Identification and Functional Expression of HAHI, a Novel Human Gene Involved in Copper Homeostasis*

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To search for a mammalian homologue of ATX1, a human liver cDNA library was screened and a cDNA clone was isolated, which encodes a protein with 47% amino acid identity to Atx1p including conservation of the MTCXGC copper-binding domain. RNA blot analysis using this cDNA identified an abundant 0.5-kilobase mRNA in all human tissues and cell lines examined. Southern blot analysis using this same clone indicated that the corresponding gene exists as a single copy in the haploid genome, and chromosomal localization by fluorescence in situ hybridization detected this locus at the interface between bands 5q32 and 5q33. Yeast strains lacking copper/zinc superoxide dismutase (SOD1) are sensitive to redox cycling agents and dioxygen and are auxotrophic for lysine when grown in air, and expression of this human ATX1 homologue (HAHI) in these strains restored growth on lysine-deficient media. Yeast strains lacking ATX1 are deficient in high affinity iron uptake and expression of IAHI in these strains permits growth on iron-depleted media and results in restoration of copper incorporation into newly synthesized Fet3p. These results identify HAHI as a novel ubiquitously expressed protein, which may play an essential role in antioxidant defense and copper homeostasis in humans.

The biological activation of dioxygen by copper is essential for the survival of all living organisms (1). In humans, copper functions in oxidative catalysis by permitting facile electron transfer reactions in a number of enzymes, which play a critical role in the biochemistry of iron homeostasis, cellular respiration, antioxidant defense, neurotransmitter biosynthesis, connective tissue formation, pigment production, and endocrine organ regulation (2). Despite the essential requirement for copper in these cellular processes, excess copper is highly toxic, and therefore cells have evolved highly specialized systems for the transport and compartmentalization of this transition metal (3).

Insight into the mechanisms of cellular copper homeostasis in humans has come from the molecular characterization of two inherited disorders of copper metabolism, Wilson disease and Menkes disease. Although patients affected with these diseases have markedly different clinical phenotypes due to either copper overload or deficiency, each disorder is due to the absence or dysfunction of a homologous member of the cation transport P-type ATPase family (4–9). These proteins reside in the trans-Golgi network of cells and transfer copper into the secretory pathway for subsequent export or incorporation into newly synthesized cuproproteins (10). Most recently, a homologous ATPase, Ccc2p, was identified in Saccharomyces cerevisiae and shown to play a central role along with the multicopper oxidase Fet3p in copper and iron metabolism in yeast (11–13). The subsequent elucidation of the essential role of the Fet3p homologue ceruleoplasmin in human iron homeostasis (14, 15) has revealed a remarkable evolutionary conservation of the mechanisms of cellular copper and iron metabolism.

Little is currently known about the pathways and mechanisms of copper trafficking in mammalian cells. ATX1 encodes a cytosolic copper-binding protein in S. cerevisiae, which is a multicyclic suppressor of sod1Δ mutants functioning to protect cells from toxicity in a copper-dependent manner (16). Yeast strains deficient in ATX1 are impaired in high affinity iron uptake, and this defect is presumably due to a failure of copper delivery to Ccc2p for subsequent transport into the secretory pathway and incorporation into Fet3p (17). As the structure and function of Ccc2p and Fet3p are highly conserved between yeast and humans, this current study was undertaken to search for a homologue of Atx1p and to elucidate any functional role for such a protein in antioxidant defense and copper homeostasis.

EXPERIMENTAL PROCEDURES

Materials—General chemicals and reagents were purchased from Sigma. DNA restriction and modifying enzymes were purchased from Promega Corp. and used according to manufacturer’s specifications. [32P]CTP and [32P]dCTP were purchased from ICN Radiochemicals. Hybridization membranes were obtained from Amersham Corp. 64Cu (600 Ci/mmol) was obtained by fast neutron bombardment of a natural zinc target as described previously (18). Yeast strains used in this study were as follows: KS107, MATα leu2-3, 112 his3Δ1 trp-289a uro3-52 GAL sod1Δ::TRP1 (19); SL215, MATα ura3-52 lys2-801 ade2-101 trpΔ−1 his3Δ−200 leu2Δ−1 atx1Δ::LEU2 (17); strain 7 (wt), MATa his3−200 leu2 trp1−101 uro3−52 ade6−15 (17); and strain 8 (ccc2Δ), MATa his3−200 leu2 trp1−101 ura3−52 ade6−15 ccc2Δ::LEU2 (13).

cDNA and Genomic Library Screening—A GenBank search for sequences homologous to yeast ATX1 identified a partial cDNA sequence (accession no. F14674) from a porcine small intestine cDNA library. To identify a human homologue of ATX1, 250,000 recombinant clones from a human liver Agt11 cDNA library were transferred to nitrocellulose membranes and hybridized utilizing a 32P-labeled cDNA probe derived from this porcine clone. Hybridized filters were washed as described previously, and positive clones were purified by subsequent rounds of screening (7). Phage DNA was isolated from positive clones by liquid

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) U70660.

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lysis, and inserts were analyzed by agarose gel electrophoresis following restriction digestion with EcoRI (20). The largest size inserts were subcloned and the nucleotide sequence determined by dyeoxy chain termination (21). To isolate genomic clones encoding HAH1, a \(5^\prime\)-labeled full-length HAH1 cDNA was amplified by polymerase chain reaction using degenerate oligonucleotides corresponding to the conserved amino- and carboxyl-terminal domains of \(\alpha\)-HAH1 (18, 19) and directionally subcloned into the EcoRI and BamHI sites of pSM703. The resulting construct pHAH703 was verified by nucleotide sequencing and transformed into KS107 (sodIΔ) and SL215 (atx1Δ) strains as described (17). The empty CEN pRS413 (25) and 2 \(\mu\) pRS-A1 plasmids containing a functional copy of the \(\alpha\)-HAH1 gene were then transformed into these same strains (16, 17). To test for reversal of lysine auxotrophy, cells were grown in air on SD1 plates with or without lysine and then transformed with or without lysine for 3 days at 30 °C (26). Iron-dependent growth was determined on SD complete media buffered with 50 \(\mu\)M Na-MES, pH 6.1, and 3 \(\mu\)M ferrozine with or without 350 \(\mu\)M ferrous ammonium sulfate for 5 days at 30 °C (26).

**HoloFet3p Biosynthesis**—To examine the biosynthesis of holoFet3p (copper incorporation into Fet3p, saturated cultures were used to inoculate 100 ml of yeast nitrogen base medium without copper, iron, and dextrose, supplemented with amino acids, 2\% glucose, 50 mM dextrose, supplemented with amino acids, 2\% glucose, 50 mM Na-MES, pH 6.1, and 100 \(\mu\)M ferrozine. All stock solutions were treated with Chelex-100. After growth to \(A_{600}\) of 0.4, cells were labeled for 2 h with 2.5 \(\mu\)Ci/ml \(\text{Cu}^{64}\). Cells were washed extensively in 150 mM NaCl, 25 mM Tris-HCl pH 7.5, and an aliquot of cells was used to determine cell-associated radioactivity. Cells were lysed by vigorous shaking with glass beads for 1 h, and protein extracts of crude membrane fractions were prepared as described (33). Samples (100 \(\mu\)g) were analyzed on 7.5\% nonreducing SDS-PAGE without heat denaturation followed by direct autoradiography of the gels as previous studies revealed that copper is retained in multicopper oxidases under these conditions (27). For Western blot analysis of Fet3p, crude membrane extracts were separated by SDS-PAGE and transferred to nitrocellulose by semidy transfer. Following blocking in 5\% nonfat dry milk, 0.1\% Tween 20, 0.02\% Nonidet P-40 in phosphate-buffered saline, membranes were incubated for 1 h with a 1:1000 dilution of an anti-peptide antiserum specific for Fet3p. Membranes were subsequently washed in phosphate-buffered saline containing 0.1\% Tween 20 and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody, and the antibody-antigen complex was detected using enhanced chemiluminescence (Amersham).

**RESULTS**

To search for a mammalian Atx1p homologue, an \(\text{ATX1}\) genomic DNA fragment was used to screen a human liver cDNA library by colony hybridization at low stringency and by polymerase chain reaction using degenerate oligonucleotides corresponding to the conserved amino- and carboxyl-terminal domains of \(\text{ATX1}\) (Fig. 1B). This approach did not result in isolation of full-length cDNA clones, and thus a partial porcine cDNA probe with homology to the 3’ region of \(\text{ATX1}\) was used to rescreen this human liver cDNA library. Seven clones were isolated by this method, and the largest size insert was characterized by nucleotide sequencing. This clone, termed \(\text{HAH1}\) (human \(\text{ATX}\) homologue 1), encompassed 113 bp of 5′ untranslated region, a 204-bp open reading frame, and 185 bp of 3′ untranslated sequence (GenBank accession no. U70660) (Fig. 1A). A polyadenylation consensus sequence was identified 18 bp from the poly(A) tail. The open reading frame of \(\text{HAH1}\) encoded a protein of 68 amino acids with 47% identity and 58% similarity to Atx1p including conservation of both the copper-binding domain (MTCXGC) and the carboxyl-terminal lysine rich region (****) indicated.

![Image](https://example.com/image.png)

**FIG. 1.** A, nucleotide sequence of \(\text{HAH1}\) cDNA clone and derived amino acid sequence of \(\text{HAH1}\) (nucleotides 114–318); polyadenylation consensus sequence is underlined. **B.** derived amino acid sequence of \(\text{HAH1}\) (H. sapiens) aligned with homologues identified in \(\text{S. cerevisiae}\) (\(\text{ATX1}\), \(\text{S. cerevisiae}\)), \(\text{Caenorhabditis elegans}\) (\(\text{C. elegans}\)), and \(\text{Arabidopsis thaliana}\) (\(\text{A. thaliana}\)). Amino acids identical to \(\text{HAH1}\) are shown in bold. The copper-binding motif (MTCXGC) and the carboxyl-terminal lysine rich region (****) are indicated.
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Fig. 2. Expression of the HAH1 gene in human tissues and cell lines. RNA blot analysis of poly(A+)
RNA from human tissues (A), regions of the central nervous system (B), and human tissue-derived cell lines (C). 2 μg of poly(A+)
RNA (A and B) or 15 μg of total RNA (C) were subjected to electrophoresis, transferred to membranes, and hy-
bridized with a 32P-labeled HAH1-specific cDNA probe as described under “Experimental Procedures.” Blots were exposed to film for 1 h at 70 °C.

2C). Although the abundance of HAH1 mRNA varied slightly in these different cell lines, all samples contained the HAH1 gene, and no change in abundance was observed with alteration of the intracellular copper content of these cells (data not shown).

To characterize the gene encoding HAH1, the full-length cDNA clone was used as a probe to analyze human genomic DNA by Southern blot analysis. As shown in Fig. 3, hybridization of restriction endonuclease-digested genomic DNA revealed a simple pattern consistent with the presence of single copy gene in the haploid genome. Three independent clones encompassing the HAH1 gene were then isolated from a genomic library and characterized using Southern blot analysis and sequencing. The largest of these clones was used to deter-
mine the chromosomal localization of the HAH1 gene. Fig. 4A shows fluorescent in situ hybridization analysis of normal metaphase chromosomes derived from peripheral blood lymphocytes utilizing this 15-kilobase genomic fragment as a probe. Consistent with the Southern blot experiments, this analysis revealed specific labeling of a single locus on the distal long arm of chromosome 5 in a total of 57 metaphase cells. Cohy-
bridization of the HAH1 probe with a probe that precisely mapped to 5q21 resulted in double-labeling of the long arm of chromosome 5. Southern blot analysis of DNA from human hamster cell hybrids confirmed this localization to chromosome 5 (data not shown). Measurements of 10 specifically labeled chromosome 5 preparations demonstrated that the HAH1 gene is located at a position that is 75% of the distance from the centromere to the telomere at an area that corresponds to the interface between bands 5q32 and 5p33 (Fig. 4B).

The ATX1 gene was isolated by virtue of its ability to reverse the aerobic lysine auxotrophy observed in sod1Δ strains. To ascertain the function of HAH1, the coding region of the HAH1 cDNA was placed under control of the S. cerevisiae phosphoglycerate kinase promoter and introduced into the sod1Δ mutant strain. This strain was subsequently analyzed for its ability to grow aerobically on plates without lysine. As can be seen in Fig. 5A, yeast strains transformed with either ATX1 or HAH1 were able to suppress the lysine auxotrophy of a sod1Δ mutant strain. Since complementation in these experiments is accomplished by over expression of these proteins, the slight differ-
ence in growth rate between the ATX1 and the HAH1 transformants (Fig. 5A, b versus c) may reflect a difference in the amount of protein expressed under these circumstances.

As revealed by studies in an atx1Δ null strain, Atx1p is essential for efficient high affinity iron uptake in S. cerevisiae (17). As shown in Fig. 5B, this atx1Δ strain is dependent upon high concentrations of exogenous iron for growth, but will grow on iron-depleted media after reintroduction of the ATX1 gene. Transformation of the atx1Δ strain with HAH1 also suppressed this iron-dependent phenotype, as evidenced by support of growth on low iron medium (Fig. 5B, c). Cells transformed with vector alone failed to grow under these conditions (Fig. 5B, b and d), indicating that the observed effect is specific for HAH1. Further studies with an HAH1 antibody will be important to ascertain the amount of HAH1 produced under these circumstances.

The role of copper in high affinity iron uptake in S. cerevisiae has been shown to be due to the plasma membrane multicopper oxidase Fet3p (11, 12), and the iron deficiency in the atx1Δ strain therefore presumably results from a failure of copper incorporation into Fet3p. To directly test this hypothesis and to obtain functional data on the role of HAH1 in copper metabolism, yeast strains were cultured in copper-free medium, pulse-
labeled with 64Cu, and then newly synthesized holoFet3p was detected in membrane fractions. Determination of radioactivity in aliquots of the cell suspensions indicated that the amount of 64Cu uptake by each strain was identical (data not shown). When membrane fractions were analyzed in this fashion, a protein with the appropriate molecular mass of Fet3p was detected in wild type (Fig. 6A, lane 1) but not in a ccc2Δ mutant.
strain (Fig. 6A, lane 2) previously shown to have Fet3p oxidase activity (13). Consistent with the partial iron deficiency observed in atx1Δ null mutants (17), a marked reduction, but not complete impairment of copper incorporation into Fet3p was observed in the atx1Δ strains transformed with either the pRS413 or pSM703 vectors alone (Fig. 6A, lanes 3 and 5). However, radiolabeled Fet3p equivalent in amount to that observed in the wild type strain was detected in the atx1Δ mutant following introduction of ATX1 containing p413-A1 (a and e), control vector pRS413 (b and f), HAH1 containing pHAH703 (c and g), or control vector pSM703 (d and h).

**Fig. 4.** A, metaphase chromosomes hybridized with a dUTP-labeled HAH1 genomic clone, followed by staining with anti-digoxigenin antibody. Arrows indicate HAH1 locus on chromosome 5. B, idiogram of HAH1 location of chromosome 5 based on multiple probe analysis as described under “Experimental Procedures.”

**Fig. 5.** A, complementation of aerobic lysine auxotrophy in sod1Δ strain KS107 grown on lysine-deficient (a–c) or SD complete media containing lysine (d–f) following transformation with pHAH-703 (b and e) or pRS-A1 (c and f). B, complementation of iron-deficient growth in atx1Δ strain SL215 grown on 3 μM ferrozine (a–d) or 3 μM ferrozine plus 350 μM ferrous ammonium sulfate (e–h) following transformation with ATX1 containing p413-A1 (a and e), control vector pRS413 (b and f), HAH1 containing pHAH703 (c and g), or control vector pSM703 (d and h).

**Fig. 6.** A, biosynthesis of holoFet3p in wild type (lane 1), ccc2Δ null mutant (lane 2), and atx1Δ null mutant (lanes 3–6) S. cerevisiae strains. atx1Δ strains were transformed with the indicated control vectors (lanes 3 and 5), p413-A1 (Atx1) (lane 4), or pHAH-703 (HAH1) (lane 6) prior to analysis as described under “Experimental Procedures.” To identify holoFet3p, cells were pulse-labeled with 64Cu, and membrane fractions analyzed by nonreducing SDS-PAGE, followed by copper autoradiography of gels as described under “Experimental Procedures.” 64Cu-labeled Fet3p is indicated by the arrow. B, Western blot analysis of Fet3p in atx1Δ strains transformed with control vectors (lanes 1 and 3), ATX1 (lane 2), or HAH1 (lane 4). Identical membrane fractions as in A were electrophoresed in 7.5% SDS-PAGE, transferred to nitrocellulose, incubated with a polyclonal antiserum to Fet3p, and developed by chemiluminescence as described under “Experimental Procedures.”

The sequence data reported in this study suggest that HAH1 is a human homologue of the S. cerevisiae copper-binding pro-
tein Atx1p. The considerable amino acid identity between these two proteins, including conservation of the MTCGXC copper-binding motif, as well as the occurrence of homologous sequences as open reading frames in the DNA of other diverse euukaryotic organisms, supports this concept and suggests a remarkable evolutionary conservation of the structure of this protein moiety similar to what has been observed for other proteins involved in copper homeostasis (2). Perhaps most importantly, the expression of HAH1 in sod1Δ and atx1Δ null yeast strains reconstitutes the defects in antioxidant defense and iron homeostasis in these mutants, indicating that the HAH1 open reading frame encodes a functional protein.

RNA blot analysis of HAH1 expression identified a single 0.5-kilobase transcript in all tissues and cell lines examined, with no evidence of qualitative differences in gene expression among tissues. These data are consistent with the size of the isolated full-length cDNA (Fig. 1A) and the structure of the HAH1 gene identified from sequencing of genomic clones (data not shown). This analysis also revealed that HAH1 is abundantly expressed in each of these tissues in amounts equivalent to that observed for copper/zinc superoxide dismutase when this transcript was similarly analyzed (data not shown).

Although no HAH1 protein expression data are yet available, the observation that HAH1 is ubiquitously and abundantly expressed supports the concept that this protein may play an important and previously unappreciated role in cellular copper homeostasis. Consistent with this concept, recent experiments in transgenic mice reveal that neither metallothionein I and II nor copper/zinc superoxide dismutase is essential for cellular copper metabolism under normal circumstances, suggesting that additional proteins must be present to fulfill this function (28–30).

Based upon the complementation of atx1Δ null strains by HAH1 and the recently defined role of Atx1p in iron metabolism in S. cerevisiae (17) it is reasonable to assume that HAH1 functions in an analogous fashion in mammalian cells. In this context, HAH1 would be responsible for the delivery of cytosolic copper to the Menkes and Wilson disease proteins in the trans-Golgi network prior to transport of this metal by these ATPases into the secretory pathway. This model is consistent with the known intracellular localization of these copper transport ATPases in mammalian cells (10), as well as the clearly defined role of the Wilson disease ATPase in providing copper for the biosynthesis of the Fet3p multicopper oxidase homologue ceruloplasmin. Although such a model is consistent with the data on Fet3p biosynthesis (Fig. 6), further understanding and confirmation of this pathway must await the development of HAH1 antibodies to define the expression and localization of this protein within the cell. The overall concept of specific proteins essential for copper trafficking within cells is also supported by recent studies in S. cerevisiae, which indicate the existence of a distinct set of intracellular proteins necessary for copper delivery to mitochondrial cytochrome c oxidase (31, 32).

In the model of cytosolic copper delivery described above inherited abnormalities in HAH1 might be sufficient to interrupt the normal traffic of copper to the secretory pathway. As such, HAH1 serves as a likely gene candidate for the genetic disorders of hepatic copper metabolism known as Indian childhood cirrhosis or idiopathic copper toxicosis, where an increase in dietary copper appears to result in cytosolic copper accumulation in genetically susceptible individuals (33–35). Although the genetic loci for such disorders have not yet been mapped, the identification of the chromosomal location of HAH1 may facilitate genetic analysis for inherited abnormalities in HAH1.

In addition to, and independent of, its role in iron homeostasis, Atx1p functions as an antioxidant protecting yeast from the toxic effects of superoxide and hydrogen peroxide (16). As Atx1p itself does not appear to have antioxidant properties, the most likely explanation for these observations is that Atx1p and HAH1 deliver copper to an as yet uncharacterized protein, which functions in cellular antioxidant defense. This hypothesis would be consistent with the observed role for Atx1p and HAH1 in the production of newly synthesized copper proteins in addition to Fet3p that are detected in wild type but not atx1Δ null strains following metabolic labeling with 64Cu (data not shown). These findings suggest a potential role for HAH1 or additional copper proteins dependent upon HAH1 in the antioxidant defense of mammalian cells. Given recent observations on the role of gain-of-function mutations in copper/zinc superoxide dismutase in patients with familial amyotrophic lateral sclerosis and data that implicate hydrogen peroxide in the pathogenesis of the neuronal degeneration resulting from these mutant enzymes, HAH1 may be a likely gene candidate for loci in other affected families as well as a potential therapeutic target for the responses to such injury (36–38).

Collectively, the data presented here suggest that HAH1 is a novel human copper-binding protein, which is abundantly and ubiquitously expressed and which functions in copper homeostasis and antioxidant defense. Although the pathways and proteins involved in copper trafficking in mammalian cells remain largely unknown, the results of this study suggest that the marked evolutionary conservation of these pathways will permit utilization of the techniques of yeast genetics to identify and characterize such proteins. Given the increasing recognition of the role of both copper and iron in inherited and acquired neurodegenerative diseases, as well as the known importance of host antioxidant defenses in human disease, such an approach is likely to continue to contribute new information to this area of biology (39, 40).

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