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Genomic organization of ATOX1, a human copper chaperone

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Abstract

Background: Copper is an essential trace element that plays a critical role in the survival of all living organisms. Menkes disease and occipital horn syndrome (OHS) are allelic disorders of copper transport caused by defects in a X-linked gene (ATP7A) that encodes a P-type ATPase that transports copper across cellular membranes, including the *trans-Golgi* network. Genetic studies in yeast recently revealed a new family of cytoplasmic proteins called copper chaperones which bind copper ions and deliver them to specific cellular pathways. Biochemical studies of the human homolog of one copper chaperone, ATOX1, indicate direct interaction with the Menkes/OHS protein. Although no disease-associated mutations have been reported in ATOX1, mice with disruption of the ATOX1 locus demonstrate perinatal mortality similar to that observed in the *brindled* mice (*Mo*br), a mouse model of Menkes disease. The cDNA sequence for ATOX1 is known, and the genomic organization has not been reported.

Results: We determined the genomic structure of ATOX1. The gene contains 4 exons spanning a genomic distance of approximately 16 kb. The translation start codon is located in the 3' end of exon 1 and the termination codon in exon 3. We developed a PCR-based assay to amplify the coding regions and splice junctions from genomic DNA. We screened for ATOX1 mutations in two patients with classical Menkes disease phenotypes and one individual with occipital horn syndrome who had no alterations detected in ATP7A, as well as an adult female with chronic anemia, low serum copper and evidence of mild dopamine-beta-hydroxylase deficiency and no alterations in the ATOX1 coding or splice junction sequences were found.

Conclusions: In this study, we characterized the genomic structure of the human copper chaperone ATOX1 to facilitate screening of this gene from genomic DNA in patients whose clinical or biochemical phenotypes suggest impaired copper transport.

Background

Copper is an essential trace element that plays a role in cellular respiration, antioxidant defense, neurotransmitter biosynthesis, connective tissue biosynthesis and pigment formation and is critical for the survival of all living organisms. A family of eukaryotic cytoplasmic proteins termed copper chaperones that bind copper ions and deliver them to specific cellular pathways was discovered recently [1]. The first copper chaperone, ATX1, is a cytosolic protein involved in an tio xidant defense and iron uptake, in addition to copper trafficking in the yeast *Saccharomyces cerevisiae* [2,3]. The human homolog of ATX1 was isolated by screening a liver cDNA library, and named HAH1 (for human ATX homolog 1) [4]. This gene maps to chromosome 5q32 [5], and is referred to synonymously as ATOX1 [6,7].
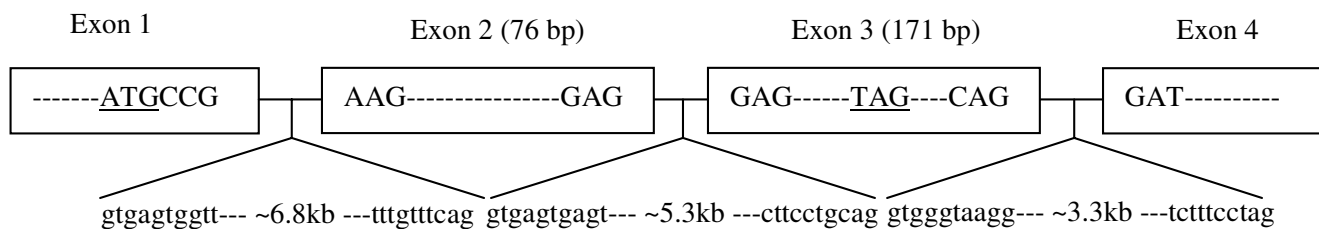
Exon 1	Exon 2 (76 bp)	Exon 3 (171 bp)	Exon 4
-------ATGCCG	AAG---------------GAG	GAG------TAG----CAG	GAT----------

gtgagtggtt--- ~6.8kb ---tttgtttcag gtgagtgagt--- ~5.3kb ---cttcctgcag gtgggtaagg--- ~3.3kb ---tctttcctag

Figure 1
The genomic structure of the *Atx1* human homologue. The translation start codon is located in the 3' end of exon 1 and the termination codon is in exon 3. The complete intronic and exonic sequences determined have been submitted to GenBank (Accession number: AY165037).

Table 1: Intron/Exon junctions of ATOX1. The nucleotide sequences of intron/exon junctions are shown by uppercase letters for exons and by lowercase letters for introns. The **GT-AG** conserved splice junction sequences are seen in all three introns.

Exon	Splice acceptor	Exon sequence	Splice donor
1		AGGCGCTGCT---(> 59bp)---AGTCATGCCG	**gt**gagtggttgcgccgtccc---
2	---tccccctgtgtttgtttc**ag**	AAGCACGAGT--- (76bp)---AAGCTTGGAG	**gt**gagtgagtggccctgagt---
3	---cccatttcctcttcctgc**ag**	GAGTTAAGTA---(171bp)---AAGGGGGCAG	**gt**gggtaaggccccagcact---
4	---taactttccatctttcct**ag**	GATGCTGATC----(136bp)---CTTTTGTTGG	

Biochemical studies of ATOX1/HAH1 [8,9] indicate copper-dependent interaction between this protein and two human copper-transporting ATPases. These latter molecules, encoded by the genes ATP7A and ATP7B, function to convey copper through the secretory pathway of cells including the *trans-Golgi* network. Mutations in ATP7A and ATP7B produce characteristic clinical phenotypes: Menkes disease and occipital horn syndrome, allelic X-linked recessive traits [10,11], and Wilson disease, an autosomal recessive trait, respectively.

Interestingly, disruption of the murine homolog (Atox1) results in perinatal mortality and biochemical abnormalities similar to that observed in the *brindled* mice (*Mo*br), a mouse model for classical Menkes disease [12]. These cumulative findings suggest that the ATOX1 and ATP7A gene products are each integral to mammalian copper transport, and that mutations in either could produce similar clinical and biochemical phenotypes. Because of our interest in the molecular basis of such phenotypes, we determined the intron-exon boundaries of ATOX1 to enable mutation screening from genomic DNA, and analyzed splice junctions and coding sequences in several relevant patients.

Results and Discussion
Genomic structure of ATOX1 and mutation analysis
We identified 4 exons and 3 introns in ATOX1 that span a genomic distance of approximately 16 kb (Figure 1). The apparent translation start codon is located in exon 1, and the termination codon in exon 3. We used 5' and 3' random amplification of cDNA ends (RACE) to obtain the 5' and 3' ends of the ATOX1 cDNA sequence. We identified 3 splice donor and 3 splice acceptor sequences and did not characterize the 5' intron/exon boundary of exon 1 and the 3' exon/intron boundary of exon 4 (Table 1). The 5' exon 1 sequence (untranslated) we obtained by 5' RACE is 60 bp shorter than the cDNA sequence initially described [5]. The three introns in ATOX1 were 6764 bp (intron 1), 5255 bp (intron 2), and 3320 bp (intron 3). Exons 2 and 3 were 76 bp and 171 bp in length, respectively. The entire genomic sequence determined was submitted to GenBank (Accession number AY165037).

Based on these findings, we designed PCR primers (Table 2) that efficiently amplify all 3 coding exons and the relevant splice junctions (Figure 2). We analyzed ATOX1 in several patients whose clinical or biochemical features were suggestive of abnormal copper transport. These included 2 of 49 patients with phenotypes consistent with classical Menkes disease [10], and a previously reported
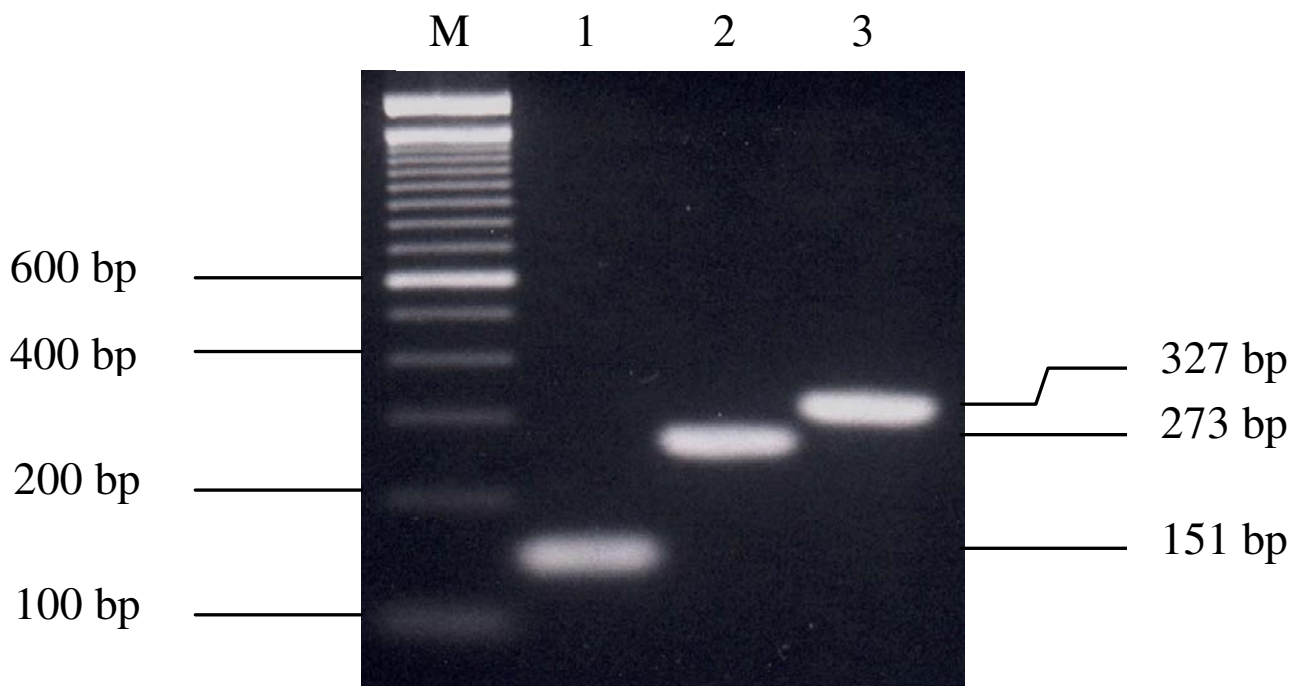
Figure 2
PCR products of 3 coding exons of ATOX1 in 2 % agarose gel. 100 bp nucleic acid markers is shown in M and exons are shown in numbers.

Table 2: Oligonucleotide primers used to amplify ATOX 1 coding sequences and splice junctions

Exon	Forward Primer (5'-3')	Reverse Primer (5'-3')	Fragment Size
Exon 1	aggcgctgctgacaccgccg	ttcaagatcagcatccggtc	151 bp
Exon 2	aggcttctgatgagtctgatgc	tctgcatgcatctgaacatg	273 bp
Exon 3	tgagtagtaatttagagcctg	aggtgttcgctctgatgagag	327 bp

patient with occipital horn syndrome [13], in none of whom ATP7A mutation have been identified. A fourth patient tested for ATOX1 alterations was a 43 year old female with a history of chronic anemia, low serum copper and mild dopamine-beta-hydroxylase deficiency, which responded to oral copper replacement (data not shown). We detected no alterations in the ATOX1 coding or splice junction sequences in any of these individuals.

Characterization of the genomic structure of ATOX1 enables rapid and efficient mutation screening from genomic DNA. Identification and clinical/biochemical delineation of patients in whom alterations in ATOX1 are found will permit better understanding of the significance of this gene product in human trace metal metabolism. In addition, functional polymorphisms in ATOX1 may be relevant in terms of phenotypic modifier effects in these copper transport disorders.

Conclusions

In this study, we determined the genomic structure of ATOX1 to facilitate screening of this gene in patients with clinical and biochemical findings suggestive of disordered copper homeostasis.

Methods

Subjects

Peripheral blood specimens or skin fibroblast cultures were obtained from two individuals diagnosed with classical Menkes disease on clinical and biochemical grounds, one patient with occipital horn syndrome [13], and one adult female with a history of chronic anemia, low serum copper and ceruloplasmin, and abnormal plasma catecholamine levels consistent with mild deficiency of dopamine-β-hydroxylase, a copper-requiring enzyme. Genomic DNA was isolated from white blood cells or fibroblasts using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. The studies were approved by the NICHD and NINDS Institutional Review Boards.

PCR amplification

Oligonucleotide primers for PCR amplification were designed from the published cDNA sequence of the human homologue of ATOX1 gene [4]. Each PCR reaction contained a final concentration of 1X optimized PCR buffer J (Invitrogen), 500 nM each PCR primer, 200 μM dNTP and 1.5 U Taq DNA polymerase (Life Technologies Inc.). 200 ng of genomic DNA was added to the total volume of 50 μl PCR reaction. These PCR reactions were then denatured for 5 minutes at 94°C in a Perkin-Elmer 9700 thermocycler; followed by 35 cycles of 94°C for 30 seconds, 55°C for 45 seconds and 72°C for 2 minutes; then with a final extension of 72°C for 7 minutes. Oligonucleotide primer sequences used to amplify ATOX 1 coding sequences and splice junctions are shown in Table 2.

5' and 3' RACE

The rapid amplification of 5' and 3' ends of cDNA (RACE) was performed with Marathon-Ready™ cDNA kit (Clontech), which is essentially an uncloned library of adaptor-ligated double-stranded cDNA. Both 5'-and 3'-RACE reactions were primed with an internal gene-specific primer (GSP) and the Marathon adaptor primer (AP). The adaptor-ligated cDNA does not contain a binding site for the AP. During the first round of thermal cycling, the GSP is extended to the end of the adaptor, creating an AP binding site at the 5' (or 3') terminus of the cDNA. In subsequent cycles, both the AP and the GSP can bind, allowing exponential amplification of the cDNA of interest.

Long PCR

To amplify the intronic regions of ATOX1, long PCR amplifications were performed in volume of 50 μl reactions by using the Expand Long Template PCR system kit (Roche). Each reaction contained 2.5 U Taq and Pwo DNA polymerases, 1X supplied buffer with 2.25 mM $MgCl_2$, 500 μM dNTP, 300 nM each forward and reverse primers and up to 500 ng genomic DNA. The reaction were optimized as follows: an initial denaturation for 2 minutes at

93°C, followed by 10 cycles of 93°C for 10 seconds, 65°C for 30 seconds and alternated with 68°C for 4 minutes. For the next 20 cycles, the temperature profile was modified by the addition of increments of 20 seconds to each extension step at 68°C. A 7 minutes final extension period at 68°C was incorporated after the last cycle of long PCR reaction.

DNA Sequencing

Sequence analysis was performed on an ABI 377 automated DNA sequencer at the National Institute of Neurological Disorders and Stroke, National Institute of Health, DNA Sequencing Facility.

Authors' contributions

All authors have read and approved the manuscript.

Acknowledgements

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