

Isolation of a murine copper transporter gene, tissue specific expression and functional complementation of a yeast copper transport mutant

Jaekwon Lee ^a, Joseph R. Prohaska ^b, Susan L. Dagenais ^c, Thomas W. Glover ^c,
Dennis J. Thiele ^{a,*}

^a Department of Biological Chemistry, The University of Michigan Medical School, Ann Arbor, MI 48109-0606, USA

^b Department of Biochemistry and Molecular Biology, School of Medicine, University of Minnesota, Duluth, MN 55812, USA

^c Departments of Pediatrics and Human Genetics, The University of Michigan Medical School, Ann Arbor, MI 48109-0618, USA

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Abstract

A polymerase chain reaction (PCR)-based strategy was used to isolate a mouse cDNA (*mCtr1*) encoding a Cu transport protein. The deduced mCtr1 protein sequence exhibits 92% identity to human Ctr1, and has structural features in common with known high affinity Cu transporters from yeast. The expression of mouse Ctr1 functionally complements baker's yeast cells defective in high affinity Cu transport. Characterization of the *mCtr1* genomic clone showed that the *mCtr1* coding sequence is encompassed within four exons and that the *mCtr1* locus maps to chromosome band 4C1–2. RNA blotting analysis demonstrated that *mCtr1* is ubiquitously expressed, with high levels in liver and kidney, and early in embryonic development. Steady state mammalian *Ctr1* mRNA levels were not changed in response to cellular Cu availability, which is distinct from the highly Cu-regulated transcription of genes encoding yeast high affinity Cu transporters. These studies provide fundamental information for further investigations on the function and regulation of Ctr1 in Cu acquisition in mammals. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Copper (Cu) is an essential cofactor for many enzymes including Cu, Zn superoxide dismutase (Cu, Zn SOD), cytochrome c oxidase, lysyl oxidase, dopamine β-monooxygenase, peptidylglycine-amidating monooxygenase and ceruloplasmin (Linder, 1991; Pena et al., 1999). Although Cu is an essential trace element, uncontrolled Cu accumulation readily facilitates the production of hydroxyl radical, which causes lipid peroxidation, oxidation of proteins and cleavage of DNA and

RNA (Halliwell and Gutteridge, 1984; Stadtman, 1992). The importance of maintaining Cu homeostasis between essential nutritional levels and toxic levels is underscored by the existence of human genetic disorders of Cu homeostasis such as Menkes syndrome and Wilson disease (Bull and Cox, 1994; DiDonato and Sarkar, 1997; Schaefer and Gitlin, 1999). The entrapment of Cu in intestinal cells and vascular endothelial cells in the blood–brain barrier in Menkes patients results in a severe deficiency in the activity of Cu-dependent enzymes that is eventually lethal. Wilson disease is characterized by excessive accumulation of Cu in the liver and brain that leads to devastating hepatic dysfunction and neurological defects. Therefore, given the critical role Cu plays in crucial biochemical reactions, and the consequences of abnormal copper homeostasis, it is important to understand the identity, mechanisms of action and regulation of cellular components responsible for the acquisition, distribution and detoxification of Cu.

Abbreviations: BAC, bacterial artificial chromosome; bp, base pair; cDNA, DNA complementary to RNA; EST, expressed sequence tag; FISH, fluorescence in situ hybridization; kb, kilobase pair; MEF, mouse embryo fibroblast; ORF, open reading frame; PCR, polymerase chain reaction.

* Corresponding author. Tel.: +1-734-763-5717;
fax: +1-734-763-7799.

E-mail address: dthiele@umich.edu (D. Thiele)

The baker's yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* have provided excellent model systems for the identification and characterization of proteins that play key roles in cellular Cu homeostasis (Eide, 1998; Labbé and Thiele, 1999; Pena et al., 1999). High affinity Cu transport in *S. cerevisiae* is mediated by two functionally redundant yet structurally distinct integral membrane proteins Ctr1 and Ctr3. Indeed, the Ctr1 and Ctr3 proteins may have fused during the course of evolution to generate the Ctr4 high affinity Cu transporter in *S. pombe* and a putative human Cu transporter, hCtr1 (Zhou and Gitschier, 1997; Labbé and Thiele, 1999; Labbé et al., 1999). As a consequence of the inability to provide Cu to the Cu-dependent high affinity Fe uptake system, to cytochrome oxidase and to Cu, Zn superoxide dismutase, yeast cells defective in high affinity Cu uptake are iron starved, unable to carry out mitochondrial respiration and sensitive to superoxide generating compounds (Dancis et al., 1994b; Labbé et al., 1999).

Important steps in understanding Cu metabolism in humans have been accomplished by the cloning of genes and through studies of the proteins defective in Wilson and Menkes diseases. The Menkes (*ATP7A*) and Wilson (*ATP7B*) disease genes encode P-type ATPases localized in trans-Golgi network (TGN) where they function to deliver Cu to proteins that traverse the secretory pathway (Bull et al., 1993; Chelly et al., 1993; Mercer et al., 1993; Tanzi et al., 1993; Vulpe et al., 1993; Yamaguchi et al., 1993). *ATP7B* expression is almost exclusive to hepatic and brain tissue, consistent with its requirement for incorporation of Cu into ceruloplasmin and for biliary excretion of Cu. The identification of mouse and rat models of Menkes and Wilson disease, and the isolation of the corresponding cDNAs and genes, have greatly facilitated comprehensive investigations of the role and mechanisms of action of ATP7A and ATP7B in Cu homeostasis.

Genes encoding additional components involved in Cu uptake and intracellular distribution in mammals have been identified due to the remarkable conservation of structure and function with yeast counterparts. Recently, a human cDNA (*hCtr1*) has been identified by functional complementation of the respiratory defect associated with *S. cerevisiae* cells with mutations that inactivate the *Ctr1* and *Ctr3* high affinity Cu transport genes (Zhou and Gitschier, 1997). Based on both functional complementation in yeast, and structural similarity to the *S. cerevisiae* and *S. pombe* high affinity Cu transport proteins, *hCtr1* has been postulated to encode a mammalian high affinity Cu transporter. Although the role and physiological importance of hCtr1 in mammalian Cu acquisition have not been demonstrated, the isolation of the murine *Ctr1* (*mCtr1*) cDNA and gene will provide important tools for biochemical and genetic investigations. We report here the isolation and charac-

terization of the *mCtr1* cDNA and gene, functional complementation in yeast, exon–intron organization and chromosomal localization of the *mCtr1* gene, tissue specific and developmental expression patterns, and steady state mRNA levels in cultured cells and rodents in response to dietary Cu deficiency or sufficiency. These studies provide fundamental information for further investigations of the function and regulation of mCtr1 in mammalian Cu acquisition.

2. Materials and methods

2.1. Yeast growth conditions

The yeast strain used in this study was MPY17 (genotype *MATa gal1 trp1-1 his3Δ200 ura3-52 ctr1::ura3::Kn^r ctr3::TRP1 his3 lys2-801 CUP1^r*) (Pena et al., 1998). Yeast cells were grown in rich medium (YPD: 1% yeast extract, 2% bactopectone, and 2% dextrose) or YPEG medium (1% yeast extract, 2% bactopectone, 2% ethanol, and 3% glycerol) in which cell growth requires functional cytochrome oxidase activity for mitochondrial respiration. Synthetic complete (SC) medium lacking specific nutrients was used for the selection and maintenance of yeast strains transformed with plasmids (Ausubel et al., 1987).

2.2. Plasmids

To express yeast *Ctr1* (*Ctr1*), human *Ctr1* (*hCtr1*), or mouse *Ctr1* (*mCtr1*) in yeast cells, polymerase chain reaction (PCR) fragments encompassing the coding regions of these genes or cDNAs were inserted into the *S. cerevisiae* expression plasmid p413-*GPD* (Mumberg et al., 1995) at the BamHI/XhoI, BamHI/XhoI, or BamHI/ EcoRI restriction enzyme sites, respectively. The sequence of all PCR products was verified by dideoxy sequencing methods. Plasmids were constructed and maintained in *Escherichia coli* DH5αF' cells with standard techniques (Ausubel et al., 1987).

2.3. Human cDNA library screen and *hCtr1* and *mCtr1* cDNA isolation

The human cDNA yeast expression library, derived from HeLa cells as described previously (Becker et al., 1991), was transformed into the MPY17 yeast strain lacking high affinity Cu transporters. Of approximately one million primary yeast transformants selected on SC-uracil medium, cells containing human cDNAs that functionally complemented the yeast Cu transport defect were identified by plating on YPEG medium. A cDNA fragment encompassing the mouse *Ctr1* coding region was amplified from a mouse brain cDNA library by PCR. Oligonucleotides for *mCtr1* PCR amplification

were designed based on mouse sequences that encode proteins having high homology with *hCtr1*.

2.4. Cloning of *mCtr1* genomic DNA

A 129/SvJ mouse genomic library (Genome Systems, St. Louis, MO) was screened with an *mCtr1* gene cDNA probe. Genomic fragments hybridizing with the *mCtr1* probe were purified, digested with BamHI, EcoRI, or SacI, and subcloned into pZErOtm-1 (Invitrogen, Carlsbad, CA). Subclones containing the *mCtr1* gene fragment were identified by Southern blotting analysis with the *mCtr1* cDNA probe.

2.5. Chromosomal localization

For chromosome preparations, mouse embryonic fibroblasts (MEF) were cultured and harvested under standard conditions. Trypsin Giemsa-banding (G-banding) and fluorescence in situ hybridization (FISH) were performed as described previously (Dagenais et al., 1999). Briefly, metaphase spreads were G-banded according to standard procedures, photographed, and destained. After air drying, slides were denatured and hybridized overnight with the biotin labeled (biotin-14-dATP) *mCtr1* BAC clone DNA, pre-annealed to mouse COT-1 (Life Technologies, Inc., Gaithersburg, MD). Signals were visualized by incubation with two layers of FITC-conjugated avidin-DCS and fluorescein conjugated anti-avidin IgG (Vector Laboratories, Burlingame, CA), and counterstained with propidium iodide (PI).

2.6. RNA blot analysis

A mouse multiple tissue RNA blot (Clontech, Palo Alto, CA) and mouse embryo tissue RNA blot (Clontech, Palo Alto, CA) were hybridized with a ³²P-labeled *mCtr1* cDNA probe. Total RNA was isolated from quick-frozen rat tissue samples using a modified guanidium thiocyanate/phenol/chloroform procedure described in detail elsewhere (Prohaska and Brokate, 1999). The RNA blots were then hybridized overnight with a purified ³²P random-primed 0.57 kb cDNA probe for *mCtr1* (1.7 ng/ml) and with a ³²P-labeled 1.2 kb probe for mouse 18S ribosomal RNA (Ambion, Austin, TX) to verify equal loading and transfer of RNA.

2.7. Experimental animals and diets

Sperm-positive Sprague–Dawley rats and male weanling Holtzman rats were purchased commercially (Harlan Sprague–Dawley, Indianapolis, IN). Rats received one of two dietary treatments, copper-deficient or copper-adequate, consisting of a Cu-deficient purified diet (Teklad Laboratories, Madison, WI) and either low

Cu drinking water or Cu-supplemented drinking water, respectively. The purified diet was formulated according to the AIN-76A diet and contained 0.44 mg Cu/kg (perinatal model) or 0.30 mg Cu/kg (postnatal model). Holtzman males, Sprague–Dawley offspring and dams on the Cu-deficient treatment drank deionized water, whereas Cu-adequate treatment groups drank water that contained 20 mg Cu/l by adding CuSO₄ to the drinking water. Rats were given free access to diet and drinking water. All animals were maintained at 24°C with 55% relative humidity on a 12 h light cycle (07:00–19:00). All protocols were approved formally by the University of Minnesota Animal Care Committee.

In the perinatal model pregnant dams were placed on the Cu-deficient treatment 7 days after they were identified as sperm-positive. Two days following parturition the litter size was adjusted to eight pups. Offspring were weaned when 21 days old, placed in stainless steel cages, and maintained on the same treatment as their respective dams for an additional 9 days. A total of 12 litters (six Cu-adequate and six Cu-deficient) were studied. Male offspring were sampled to evaluate the effects of Cu deficiency on *Ctrl* mRNA expression in brain tissue. In the postnatal model 16 male weanling Holtzman rats were divided equally and randomly assigned to either Cu-deficient or Cu-adequate treatments. Rats were maintained on their respective treatments for 4 weeks in stainless steel cages. Features of the 7 week-old Holtzman male rats were described previously (Prohaska and Brokate, 1999).

2.8. Chemical analyses of Cu concentrations

Portions of liver, cerebella, and 1 g samples of diets were wet-digested with 4 ml of concentrated HNO₃ (AR select grade, Mallinckrodt, St. Louis, MO), and the residue was brought to 4 ml with 0.1 mol/l HNO₃. Samples were then analyzed for Cu by flame atomic absorption spectroscopy (Model 2380, Perkin-Elmer, Norwalk, CT).

3. Results

3.1. Cloning of *hCtr1* and *mCtr1* cDNAs

A previous report described the isolation of a human cDNA encoding a putative Cu transport protein, *hCtr1*, identified by functional complementation of the respiratory defect in yeast cells lacking both high affinity Cu transporters (Zhou and Gitschier, 1997). Using a similar approach, we identified 26 independent human cDNA isolates which, when expressed in *ctr1Δ ctr3Δ* yeast cells (strain MPY17), complemented the respiratory defect and allowed growth on YPEG medium. Restriction enzyme mapping and DNA sequence analysis of these

cDNAs demonstrated that all encode proteins identical to that of hCtrl. To begin to understand the potential physiological importance of Ctrl in mammalian Cu acquisition, we searched the database for cDNA sequences encoding mouse proteins with high identity to hCtrl and found overlapping sequences (AI787127, AA116457, AA107389, AA250186, AA726438) from mouse expressed sequence tag (EST) databases which encode proteins with high homology to hCtrl. The mouse *mCtrl* open reading frame (ORF) was isolated by PCR from a mouse brain cDNA library. The sequence of the *mCtrl* ORF encodes a 188 amino acid protein with 92% identity with *hCtrl* (Fig. 1). Both proteins have the same predicted topological features, with three transmembrane domains and a putative hydrophilic extracellular amino-terminal domain encompassing two histidine-rich regions and two methionine-rich regions.

3.2. *mCtrl* functionally complements yeast cells defective in high affinity Cu transport

To gain insight into whether mCtrl may function in Cu acquisition, we ascertained whether expression of *mCtrl* in yeast cells defective in high affinity Cu trans-

port would restore growth on respiratory carbon sources. The *mCtrl* ORF was expressed in MPY17 cells under the control of the constitutive glyceraldehyde 3-phosphate dehydrogenase (*GPD*) gene promoter and transformants were tested for growth on YPEG. As shown in Fig. 2, similar to yeast *Ctrl*, expression of mouse or human *Ctrl* complements the respiratory growth phenotype of baker's yeast defective in high affinity Cu transport. This functional complementation of yeast Cu transporters by mCtrl and hCtrl, as well as their structural homology, strongly suggests that they are mammalian counterparts of yeast high affinity Cu transporters.

3.3. Isolation of the *mCtrl* genomic clone and analysis of exon–intron organization

The *mCtrl* genomic clone was isolated from a 129/SvJ mouse genomic library using the *mCtrl* coding region cDNA as a probe. Genomic DNA fragments which hybridized with the *mCtrl* probe were subcloned and DNA fragments encompassing *mCtrl* coding sequences were identified by Southern hybridization with the *mCtrl* cDNA coding region probe. The composite restriction map of the *mCtrl* genomic clone is

mCtrl1	MEMHHHGMNH	TTDDNITM	PPHHHPTTSASHSHGGGDS	MMMMPM	TFYFD	48
hCtrl1	MDHSHHMGMSY	MDSNSTMQPS	HHHPTTSASHSHGGGDSS	MMMMPM	TFYFG	50
	***	***	*	*	*	
mCtrl1	FKNVNLLFSG	LVIINTPGEMAGAF	VAVFLLAMFY	EGLKIA	REGLLRKSQVS	98
hCtrl1	FKNVELLLFSG	LVIINTAGEMAGAF	VAVFLLAMFY	EGLKIA	RESLLRKSQVS	100
	*	*		*		
mCtrl1	IRYNSMPVPG	PNGTILMETHKTV	GOQMLSFPHLLQ	TVLHIIQV	VISYFLM	148
hCtrl1	IRYNSMPVPG	PNGTILMETHKTV	GOQMLSFPHLLQ	TVLHIIQV	VISYFLM	150
mCtrl1	LIFMTYNGYL	LCIAVAAGAGTGY	FLFSWKKAV	VVDITE	HCH	188
hCtrl1	LIFMTYNGYL	LCIAVAAGAGTGY	FLFSWKKAV	VVDITE	HCH	190

Fig. 1. Amino acid sequences of mouse Ctrl and human Ctrl and their alignment. The predicted mouse Ctrl (mCtrl) protein contains 188 amino acids with nine methionines (M) and 10 histidines (H) in the hydrophilic amino-terminal region as potential Cu binding sites. The methionine and histidine residues are in bold, and the predicted transmembrane regions are underlined. Human Ctrl (hCtrl), which is composed of 190 amino acids, and mCtrl share 92% identity in amino acid sequence. The amino acids that are unique to hCtrl and mCtrl are marked with an asterisk.

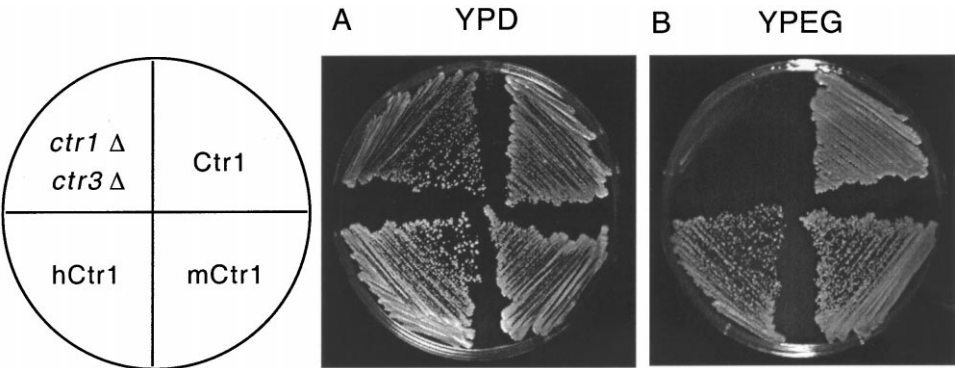


Fig. 2. Expression of mouse *Ctrl* complements the respiratory deficiency of yeast cells defective in high affinity Cu transport. (A) Yeast strain MPY17, defective in Cu transport (*ctr1Δ*, *ctr3Δ*) can grow well on the fermentable carbon source dextrose (YPD medium). (B) MPY17 fails to grow on non-fermentable carbon sources such as glycerol and ethanol (YPEG medium) due to a mitochondrial respiratory deficiency. Human *Ctrl* (*hCtrl*) and mouse *Ctrl* (*mCtrl*) expression under the control of the *GPD* gene promoter complements the respiratory growth defect of MPY17. Yeast *Ctrl* (*Ctrl*) expressed from the same promoter is used as a positive control. Plates were photographed after 3 days of growth at 30°C.

shown in Fig. 3A. PCR analysis with *mCtrl* ORF specific primers and partial sequencing showed that the *mCtrl* protein is completely encoded in four exons that are distributed over 3.3 kb of genomic DNA (Fig. 3B). Intron–exon borders were determined by comparing the *mCtrl* genomic sequence with the cDNA sequence encompassing the *mCtrl* ORF, and showed the expected GT–AG intron–exon boundary sequences.

3.4. Chromosomal localization of *mCtrl* gene

An *mCtrl* genomic clone in a Bacmid was used to determine the chromosomal localization of the *mCtrl*

gene. Fig. 4 shows the results of FISH analysis of normal metaphase chromosomes derived from mouse embryonic fibroblasts. G-banding and FISH techniques were used to localize the *mCtrl* locus to chromosome band 4C1–2. This location was based on the standard mouse ideogram and karyotype (Evans, 1996). Hybridization efficiency was >90% with no other specific hybridization signals observed for more than 30 metaphases scored. Two-color FISH with mouse BAC clone 13J21 (Research Genetics, Huntsville, AL), containing STS markers MT2058 and D4Mit225.1, was also performed to verify the mapping of *mCtrl* to chromosome 4 (data not shown). It was reported previously that *hCtrl* maps

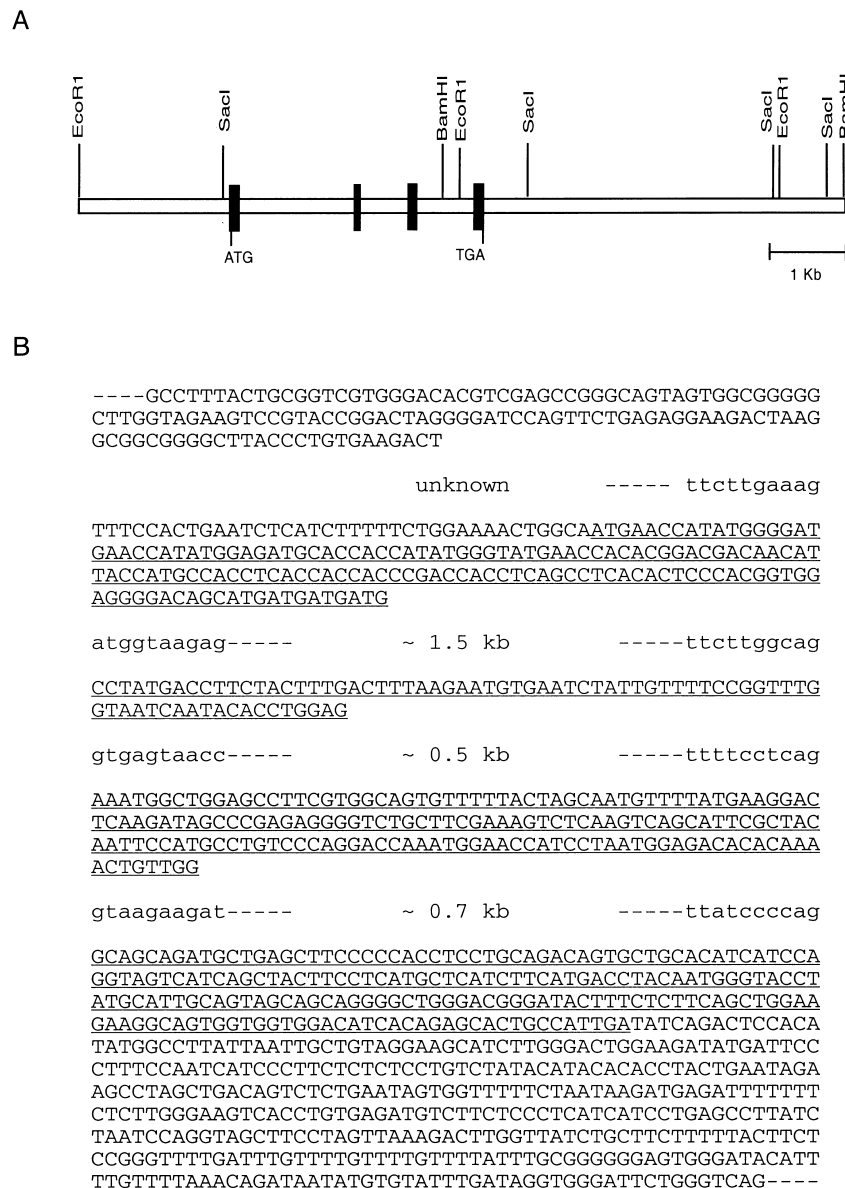


Fig. 3. Structure and exon–intron organization of the mouse *Ctrl* gene. (A) A composite restriction map of the genomic clone encompassing the mouse *Ctrl* (*mCtrl*) coding region is diagrammed. Coding exons indicated with black rectangles, restriction enzyme sites shown, and the scale indicated to the right. (B) Partial nucleotide sequence of the *mCtrl* gene is shown. Capital letters indicate exonic sequences, while lowercase letters indicate intronic sequences. The 567 bp *mCtrl* coding sequence is underlined and the length of the introns estimated in kilobase pairs.

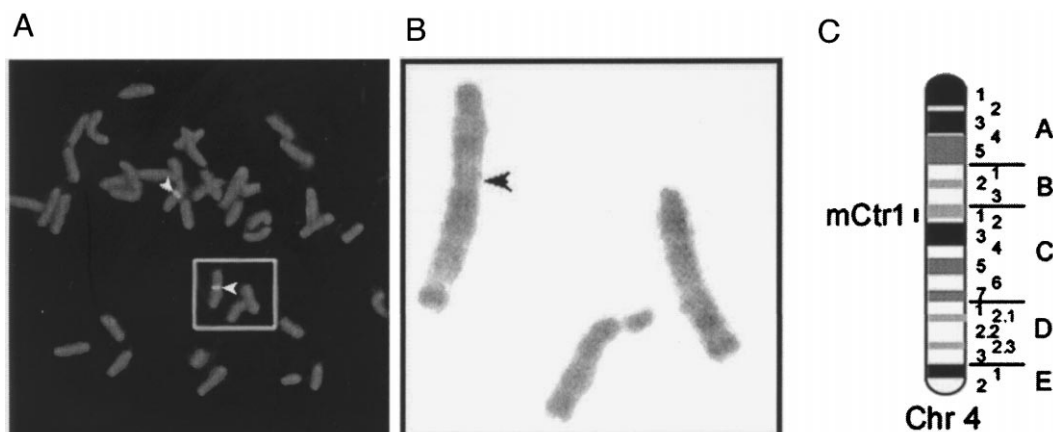


Fig. 4. Chromosomal localization of the mouse *Ctrl* gene by FISH. Hybridization of a biotin labeled mouse *Ctrl* (*mCtrl*) BAC probe (A) to mouse embryonic fibroblast (MEF) metaphase chromosomes. (B) A G-banded image of the boxed chromosomes from (A) for the same metaphase spread. An ideogram of the mapping location is depicted in (C). Arrows indicate hybridization and localization of *mCtrl* BAC clone in (A) and (B), respectively.

to 9q31–q32. Given that human chromosomal region 9q22–q34 is syntenic to mouse chromosome region 4C, these observations, and other data, support the notion that *hCtrl* and *mCtrl* represent orthologues with similar structure and function.

3.5. Tissue specific and developmental expression of *mCtrl*

If *mCtrl* functions as an important high affinity Cu transport protein, we expect that it would be expressed in many mouse tissues. Expression of *mCtrl* in specific organs and tissues was examined by RNA blotting using a mouse multiple tissue RNA blot probed with a ^{32}P -labeled cDNA fragment encompassing the *mCtrl* coding sequence and the mouse cyclophilin cDNA as a control (Fig. 5A). Although two *mCtrl* transcripts (approximately 1.8 and 5.0 kb) are detected in RNA isolated from all organs examined, quantitative analysis indicates that liver and kidney have approximately 20-fold higher levels of *mCtrl* mRNA compared with other organs (Fig. 5A). These results suggest that *mCtrl* protein may function in Cu acquisition for all tissues examined, and in particular for liver and kidney. Although previous studies have shown that *hCtrl* is expressed in many human tissues, the elevated levels of *hCtrl* mRNA in liver and kidney, relative to other human tissues, were not as pronounced as what we have observed for *mCtrl*. Furthermore, although we do not demonstrate any functional significance of the two distinct sized mRNAs detected by the *mCtrl* probe, two distinct mRNAs were also detected by the *hCtrl* coding region in human tissues (Zhou and Gitschier, 1997; Lee and Thiele, unpublished results), and by the *mCtrl* probe in rat (see below). However, only one *Ctrl* transcript is observed in yeast cells (Dancis et al., 1994b). Because we detected only one band by high stringency Southern blot analysis

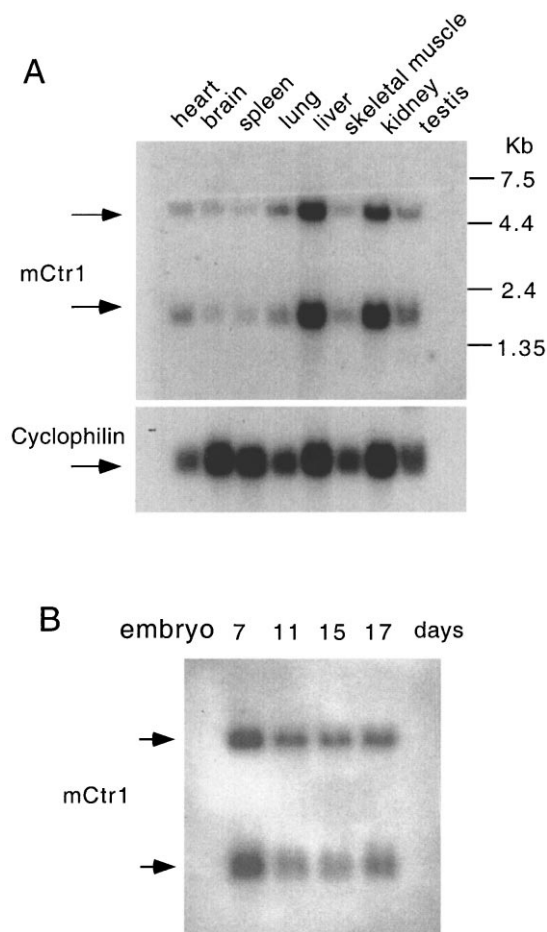


Fig. 5. Expression of mouse *Ctrl* mRNA in mouse organs, tissues and in embryos. A mouse multiple tissue RNA blot (A) and a mouse developmental blot (in days post-fertilization) (B) were hybridized with a mouse *Ctrl* (*mCtrl*) specific ^{32}P -labeled cDNA probe. Two *mCtrl* transcripts of approximately 1.8 and 5 kb are indicated with arrows. The mouse cyclophilin cDNA was used to detect the cyclophilin mRNA as a loading control.

of mouse genomic DNA with the *mCtr1* cDNA probe, it is unlikely that the mouse genome contains multiple *mCtr1* genes. These two *Ctr1* mRNA species may represent alternately spliced mRNAs or RNAs in which two distinct sites of poly A addition have been utilized, and their biological significance will require further investigation.

Because many studies have documented the critical role that Cu plays in normal mammalian growth and development (Brinster and Cross, 1972; Hurley et al., 1980; Menino et al., 1986), we have investigated the expression of *mCtr1* mRNA in developing whole mouse embryos from day 7 to day 17 post-fertilization. As shown in Fig. 5B, *mCtr1* mRNA is readily detectable from day 7 embryos through day 17, indicating very early and sustained expression in mouse development. Furthermore, we have demonstrated that *mCtr1* is strongly expressed in mouse embryonic stem cells as two mRNA species (Lee and Thiele, data not shown).

3.6. Mammalian *Ctr1* steady state mRNA levels are not changed by dietary Cu status

Previous reports have indicated that liver parenchymal cells isolated from nutritionally Cu-deficient rats or mice exhibit elevated Cu influx but decreased Cu efflux relative to Cu-sufficient control cells (Berg et al., 1991; Darwish et al., 1983). These and other results suggest that mammalian cells possess the ability to adapt to Cu availability to modulate the efficiency of Cu accumulation. Furthermore, it has been demonstrated for yeast cells that the high affinity Cu transporters are regulated at the level of transcription (Dancis et al., 1994b; Labbé et al., 1997), stability (Ooi et al., 1996), and protein trafficking as a function of environmental Cu levels (Ooi et al., 1996). To begin to investigate the molecular mechanisms underlying changes in Cu accumulation in mammals as a function of dietary Cu status, we measured steady state *Ctr1* mRNA levels in organs and tissues of Cu-deficient and Cu-adequate rats. We utilized the *mCtr1* cDNA as a probe for these experiments, given the typically high DNA sequence homology between mouse and rat genes.

Thirty-day-old male Sprague–Dawley rat offspring in the perinatal model (see Materials and methods) exhibited signs consistent with severe Cu deficiency. Compared with Cu-adequate rats, the liver Cu concentrations in Cu-deficient rats were 89% lower [$0.77 \pm 0.18 \mu\text{g/g}$ ($n=6$) in Cu-deficient rats compared with 7.06 ± 0.19 ($n=6$) in Cu-adequate rats], as determined by atomic absorption spectroscopy. Concentrations of Cu in the cerebellum were 84% lower in Cu-deficient than Cu-adequate males ($0.32 \pm 0.01 \mu\text{g/g}$ compared with 2.05 ± 0.07). The Cu-deficient 49-day-old male Holtzman rats in the postnatal model also exhibited signs consistent with severe Cu deficiency

compared with Cu-adequate males. Liver Cu concentrations were 90% lower in Cu-deficient rats than Cu-adequate rats [$0.37 \pm 0.05 \mu\text{g/g}$ ($n=8$) versus 3.71 ± 0.14 ($n=8$)]. Small intestinal Cu concentrations were not measured in these rats, but in a similar study the Cu concentration of the first 15 cm of small intestine

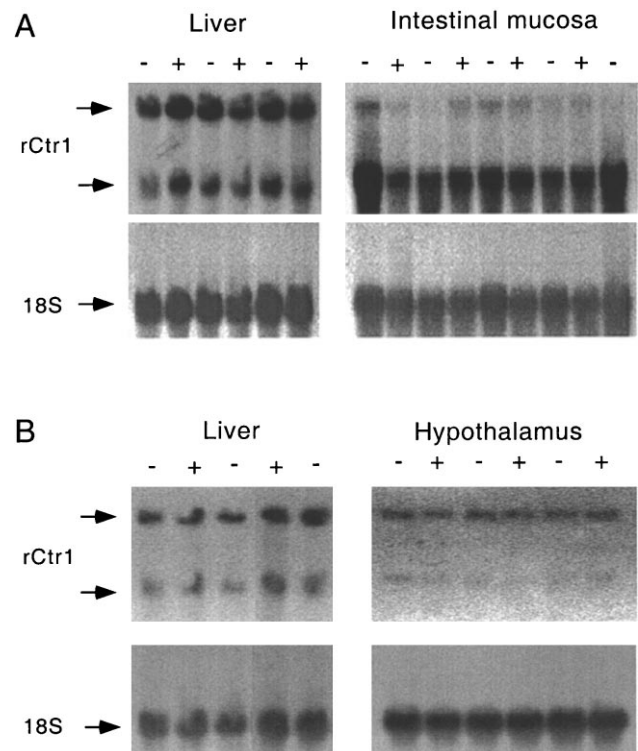


Fig. 6. Rat *Ctr1* mRNA analysis in Cu-adequate and Cu-deficient rat models. (A) Total RNA was isolated from rat liver and small intestine of 49-day-old male Holtzman rats following postnatal Cu adequacy (+) or Cu deficiency (-). Each lane contains 15 µg of total RNA and blots were probed with ^{32}P -labeled cDNA probes specific for mouse *Ctr1* and 18S ribosomal RNA. Arrows indicate the migration positions of 28S and 18S ribosomal RNA visualized with acridine orange. The rat *Ctr1* (*rCtr1*)/18S density ratio was determined and the mean $\pm$ SEM for the larger liver transcript of Cu-deficient rats (1.34 ± 0.11) was not different from the ratio for Cu-adequate rats [1.14 ± 0.07 ($P>0.05$)]. A similar outcome was detected for the smaller liver transcript, Cu-deficient 1.57 ± 0.33 compared with Cu-adequate 1.82 ± 0.11 . The *rCtr1*/18S density ratio was determined for small intestine and the mean $\pm$ SEM for the larger transcript of Cu-deficient rats (0.08 ± 0.02) was not different from the ratio for Cu-adequate rats [0.11 ± 0.01 ($P>0.05$)]. A similar finding was detected for the smaller intestinal transcript, Cu-deficient 2.68 ± 0.43 compared with Cu-adequate 1.95 ± 0.14 . (B) *rCtr1* mRNA analysis of rat liver and hypothalamus of 30-day-old male Sprague Dawley rats following perinatal Cu adequacy (+) or Cu deficiency (-). Each lane contains 15 µg of total RNA and was analyzed as described in (A). The *Ctr1*/18S density ratio was determined and the mean $\pm$ SEM for the larger liver transcript of Cu-deficient rats (0.99 ± 0.08) was not different from the ratio for Cu-adequate rats [0.92 ± 0.02 ($P>0.05$)]. A similar outcome was detected for the smaller liver transcript, Cu-deficient 0.72 ± 0.03 compared with Cu-adequate 0.86 ± 0.03 . The *rCtr1*/18S density ratio was determined for the larger transcript of hypothalamus and the mean $\pm$ SEM for the Cu-deficient rats (0.52 ± 0.07) was not different from the ratio for Cu-adequate rats [0.50 ± 0.03 ($P>0.05$)].

of Cu-deficient rats was 69% lower than that in small intestine of Cu-adequate rats. Collectively, these data suggest that the rat tissues used for RNA isolation represented two different states, Cu-adequate and Cu-deficient.

Despite the severe Cu-deficient state exhibited in the 49-day-old male Holtzman rats, there was no impact of Cu deficiency on steady state rat *Ctr1* (*rCTR1*) mRNA levels for either liver or small intestine (Fig. 6A). The abundance of the 18S rRNA signal was similar for both treatment groups. The $-Cu/+Cu$ density value (18S rRNA/*rCtr1* mRNA) for liver was 0.97, and for small intestine 1.05. The abundance of the two *rCtr1* transcripts, approximately the same sizes as those detected in both mouse (see above) and human tissues (Zhou and Gitschier, 1997; Lee and Thiele, unpublished results), appears to be tissue specific, with the larger transcript more abundant in liver and the smaller transcript more abundant in small intestine. In the 30-day-old male rat offspring, there was also no significant impact of Cu deficiency on *Ctr1* mRNA abundance in liver or hypothalamus (Fig. 6B). The 18S rRNA/ $-Cu/+Cu$ density value for liver was 0.81, and for hypothalamus 1.05. The hypothalamus mRNA level for *Ctr1* is low compared with liver (film was exposed 5 days versus 1 day for liver), and the hypothalamus was the only brain subregion in which a reliable signal was detected when compared with corpus striatum, mid-brain, and medulla oblongata/pons by RNA blotting (data not shown). Furthermore, we could not observe any changes in *hCtr1* steady state mRNA levels in the Hek 293 cell line under Cu starvation conditions using Cu chelator treatment, or in cultures supplemented with Cu (Lee and Thiele, data not shown). Therefore, results obtained in animal models and in cultured human cells suggest that there is no regulation of mammalian *Ctr1* steady state mRNA levels in response to Cu availability, however, mammalian *Ctr1* mRNA could be responsive to Cu availability in other tissues or under other conditions.

4. Discussion

Cu acquisition from dietary sources and its subsequent distribution to organs and tissues is critical to provide adequate Cu to an array of important Cu-dependent enzymes. Recent investigations have begun to shed light on the identity and mechanisms of action of proteins involved in the distribution, compartmentalization and efflux of Cu ions in yeast and mammalian systems, and clearly demonstrate remarkable conservation of the structure and function of Cu homeostasis proteins in eukaryotes. However, little is known about the identity of mammalian proteins that transport Cu inwardly across the plasma membrane, or their

mechanisms of action and regulation. Toward that goal we have isolated and characterized cDNAs and genomic DNA fragments encompassing the murine *Ctr1* coding sequence. Based on the sequence homology and predicted topological similarity with high affinity Cu transport proteins from yeast, and a putative Cu transporter from humans, mCtr1 is likely to serve as a mammalian high affinity Cu transporter. Furthermore, we demonstrate here that the expression of *mCtr1* in yeast cells is able to functionally complement the loss of yeast high affinity Cu transport systems.

The yeast high affinity Cu transporters in the Ctr1/Ctr4 class, and the putative mammalian Ctr1 high affinity Cu transporters, have very similar primary structural features. They possess three potential transmembrane domains and are rich in methionine and histidine residues within an amino-terminal hydrophilic region predicted to be extracellular. The methionine-rich motifs (Mets motifs), repeated eight times in *S. cerevisiae* Ctr1 (Dancis et al., 1994a) and five times in the *S. pombe* Ctr4 (Labbé et al., 1999) amino-terminus, are arranged as Met-X-Met-X-X-Met. Based on the ability of methionine thioether groups to serve as ligands for Cu, it is possible that these methionine-rich domains may serve as extracellular Cu binding sites important for high affinity Cu uptake for Ctr1/4 proteins. Similar motifs have been identified in CopA and CopB bacterial Cu transporters (Cha and Cooksey, 1991; Odermatt et al., 1993). However, the possibility that the Mets motifs play a direct role in Cu uptake must be experimentally tested by genetic and biochemical approaches, and by protein structure and Cu binding analyses.

RNA blotting analysis of RNA isolated from mouse tissues demonstrated that *mCtr1* is expressed in all organs examined, with particularly high levels in liver and kidney. This is consistent with a number of reports demonstrating that the liver is a central organ for Cu metabolism (Linder, 1991; Linder and Hazegh-Azam, 1996). Most Cu absorbed from dietary sources is rapidly transported into the liver, where hepatocytes transport Cu across the plasma membrane for delivery to the secretory pathway by the HAH1 Cu chaperone (Klomp et al., 1997), as well as to Cu, Zn SOD and the mitochondria by the Ccs and Cox17 Cu chaperones, respectively (Culotta et al., 1997; Amaravadi et al., 1997). Determining the subcellular localization of the mCtr1 protein in hepatocytes and in other cell types will be important for understanding the potential role of Ctr1 in Cu uptake in these and other tissues.

The presence of two distinct *Ctr1* transcripts in humans, mouse and rat suggests the possible existence of *Ctr1* isoform genes, alternatively spliced mRNAs or RNAs in which two distinct sites of poly A addition have been utilized. Given that we isolated only one genomic *Ctr1* locus in the screening of a mouse genomic library, and the single band of genomic DNA identified

by probing SacI digested mouse genomic DNA with the *mCtr1* cDNA, this suggests the presence of a single copy of the *mCtr1* gene in the mouse genome. The intensity of the two *Ctrl* transcripts appears similar in all mouse and human organs and tissues analyzed (Fig. 5A). However, in rat the smaller mRNA species is highly expressed in intestinal mucosa, but the larger species is more abundant in the hypothalamus and liver (Fig. 6). The functional significance, if any, of these two *Ctrl* mRNA species must await further investigation.

Because Cu is an essential yet toxic trace element, regulation of its uptake and distribution in response to changes in available Cu is critical to provide an adequate amount of Cu without Cu overload. It has been reported in animal studies that higher levels of Cu are absorbed after a Cu restriction period, and in yeast that genes encoding the high affinity Cu transporters are transcriptionally regulated by Cu availability (Dancis et al., 1994b; Labbé et al., 1997); increased under conditions of Cu limitation and repressed under conditions of Cu adequacy. However, we did not observe regulation of mammalian *Ctrl* mRNA levels in rats following dietary Cu restriction (Fig. 6), or in cultured cells exposed to variable Cu conditions (data not shown). If *Ctrl* is a mammalian high affinity Cu uptake protein, these results suggest other possible modes of regulation of Cu uptake. First, other regulatory mechanisms could be responsive to Cu, such as Cu-dependent degradation, as has been reported for the *S. cerevisiae* *Ctrl* protein (Ooi et al., 1996), or Cu-dependent protein trafficking mechanisms, as has been reported for the *S. cerevisiae* *Ctrl* protein and the mammalian ATP7A and ATP7B proteins (Ooi et al., 1996; Petris et al., 1996; Hung et al., 1997). Second, perhaps *Ctrl* mRNA levels are responsive to Cu availability, but only in specific cell types or under unknown conditions. Third, it is possible that the regulation of Cu uptake in response to Cu availability occurs via mechanisms that are *Ctrl*-independent. Studies are currently underway to identify how Cu levels may regulate *Ctrl* function, expression, or localization.

The *mCtr1* coding region is encompassed within four exons. The exon containing the translation initiation site does not contain all of the *mCtrl* EST sequences available in the database, indicating that the *mCtr1* gene has at least one 5' non-coding exon. The identification of this exon, as well as the localization of the *mCtr1* promoter and transcriptional regulatory elements, is currently underway. *mCtr1* mRNA is detectable in embryos 7 days post-fertilization and in embryonic stem cells (Lee and Thiele, data not shown), suggesting the possibility that *mCtrl* may play an important role at a very early stage of embryonic development. The generation of a mouse model with a targeted deletion of the *mCtr1* gene will allow genetic and biochemical studies of the physiological role of *Ctrl* in mammalian Cu acquisition.

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