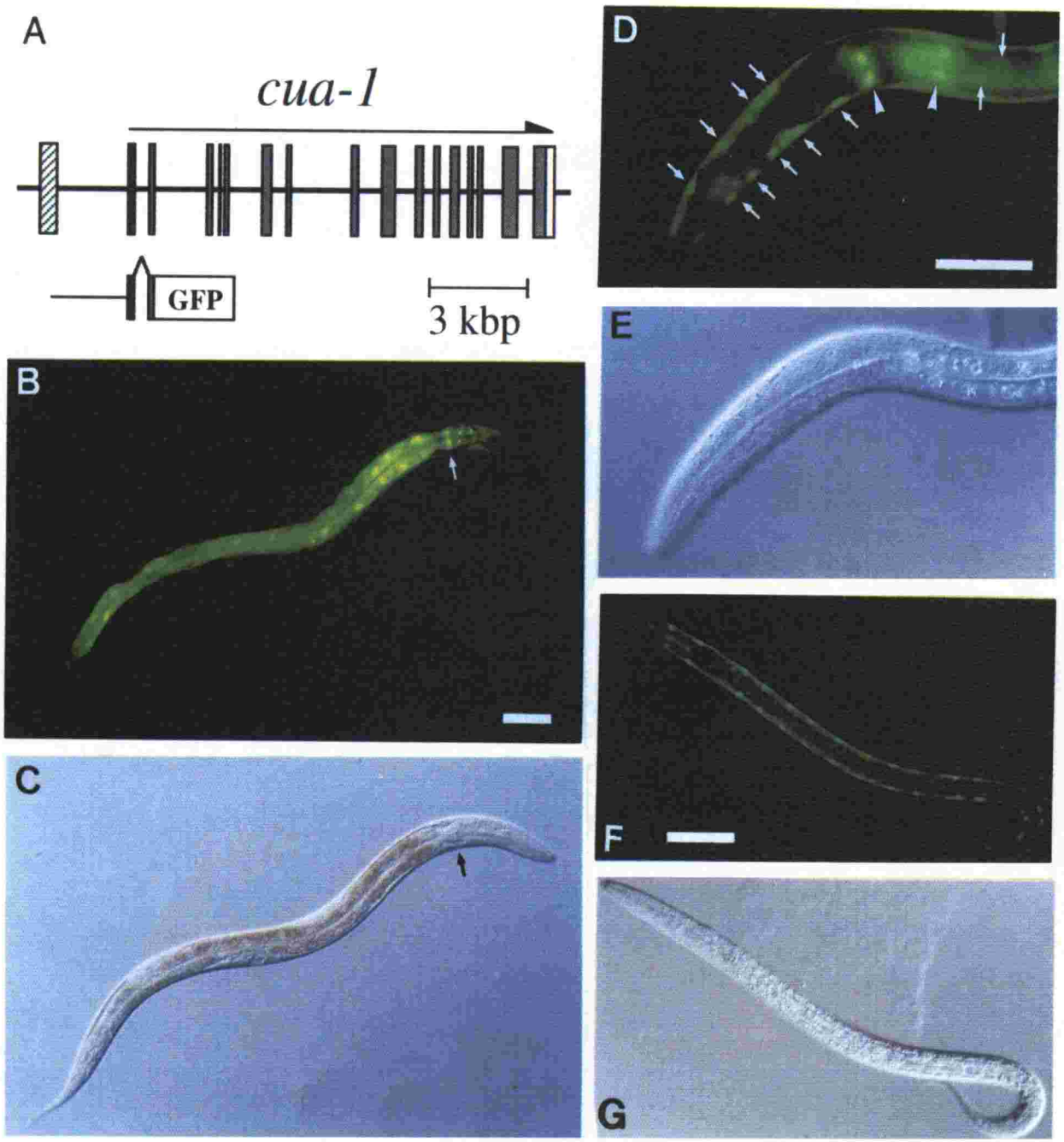


Fig. 20 Expression of *cua-1* in intestinal and hypodermal cells.

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.

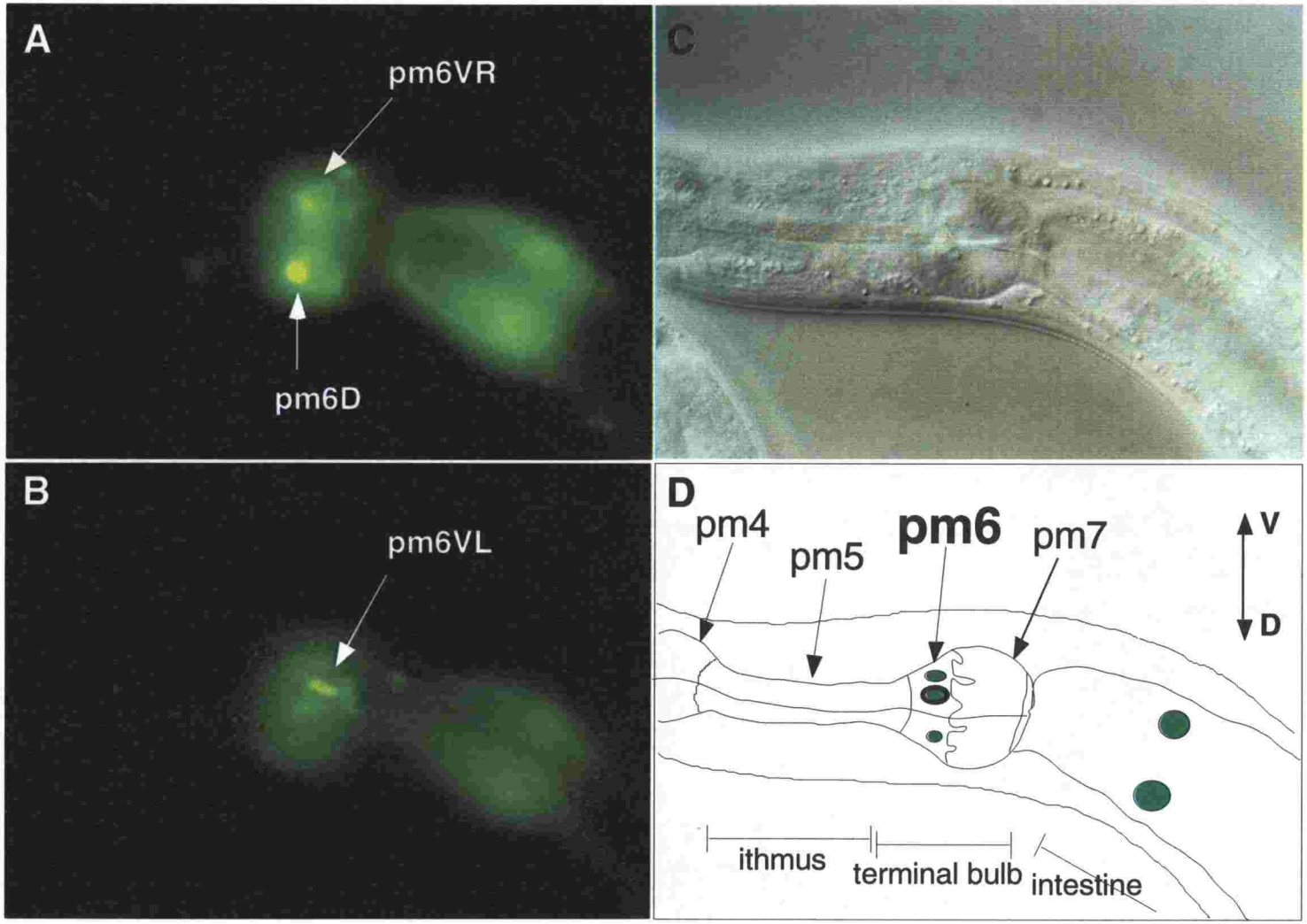


Fig. 21 Expression of *cua-1* gene in pharyngeal muscle cell, pm6

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 mononu cleated 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 *Δatx1*) [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 *HAH1* 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, *HAH1* 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 exoskeltal 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*.

REFERENCES

[1] Crapo, J.D., Oury, T., Rabouille, C., Slot, J.W. and Chang, L.Y. (1992). Copper,zinc superoxide dismutase is primarily a cytosolic protein in human cells. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 10405-10409.

[2] Kodama, H., Okabe, I., Yanagisawa, M. and Kodama, Y. (1989). Copper deficiency in the mitochondria of cultured skin fibroblasts from patients with Menkes syndrome. *J. Inherit. Metab. Dis.* **12**, 386-389.

[3] Kuivaniemi, H., Ala-Kokko, L. and Kivirikko, K.I. (1986). Secretion of lysyl oxidase by cultured human skin fibroblasts and effects of monensin, nigericin, tunicamycin and colchicine. *Biochim. Biophys. Acta.* **883**, 326-334.

[4] Janssens, A.R., Bosman, F.T., Ruiter, D.J. and Van den Hamer, C.J. (1984). Immunohistochemical demonstration of the cytoplasmic copper-associated protein in the liver in primary biliary cirrhosis: its identification as metallothionein. *Liver* **4**, 139-147.

[5] Janssens, A.R., Van Noord, M.J., Van Hoek, C.J., Ruiter, D.J., Mauw, B.J. and Van den Hamer, C.J. (1984). The lysosomal copper concentration in the liver in primary biliary cirrhosis. *Liver* **4**, 396-401.

[6] Uauy, R., Olivares, M. and Gonzalez, M. (1998). Essentiality of copper in humans. *Am. J. Clin. Nutr.* **67**, 952S-959S.

[7] Fridovich, I. (1978). The biology of oxygen radicals. *Science* **201**, 875-880.

[8] Imlay, J.A. and Linn, S. (1988). DNA damage and oxygen radical toxicity. *Science* **240**, 1302-1309.

[9] Halliwell, B. and Gutteridge, J.M. (1984). Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* **219**, 1-14.

[10] Predki, P.F. and Sarkar, B. (1992). Effect of replacement of "zinc finger" zinc on estrogen receptor DNA interactions. *J. Biol. Chem.* **267**, 5842-5846.

[11] Predki, P.F. and Sarkar, B. (1994). Metal replacement in "zinc finger" and its effect on DNA binding. *Environ. Health Perspect.* **3**, 195-198.

[12] Menkes, J.H., Alter, M., Steigleder, G.K., Weakley, D.R. and Sung, J.H. (1962). A sex-linked reccessive disorder with reterdation of growth, peculiar hair,

and focal cerebral and cerebellar degeneration. *Pediatrics* **29**, 764-779.

[13] Danks, D.M., Campbell, P.E., Walker-Smith, J., Stevens, B.J., Gillespie, J.M., Blomfield, J. and Turner, B. (1972). Menkes' kinky-hair syndrome. *Lancet* **1**, 1100-1102.

[14] Danks, D.M., Stevens, B.J., Campkell, P.E., Cartwright, E.C., Gillespie, J.M., Townley, R.R., Blomfield, J., Turner, B.B., Mayne, V. and Walker-Smith, J.A. (1974). Menkes kinky-hair syndrome. An inherited defect in the intestinal absorption of copper with widespread effects. *Birth Defects Orig. Artic. Ser.* **10**, 132-7.

[15] Danks, D.M. (1989). Disorders of copper transport. In *Metabolic basis of inherited disease*, Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D., eds. (New York: McGraw Hill), pp. 1411-1432.

[16] Camakaris, J., Danks, D.M., Ackland, L., Cartwright, E., Borger, P. and Cotton, R.G. (1980). Altered copper metabolism in cultured cells from human Menkes' syndrome and *mottled* mouse mutants. *Biochem. Genet.* **18**, 117-131.

[17] Darwish, H.M., Hoke, J.E. and Ettinger, M.J. (1983). Kinetics of Cu(II) transport and accumulation by hepatocytes from copper-deficient mice and the *brindled* mouse model of Menkes disease. *J. Biol. Chem.* **258**, 13621-13626.

[18] Goka, T.J., Stevenson, R.E., Hefferan, P.M. and Howell, R.R. (1976). Menkes disease: a biochemical abnormality in cultured human fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **73**, 604-606.

[19] Horn, N. (1976). Copper incorporation studies on cultured cells for prenatal diagnosis of Menkes' disease. *Lancet* **1**, 1156-1158.

[20] Sternlieb, I. (1978). Diagnosis of Wilson's disease. *Gastroenterology* **74**, 787-789.

[21] Wilson, S.A.K. (1912). Progressive lenticular degeneration: a familial nervous disease associated with cirrhosis of the liver. *Brain* **34**, 295-309.

[22] Mori, N. (1994). Genetic approaches toward understanding of the mechanisms of neurological disoerders: Mutations in copper transporter genes cause neurode generation during brain development. *Saibou Kougaku* **13**, 816-822.

[23] Sternlieb, I. (1990). Perspectives on Wilson's disease. *Hepatology* **12**, 1234-1239.

[24] Brewer, G.J. and Yuzbasiyan-Gurkan, V. (1992). Wilson disease.

Medicine **71**, 139-164.

[25] Chelly, J., Tumer, Z., Tonnesen, T., Petterson, A., Ishikawa-Brush, Y., Tommerup, N., Horn, N. and Monaco, A.P. (1993). Isolation of a candidate gene for Menkes disease that encodes a potential heavy metal binding protein. *Nature Genetics* **3**, 14-19.

[26] Mercer, J.F., Livingston, J., Hall, B., Paynter, J.A., Begy, C., Chandrasekharappa, S., Lockhart, P., Grimes, A., Bhave, M., Siemieniak, D. and Glover, T.W. (1993). Isolation of a partial candidate gene for Menkes disease by positional cloning. *Nature Genetics* **3**, 20-25.

[27] Vulpe, C., Levinson, B., Whitney, S., Packman, S. and Gitschier, J. (1993). Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. *Nature Genetics* **3**, 7-13.

[28] Bull, P.C., Thomas, G.R., Rommens, J.M., Forbes, J.R. and Cox, D.W. (1993). The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene. *Nature Genetics* **5**, 327-337.

[29] Tanzi, R.E., Petrukhin, K., Chernov, I., Pellequer, J.L., Wasco, W., Ross, B., Romano, D.M., Parano, E., Pavone, L., Brzustowicz, L.M., Devote, M., Peppercorn, J., Bush, A.I., Sternlieb, I., Pirastu, M., Gusella, J.F., Evgrafov, O., Penchaszadeh, G.K., Honig, B., Edelman, I.S., Soares, M.B., Scheinberg, I.H. and Gilliam, T.C. (1993). The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. *Nature Genetics* **5**, 344-350.

[30] Yamaguchi, Y., Heiny, M.E. and Gitlin, J.D. (1993). Isolation and characterization of a human liver cDNA as a candidate gene for Wilson disease. *Biochem. Biophys. Res. Commun.* **197**, 271-277.

[31] Bayle, D., Wangler, S., Weitzenegger, T., Steinhilber, W., Volz, J., Przybylski, M., Schafer, K.P., Sachs, G. and Melchers, K. (1998). Properties of the P-type ATPases encoded by the *copAP* operons of *Helicobacter pylori* and *Helicobacter felis*. *J. Bacteriol* **180**, 317-329.

[32] Odermatt, A., Suter, H., Krapf, R. and Solioz, M. (1992). An ATPase operon involved in copper resistance by *Enterococcus hirae*. *Ann. N. Y. Acad. Sci.* **671**, 484-486.

[33] Odermatt, A., Suter, H., Krapf, R. and Solioz, M. (1993). Primary structure of

two P-type ATPases involved in copper homeostasis in *Enterococcus hirae*. *J. Biol. Chem.* **268**, 12775-12779.

[34] Carafoli, E. (1992). P-type ATPases. Introduction. *J. Bioenerg. Biomembr.* **24**, 245-247.

[35] Solioz, M. and Vulpe, C. (1996). CPx-type ATPases: a class of P-type ATPases that pump heavy metals. *Trends Biochem. Sci.* **21**, 237-241.

[36] Silver, S. (1998). Genes for all metals--a bacterial view of the periodic table. *J. Ind. Microbiol. Biotechnol.* **20**, 1-12.

[37] Solioz, M., Odermatt, A. and Krapf, R. (1994). Copper pumping ATPases: common concepts in bacteria and man. *FEBS Lett.* **346**, 44-47.

[38] Lutsenko, S. and Kaplan, J.H. (1995). Organization of P-type ATPases: significance of structural diversity. *Biochemistry* **34**, 15607-15613.

[39] Payne, A.S. and Gitlin, J.D. (1998). Functional expression of the Wilson disease protein reveals mislocalization and impaired copper-dependent trafficking of the common H1069Q mutation. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 10854-10859.

[40] Petris, M.J., Mercer, J.F., Culvenor, J.G., Lockhart, P., Gleeson, P.A. and Camakaris, J. (1996). Ligand-regulated transport of the Menkes copper P-type ATPase efflux pump from the Golgi apparatus to the plasma membrane: a novel mechanism of regulated trafficking. *EMBO J.* **15**, 6084-6095.

[41] Hung, I.H., Suzuki, M., Yamaguchi, Y., Yuan, D.S., Klausner, R.D. and Gitlin, J.D. (1997). Biochemical characterization of the Wilson disease protein and functional expression in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* **272**, 21461-21466.

[42] Yang, X.L., Miura, N., Kawarada, Y., Terada, K., Petrukhin, K., Gilliam, T. and Sugiyama, T. (1997). Two forms of Wilson disease protein produced by alternative splicing are localized in distinct cellular compartments. *Biochem. J.* **326**, 897-902.

[43] Thiele, D.J. (1988). *ACE1* regulates expression of the *Saccharomyces cerevisiae* metallothionein gene. *Mol. Cell. Biol.* **8**, 2745-2752.

[44] Lin, C.M. and Kosman, D.J. (1990). Copper uptake in wild type and copper metallothionein-deficient *Saccharomyces cerevisiae*. Kinetics and mechanism. *J. Biol. Chem.* **265**, 9194-9200.

[45] Vulpe, C.D. and Packman, S. (1995). Cellular copper transport.

Ann. Rev. Nutr. **15**, 293-322.

[46] Georgatsou, E., Mavrogiannis, L.A., Fragiadakis, G.S. and Alexandraki, D. (1997). The yeast Fre1p/Fre2p cupric reductases facilitate copper uptake and are regulated by the copper-modulated Mac1p activator.
J. Biol. Chem. **272**, 13786-13792.

[47] Dancis, A., Yuan, D.S., Haile, D., Askwith, C., Elde, D., Moehle, C., Kaplan, J. and Klausner, R.D. (1994). Molecular characterization of a copper transport protein in *S. cerevisiae*: an unexpected role for copper in iron transport. *Cell* **76**, 393-402.

[48] Dancis, A., Haile, D., Yuan, D.S. and Klausner, R.D. (1994). The *Saccharomyces cerevisiae* copper transport protein (Ctr1p). Biochemical characterization, regulation by copper, and physiologic role in copper uptake.
J. Biol. Chem. **269**, 25660-25667.

[49] Glerum, D.M., Shtanko, A. and Tzagoloff, A. (1996). Characterization of *COX17*, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase.
J. Biol. Chem. **271**, 14504-14509.

[50] Beers, J., Glerum, D.M. and Tzagoloff, A. (1997). Purification, characterization, and localization of yeast Cox17p, a mitochondrial copper shuttle.
J. Biol. Chem. **272**, 33191-33196.

[51] Culotta, V.C., Klomp, L.W., Strain, J., Casareno, R.L., Krems, B. and Gitlin, J.D. (1997). The copper chaperone for superoxide dismutase.
J. Biol. Chem. **272**, 23469-23472.

[52] Lin, S.J., Pufahl, R.A., Dancis, A., O'Halloran, T.V. and Culotta, V.C. (1997). A role for the *Saccharomyces cerevisiae ATX1* gene in copper trafficking and iron transport. *J. Biol. Chem.* **272**, 9215-9220.

[53] Lin, S.J. and Culotta, V.C. (1995). The *ATX1* gene of *Saccharomyces cerevisiae* encodes a small metal homeostasis factor that protects cells against reactive oxygen toxicity. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 3784-3788.

[54] Yuan, D.S., Stearman, R., Dancis, A., Dunn, T., Beeler, T. and Klausner, R.D. (1995). The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake.
Proc. Natl. Acad. Sci. U. S. A. **92**, 2632-2636.

[55] Fu, D., Beeler, T.J. and Dunn, T.M. (1995). Sequence, mapping and disruption of

CCC2, a gene that cross-complements the Ca^{2+}-sensitive phenotype of csg1 mutants and encodes a P-type ATPase belonging to the Cu^{2+}-ATPase subfamily. *Yeast* **11**, 283-292.

[56] Yuan, D.S., Dancis, A. and Klausner, R.D. (1997). Restriction of copper export in *Saccharomyces cerevisiae* to a late Golgi or post-Golgi compartment in the secretory pathway. *J. Biol. Chem.* **272**, 25787-25793.

[57] Klomp, L.W., Lin, S.J., Yuan, D.S., Klausner, R.D., Culotta, V.C. and Gitlin, J.D. (1997). Identification and functional expression of *HAH1*, a novel human gene involved in copper homeostasis. *J. Biol. Chem.* **272**, 9221-9226.

[58] Zhou, B. and Gitschier, J. (1997). *hCTR1*: a human gene for copper uptake identified by complementation in yeast. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 7481-7486.

[59] Sambongi, Y., Wakabayashi, T., Yoshimizu, T., Omote, H., Oka, T. and Futai, M. (1997). *Caenorhabditis elegans* cDNA for a Menkes/Wilson disease gene homologue and its function in a yeast *CCC2* gene deletion mutant. *J. Biochem.* **121**, 1169-1175.

[60] Wakabayashi, T., Nakamura, N., Sambongi, Y., Wada, Y., Oka, T. and Futai, M. (1998). Identification of copper chaperone, CUC-1, in *Caenorhabditis elegans*: tissue specific co-expression with the copper transporting ATPase, CUA-1. *FEBS Lett.* in press.

[61] Stearman, R., Yuan, D.S., Yamaguchi-Iwai, Y., Klausner, R.D. and Dancis, A. (1996). A permease-oxidase complex involved in high-affinity iron uptake in yeast. *Science* **271**, 1552-1557.

[62] Sulston, J. and Hodgkin, J. (1988). Methods. In *The Nematode Caenorhabditis elegans*, Woods, W.B., ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 587-606.

[63] Sambrook, J., Fritisch, E.F. and Maniatis, T. (1989). *Molecular Cloning. A laboratory manual, 2nd ed.* (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

[64] Spieth, J., Brooke, G., Kuersten, S., Lea, K. and Blumenthal, T. (1993). Operons in *C. elegans*: polycistronic mRNA precursors are processed by trans-splicing of SL2 to downstream coding regions. *Cell* **73**, 521-532.

[65] Rothstein, R.J. (1983). One step gene disruption in yeast. In *Methods in Enzymology*, Wu, R., Grossman, L. and Moldav, K., eds. (New York: Academic Press), pp. 202-211.

[66] Kaiser, C., Michaelis, S. and Mitchell, A. (1994). *Methods in yeast genetics* (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

[67] Nishikawa, S. and Nakano, A. (1991). The GTP-binding Sar1 protein is localized to the early compartment of the yeast secretory pathway. *Biochim. Biophys. Acta.* **1093**, 135-143.

[68] Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4350-4354.

[69] Coulson, A., Waterston, R., Kiff, J., Sulston, J. and Kohara, Y. (1988). Genome linking with yeast artificial chromosomes. *Nature* **335**, 184-186.

[70] Krause, M. (1995). Transcription and translation. In *Caenorhabditis elegans: Modern Biological Analysis of an Organizm*, Epstein, H.F. and Shakes, D.C., eds. (San Diego: Academic Press), pp. 483-512.

[71] Serrano, R. (1988). Structure and function of proton translocating ATPase in plasma membranes of plants and fungi. *Biochim. Biophys. Acta.* **947**, 1-28.

[72] Maruyama, K. and MacLennan, D.H. (1988). Mutation of aspartic acid-351, lysine-352, and lysine-515 alters the Ca2+ transport activity of the Ca^{2+}-ATPase expressed in COS-1 cells. *Proc. Natl. Acad. Sci. U. S. A.* **85**, 3314-3318.

[73] Kyte, J. and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105-132.

[74] Iida, M., Terada, K., Sambongi, Y., Wakabayashi, T., Miura, N., Koyama, K., Futai, M. and Sugiyama, T. (1998). Analysis of functional domains of Wilson disease protein (ATP7B) in *Saccharomyces serevisiae*. *FEBS Lett.* **428**, 281-285.

[75] Thomas, G.R., Forbes, J.R., Roberts, E.A., Walshe, J.M. and Cox, D.W. (1995). The Wilson disease gene: spectrum of mutations and their consequences. *Nature Genetics* **9**, 210-217.

[76] Ge, Z., Hiratsuka, K. and Taylor, D.E. (1995). Nucleotide sequence and mutational analysis indicate that two *Helicobacter pylor*i genes encode a P-type ATPase and a cation-binding protein associated with copper transport.

Mol. Microbiol. **15**, 97-106.

[77] Brandl, C.J., Green, N.M., Korczak, B. and MacLennan, D.H. (1986). Two Ca^{2+} ATPase genes: homologies and mechanistic implications of deduced amino acid sequences. *Cell* **44**, 597-607.

[78] Vilsen, B., Andersen, J.P., Clarke, D.M. and MacLennan, D.H. (1989). Functional consequences of proline mutations in the cytoplasmic and transmembrane sectors of the Ca^{2+}-ATPase of sarcoplasmic reticulum. *J. Biol. Chem.* **264**, 21024-21030.

[79] Wilcox, C.A., Redding, K., Wright, R. and Fuller, R.S. (1992). Mutation of a tyrosine localization signal in the cytosolic tail of yeast Kex2 protease disrupts Golgi retention and results in default transport to the vacuole. *Mol. Biol. Cell.* **3**, 1353-1371.

[80] Cooper, A. and Bussey, H. (1992). Yeast Kex1p is a Golgi-associated membrane protein: deletions in a cytoplasmic targeting domain result in mislocalization to the vacuolar membrane. *J. Cell Biol.* **119**, 1459-1468.

[81] Roberts, C.J., Nothwehr, S.F. and Stevens, T.H. (1992). Membrane protein sorting in the yeast secretory pathway: evidence that the vacuole may be the default compartment. *J. Cell Biol.* **119**, 69-83.

[82] Tsai, K.J., Yoon, K.P. and Lynn, A.R. (1992). ATP-dependent cadmium transport by the *cadA* cadmium resistance determinant in everted membrane vesicles of *Bacillus subtilis*. *J. Bacteriol.* **174**, 116-121.

[83] Tsai, K.J. and Linet, A.L. (1993). Formation of a phosphorylated enzyme intermediate by the cadA Cd^{2+}-ATPase. *Arch. Biochem. Biophys.* **305**, 267-270.

[84] Solioz, M. and Odermatt, A. (1995). Copper and silver transport by *CopB*-ATPase in membrane vesicles of *Enterococcus hirae*. *J. Biol. Chem.* **270**, 9217-9221.

[85] Clarke, D.M., Maruyama, K., Loo, T.W., Leberer, E., Inesi, G. and MacLennan, D.H. (1989). Functional consequences of glutamate, aspartate, glutamine, and asparagine mutations in the stalk sector of the Ca^{2+}-ATPase of sarcoplasmic reticulum. *J. Biol. Chem.* **264**, 11246-11251.

[86] Lutsenko, S., Petrukhin, K., Cooper, M.J., Gilliam, C.T. and Kaplan, J.H. (1997). N-terminal domains of human copper-transporting adenosine

triphosphatases (the Wilson's and Menkes disease proteins) bind copper selectively *in vivo* and *in vitro* with stoichiometry of one copper per metal-binding repeat. *J. Biol. Chem.* **272**, 18939-18944.

[87] Hamlett, N.V., Landale, E.C., Davis, B.H. and Summers, A.O. (1992). Roles of the *Tn21 merT, merP*, and *merC* gene products in mercury resistance and mercury binding. *J. Bacteriol.* **174**, 6377-6385.

[88] Payne, A.S. and Gitlin, J.D. (1998). Functional expression of the menkes disease protein reveals common biochemical mechanisms among the copper-transporting P-type ATPases. *J. Biol. Chem.* **273**, 3765-3770.

[89] Shah, A.B., Chernov, I., Zhang, H.T., Ross, B.M., Das, K., Lutsenko, S., Parano, E., Pavone, L., Evgrafov, O., Ivanova-Smolenskaya, I.A., Anneren, G., Westermark, K., Urrutia, F.H., Penchaszadeh, G.K., Sternlieb, I., Scheinberg, I.H., Gilliam, T.C. and Petrukhin, K. (1997). Identification and analysis of mutations in the Wilson disease gene (ATP7B): population frequencies, genotype-phenotype correlation, and functional analyses. *Am. J. Hum. Genet.* **61**, 317-328.

[90] Pufahl, R.A., Singer, C.P., Peariso, K.L., Lin, S., Schmidt, P.J., Fahrni, C.J., Culotta, V.C. and Penner-Hahn, J.E. (1997). Metal ion chaperone function of the soluble Cu(I) receptor Atx1. *Science* **278**, 853-856.

[91] Yoshimizu, T., Omote, H., Wakabayashi, T., Sambongi, Y. and Futai, M. (1998). Essential Cys-Pro-Cys motif of *Caenorhabditis elegans* copper transport ATPase. *Biosci. Biotechnol. Biochem.* **62**, 1258-1260.

[92] Wilson, R., Ainscough, R., Anderson, K., Baynes, C., Berks, M., Bonfield, J., Burton, J., Connell, M., Copsey, T., Cooper, J., Coulson, A., Craxton, M., Dear, S., Du, Z., Durbin, R., Favello, A., Fulton, L., Gardner, A., Green, P., Hawkins, T., Hillier, L., Jier, M., Johnston, L., Jones, M., Kershow, J., Kirsten, J., Laister, N., Latreille, P., Lightning, J., Lloyd, C., McMurray, A., Mortimore, B., O'Callaghan, M., Parsons, J., Percy, C., Rifken, L., Roopra, A., Saunders, D., Shownkeen, R., Smaldon, N., Smith, A., Sonnhammer, E., Staden, R., Sulston, J., Thierry-Mieg, J., Thomas, K., Vaudin, M., Vaughan, K., Waterston, R., Watson, A., Weinstock, L., Wilkinson-Sproat, J. and Wohldman, P. (1994). 2.2 Mb of contiguous nucleotide sequence from chromosome III of *C. elegans*. *Nature* **368**, 32-38.

[93] Hung, I.H., Casareno, R.L., Labesse, G., Mathews, F.S. and Gitlin, J.D. (1998). HAH1 is a copper-binding protein with distinct amino acid residues mediating copper homeostasis and antioxidant defense. *J. Biol. Chem.* **273**, 1749-1754.

[94] Tanaka, K., Nakafuku, M., Tamanoi, F., Kaziro, Y., Matsumoto, K. and Toh-e, A. (1990). *IRA2*, a second gene of *Saccharomyces cerevisiae* that encodes a protein with a domain homologous to mammalian ras GTPase-activating protein. *Mol. Cell. Biol.* **10**, 4303-4313.

[95] Kreis, T.E. (1986). Microinjected antibodies against the cytoplasmic domain of vesicular stomatitis virus glycoprotein block its transport to the cell surface. *EMBO J.* **5**, 931-941.

[96] Kolodziej, P.A. and Young, R.A. (1991). Epitope tagging and protein surveillance. In *Methods Enzymol*, Wu, R., Grossman, L. and Moldav, K., eds. (New York: Academic Press), pp. 508-519.

[97] Sikorski, R.S. and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19-27.

[98] Jones, J.S. and Prakash, L. (1990). Yeast *Saccharomyces cerevisiae* selectable markers in pUC18 polylinkers. *Yeast* **6**, 363-366.

[99] Gietz, D., St. Jean, A., Woods, R.A. and Schiestl, R.H. (1992). Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.* **20**, 1425.

[100] Nakamura, N., Hirata, A., Ohsumi, Y. and Wada, Y. (1997). Vam2/Vps41p and Vam6/Vps39p are components of a protein complex on the vacuolar membranes and involved in the vacuolar assembly in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* **272**, 11344-11349.

[101] Mello, C.C., Kramer, J.M., Stinchcomb, D. and Ambros, V. (1991). Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* **10**, 3959-3970.

[102] Zorio, D.A., Cheng, N.N., Blumenthal, T. and Spieth, J. (1994). Operons as a common form of chromosomal organization in *C. elegans*. *Nature* **372**, 270-272.

[103] Yamaguchi-Iwai, Y., Stearman, R., Dancis, A. and Klausner, R.D. (1996). Iron-regulated DNA binding by the Aft1 protein controls the iron regulon in yeast.

EMBO J. **15**, 3377-3384.

[104] Riddle, D.L. (1988). The Dauer Larva. In *The Nematode Caenorhabditis elegans*, Woods, W.B., ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 393-414.

[105] Jungmann, J., Reins, H.A., Lee, J., Romeo, A., Hassett, R., Kosman, D. and Jentsch, S. (1993). Mac1, a nuclear regulatory protein related to Cu-dependent transcription factors is involved in Cu/Fe utilization and stress resistance in yeast. *EMBO J.* **12**, 5051-5056.

[106] Kaler, S.G., Gallo, L.K., Proud, V.K., Percy, A.K., Mark, Y., Segal, N.A., Goldstein, D.S., Holmes, C.S. and Gahl, W.A. (1994). Occipital horn syndrome and a mild Menkes phenotype associated with splice site mutations at the MNK locus. *Nature Genetics* **8**, 195-202.

PUBLICATIONS RELATED TO THIS THESIS

CHAPTER 2

Yoshihiro Sambongi, **Tokumitsu Wakabayashi**, Takao Yoshimizu, Hiroshi Omote, Toshihiko Oka, and Masamitsu Futai

Caenorhabditis elegans cDNA for a Menkes/Wilson disease gene homologue and its function in a yeast *CCC2* deletion mutant.

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

Masatake Iida, Kunihiko Terada, Yoshihiro Sambongi, **Tokumitsu Wakabayashi**, Naoyuki Miura, Kenji Koyama, Masamitsu Futai, and Toshihiro Sugiyama

Analysis of functional domains of Wilson disease protein (ATP7B) in *Saccharomyces cerevisiae.*

FEBS Lett. 428, 281-285 (1998)

Takao Yoshimizu, Hiroshi Omote, **Tokumitsu Wakabayashi**, Yoshihiro Sambongi, and Masamitsu Futai

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

Biosci. Biotechnol. Biochem. 62, 1258-1260 (1998)

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)