

SHORT COMMUNICATION

Structure, Expression, and Chromosomal Localization of the Mouse *Atox1* Gene

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Copper trafficking in eukaryotes involves small proteins termed metallochaperones, which mediate copper delivery to specific intracellular sites. Previous studies in yeast and human cell lines have suggested that *Atox1* plays a critical role in copper delivery to the secretory pathway. In the present study, a mouse *Atox1* (*mAtox1*) cDNA was cloned and shown to encode an open reading frame with 85% amino acid identity to human *Atox1*. RNA blot analysis revealed that *mAtox1* was expressed as a single transcript in multiple tissues, and immunoblotting indicated that the relative abundance of *mAtox1* mRNA directly correlated with *mAtox1* protein. Analysis of the *mAtox1* gene locus revealed a genomic structure with four exons encompassing a total of 14.5 kb. RFLP and haplotype analyses indicated that the *mAtox1* locus was tightly linked to the *Trhr* and *D15Bir7* loci on mouse chromosome 15. Taken together, these data reveal marked evolutionary conservation of *Atox1* structure and provide a genomic organization and localization that will aid in the genetic deciphering of the molecular role of this protein in copper homeostasis. © 2000 Academic Press

Copper chaperones belong to a class of metalloproteins that deliver copper to specific intracellular targets. Copper is an essential micronutrient for all aerobic organisms (8), and a role for this metal in mammalian biology is underscored in genetic disorders of copper metabolism, e.g., Menkes and Wilson disease (13). Because copper is reactive with cellular macromolecules and therefore toxic to cells (3), there is little

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or no free intracellular copper (11). Within cells, copper is bound by specific proteins called chaperones that were first identified in the yeast *Saccharomyces cerevisiae*, and homologues have been identified in other species (14).

The yeast *Atx1p* encodes a small cytosolic protein that binds to copper via the copper-binding MXCXXC motif and delivers this metal to the secretory pathway for incorporation into the multicopper oxidase, Fet3Pp (6, 10). Human *Atox1* (previously termed HAH1) is the human homologue of *Atx1p* and has been shown to complement functionally the *atx1Δ* mutant strain (5). The cysteines in the MXCXXC motif of human *Atox1* are essential for copper binding and for human *Atox1* interaction with the Menkes and Wilson copper transporting P-type ATPases in the *trans*-Golgi network of cells (2, 4).

In an effort to understand the physiological function of *Atox1* in mammalian copper trafficking, the mouse homologue was identified. A BLAST search (1) with human *Atox1* sequence revealed 72.34% identity with a mouse EST (Accession No. W49206). Based on the DNA sequence of this EST, 5' and 3' primers were generated, and an adult BALB/c mouse lung library (Clontech) was used to PCR-amplify and clone a 432-bp DNA fragment. Dideoxy sequencing confirmed that this clone was identical to the EST and was named *mAtox1* (HUGO Nomenclature Committee). *mAtox1* encoded a 68-amino-acid protein that was 85.3% identical to human *Atox1* (Fig. 1A). The *Atx1p* crystal structure reveals a hydrophobic core made up of residues that may be essential for maintaining the protein's overall three-dimensional structure (12). All of those residues, as well as the N-terminal metal-binding motif, are conserved in *mAtox1* (Fig. 1A), suggesting that *mAtox1* may play an analogous role in copper binding and trafficking to the secretory pathway. To determine the tissue-specific distribution of *mAtox1* in mice, RNA blot analysis was performed using the cDNA as the radiolabeled probe. A single 0.6-kb tran-

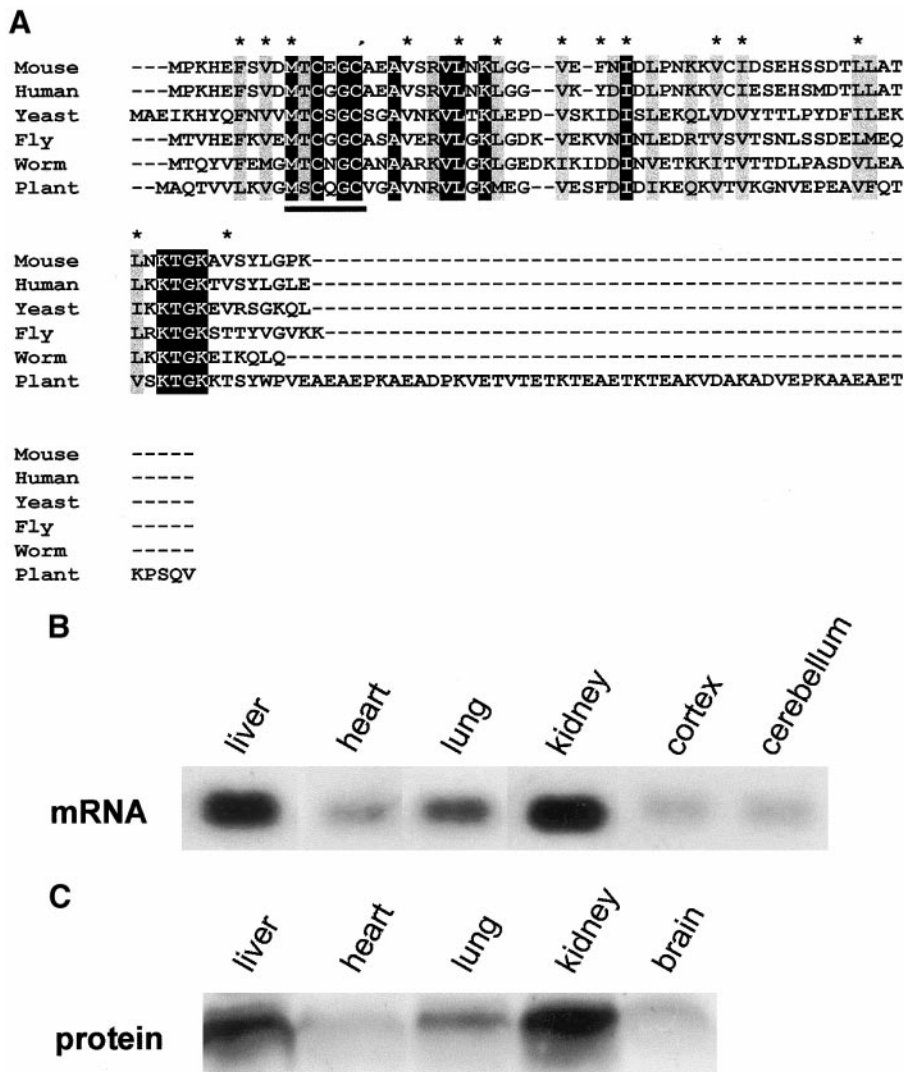


FIG. 1. (A) Multiple sequence alignment of the copper chaperone Atox1 with that of the human (U70660), yeast (*S. cerevisiae*) (L23520), fly (*D. melanogaster*) (AA392844), worm (*C. elegans*) (AB017201), and plant (*A. thaliana*) (U88711) protein sequence using the ClustalW (v. 1.7) program. Accession numbers are given in parentheses. Identical amino acids and conservative changes are indicated by reversed and shaded characters, respectively. Asterisks indicate residues comprising the hydrophobic core, and the horizontal bar denotes the metal-binding MXCXXC motif. (B) Expression of *Atox1* mRNA in tissues. RNA was prepared from mouse tissue using standard protocols. 15 μ g of total RNA was loaded per sample and subjected to Northern blot analysis using the *Atox1* cDNA as a probe. (C) Immunoblot analysis of Atox1. Tissue protein was prepared from mice by dounce homogenization, and protein was estimated by Bradford's assay. 85 μ g of postnuclear supernatant was separated on a 4–20% SDS-PAGE gradient gel, transferred to nitrocellulose, and examined using HAH1 antiserum and chemiluminescence.

script was revealed in peripheral tissues, in the central nervous system (Fig. 1B), and in cell lines (data not shown). *mAtox1* mRNA expression accumulated to varying levels in different tissues; the transcripts were most abundant in the kidney and liver. Immunoblot analysis with human Atox1 antisera detected in mouse organs a single polypeptide that correlated with the transcript abundance in those tissues (Fig. 1C).

To determine the organization of *Atox1* in the mouse genome, a radiolabeled cDNA probe was used to screen a mouse SVJ129 genomic DNA library in the bacteriophage Lambda FIX II (Stratagene). Two phage clones that were positive for probes generated from the 5' and 3' ends of the cDNA were isolated, and Southern blot analysis revealed 14- and 18-kb inserts, respectively.

Using the phage clones as template, PCR amplification was performed with primers flanking the putative intron-exon splice junctions to map the exact intronic locations in *mAtox1* (Fig. 2A). Four fragments were identified, isolated, subcloned, and sequenced to define the location of the splice junctions. Restriction mapping and Southern blot analyses of each clone revealed that *mAtox1* is organized into four exons interspersed by a total of 14.5 kb of intronic DNA (Fig. 2A). This structure was confirmed by PCR amplification of SVJ129 mouse genomic DNA as well as by Southern blot analysis (data not shown).

To determine the chromosomal location of *mAtox1*, RFLP analysis was performed. Genomic DNA from C57BL/6J and *M. spretus* was digested with eight



FIG. 2. Mapping of the *Atox1* locus. (A) Structural organization of *Atox1* gene based on genomic DNA and phage clones isolated from a SVJ129 mouse strain. Introns and restriction sites are depicted as horizontal and vertical lines, respectively. Exons are not drawn to scale, and shaded portions within exons indicate the open reading frames. Arrows indicate primers generated for PCR, based on *Atox1* cDNA, to map the intron-exon junctions. (B) Southern blot RFLP analysis of *Atox1*. C57BL/6J (B) and *M. spretus* (S) genomic DNAs were digested with *Bam*HI. Probing with a 430-bp *Atox1* cDNA revealed a RFLP with bands of 6.1 kb for C57BL/6J and 14 kb for *M. spretus*. (C) Haplotype analysis of Chr. 15 genetic markers in the (C57BL/6J x *M. spretus*)F₁ x *M. spretus* type backcross mice (BSS type) showing linkage and relative position of *Atox1*. Black boxes indicate the inheritance of the C57BL/6J (B) allele, and white boxes indicate the inheritance of the *M. spretus* (S) allele from the (C57BL/6J x *M. spretus*)F₁ parent. The first two columns indicate the number of backcross progeny with no recombinants. The following columns show the recombinational events between adjacent loci (signified by the change from white to black box). The number of recombinants is listed below each column, and the frequency of each recombination between adjacent loci is indicated.

different restriction enzymes, and high-stringency Southern blots performed with a cDNA probe identified a RFLP for *Bam*HI of 6.1- and 14-kb fragments for C57BL/6J and *M. spretus*, respectively (Fig. 2B). Ninety six genomic DNAs from C57BL/6J, *M. spretus*, and the (C57BL/6J x *M. spretus*)F₁ x *M. spretus* type backcross DNA panel (BSS; The Jackson Laboratory) were digested with *Bam*HI and probed with the *Atox1* cDNA. Segregation of the *mAtox1* alleles was compared to the other loci from the mouse genome database by The Jackson Laboratory backcross DNA map service.

Haplotype analysis indicated the mapping of the *Atox1* locus to mouse chromosome 15 (Fig. 2C). Perfect cosegregation with *D15Wsu126e* (DNA segment, Chr. 15, Wayne State University 126, expressed) on chromosome 15 was observed. No recombinants were found between *mAtox1* and *D15Wsu126e*, indicating a distance of less than 1 cM between the two loci. The *mAtox1* locus also mapped between the *Trhr* (thyrotropin-releasing hormone receptor) and the *D15bir7* (Birkenmeier anonymous DNA fragment 7) loci. Map distances including standard errors were determined

and are as follows: Trhr- 2.1 ± 1.5 cM-Atox1- 4.3 ± 2.9 cM-D15Bir7. Thus, *mAtox1* maps to a region on Chr. 15 that is distal to a region of homology to human chromosome 5, the location of human *Atox1* (5).

Taken together, the results of these studies indicate evolutionary conservation of the sequence of mAtox1 with preservation of all amino acid residues essential for copper binding and function in the yeast (9) and human homologues (4). Given recent data indicating that the interaction of human Atox1 with the Wilson disease ATPase is essential for hepatic copper homeostasis in humans (2), as well as studies demonstrating region specific expression of *mAtox1* in the adult brain (7), the chromosomal localization, genomic structure, and expression data presented here should permit further elucidation of the role of this chaperone in copper metabolism and human diseases.

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