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ISOLATION AND CHARACTERIZATION OF A HUMAN LIVER cDNA AS A CANDIDATE GENE FOR WILSON DISEASE+

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The putative copper and ATP-binding domains of the human Menkes disease gene were used as probes to screen a human liver cDNA library at reduced stringency. Sixty-five clones which remained positive after tertiary screening were subcloned and sequenced. One of these cDNA clones contains an open reading frame with 65% amino acid homology to the Menkes protein. Southern blot analysis localizes this cDNA to the region of the Wilson disease locus on chromosome 13. This cDNA detects a 7.5 kB transcript which is present in human liver and cell lines devoid of the Menkes transcript and which is absent in liver from a patient with Wilson disease. These data suggest that this cDNA is a candidate gene for Wilson disease and that the protein encoded at this locus is a member of the P-type ATPase family.

Wilson disease is an inherited metabolic disorder resulting in hepatic cirrhosis and neuronal degeneration (1). Early pathological studies identified a marked increase in the copper content of liver and brain tissue from patients with Wilson disease (2,3). These studies identified this disease as a unique disorder of copper metabolism revealing that specific biochemical pathways exist which control the cellular metabolism of this transition metal. Immunochemical studies showed that most patients have a significant decrease in the serum concentration of ceruloplasmin thus providing a presymptomatic diagnostic marker and suggesting impaired transport of copper into an intracellular pool essential for biliary copper excretion and incorporation of copper during the biosynthesis of ceruloplasmin (4). Genetic analysis has localized the Wilson disease gene to chromosome 13 allowing diagnosis in families with informative polymorphisms and providing a potential avenue for molecular analysis of the disease (5). Despite these investigations the etiology of the abnormality in copper metabolism in Wilson disease remains unknown.

+Sequence data from this article have been deposited with the EMBL/Genbank Data Libraries under Accession No. L25442.

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Menkes disease is an X-linked metabolic disorder which results in abnormal depigmented hair and arterial and neuronal degeneration (6). This disease is also the result of a specific defect in cellular copper metabolism although the tissues affected and the hypocupremic phenotype are distinct from that observed in Wilson disease (7). The Menkes disease gene encodes a putative intracellular copper transport protein which is a member of the cation-transporting P-type ATPase family (8-10). This protein is normally expressed in multiple nonhepatic tissues and loss of the transport function apparently results in the abnormal intracellular copper accumulation characteristic of Menkes disease. The Menkes protein is the first eucaryotic copper transporter to be characterized but the sequence conservation between this protein and known procaryotic copper transporters suggests that the protein structure necessary for intracellular copper transport is highly conserved (11). Studies of hepatocyte holoceruloplasmin biosynthesis suggested to us that Wilson disease may result from a deficient intracellular copper transporter (12). Therefore to identify potential candidates for the Wilson disease gene we used the Menkes cDNA to screen a human liver cDNA library at low stringency.

Materials and Methods

Three sets of oligonucleotide primers (Fig. 1)

M5- 5'ACCCGGGAATGGATCCAAGTATGGGTGTGAATTCTGTT3'
M6- 5'ACCCGGGTTCTGGAGTGACTGAGCTTGCATTATACTT3'
M7- 5'ACCCGGGATATAGAGTTAGTATCACAAGTGAAGTTGAG3'
M8- 5'ACCCGGGTAAAGTGACTTGCTGACCGATCCTTCTTGAC3'
M9- 5'ACCCGGGAGTAAAGGTTCTAACTGAAAGTAACAGAATA3'
M10-5'ACCCGGGGTCAATACTTGCCACTACATCCAGAAGATC3'

were synthesized with 5' Sma I sites and used to amplify fragments from a human spleen cDNA library (Clonetech) as previously described (13). Amplified DNA was sequenced (14), labelled by random priming (15) and used to screen 10^6 individual plaques from a human liver cDNA library (Clonetech) under reduced stringency at 37°C (16). Duplicate positive clones were processed through tertiary screening, and insert DNA was amplified by polymerase chain reaction using λgt11 primers, subcloned and sequenced (17). Protein sequence similarities were determined using the Lipman-Pearson algorithm (18). RNA was obtained from tissue and cell-lines by dissolution in guanidinium isothiocyanate followed by CsCl density gradient centrifugation (19). RNA samples were electrophoresed in 0.7% formaldehyde-agarose denaturing gels, transferred to nylon membranes and hybridized as described (20). DNA was isolated from human placenta and cell lines by phenol extraction and Southern blots of restriction endonuclease digested DNA from these sources and a panel

of human/rodent somatic cell hybrids (Bios) were hybridized as described (20). A human/rodent somatic cell hybrid containing chromosome 13 (GM 10898) was obtained from NIGMS Human Genetic Mutant Cell Respiratory. Human/rodent somatic cell hybrids containing human chromosome 13 deletions were obtained from Webster Cavanee (21). RNA samples from hepatic transplant patients were obtained from Colin Bingle (22).

Results

Simultaneous low stringency screening of a human liver cDNA library with three specific regions from the Menkes cDNA (Fig. 1) resulted in the isolation of 65 individual cDNA clones. The nucleotide sequence of these clones was determined and one clone designated Y2 was found to contain an open reading frame with 68% amino acid homology to the Menkes protein. The translated amino acid sequence of clone Y2 was aligned with the Menkes protein revealing homology in the putative fifth and sixth copper-binding domains as well as the first two transmembrane regions (Fig. 2).

The tissue origin and degree of sequence similarity between clone Y2 and the Menkes protein suggested that this clone might encode a putative Wilson disease copper transporter. Therefore this clone was used in Southern blot analysis of human/rodent somatic cell hybrids. Analysis of a panel of such hybrids revealed a single BamHI fragment in human genomic DNA and hybrids containing chromosome 13 (Fig. 3A). Similarly a single EcoRI band was detected in human genomic DNA and a chromosome 13 specific hybrid (Fig. 3B, lanes 1,2 and 5) but not in DNA from the parent rodent cell line (lane 6) or two somatic cell hybrids with chromosome 13 deletions in the region of the Wilson locus (lanes 3,4). A single but distinct band was also detected in all samples containing rodent DNA which is presumed to be the hamster equivalent of the locus identified by Y2 (Fig. 3).

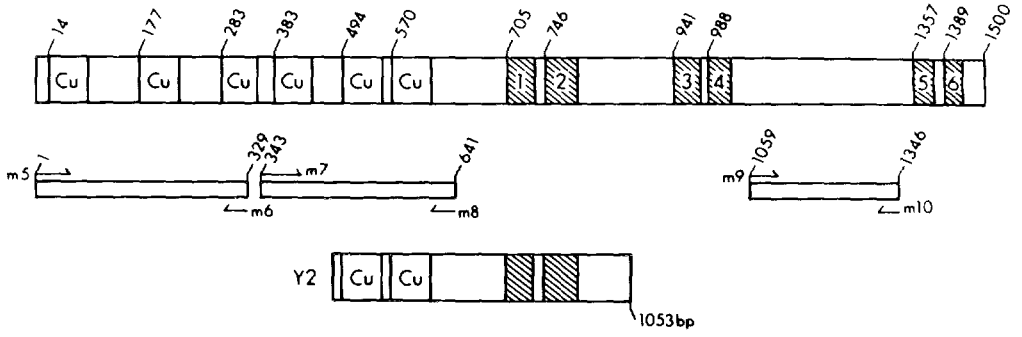

Figure 1. Diagram of the Menkes protein showing the location of the putative copper-binding (Cu) and transmembrane (hatched) domains. Numbering system for amino acids are from Vulpe et al. (8). Shown beneath is the location of the specific primers (m5-m6, m7-m8, m9-m10) and the amplified DNA fragments used to screen a liver cDNA library. Also shown is the structure of clone Y2 (1053bp) aligned by maximum sequence homology to the Menkes protein.

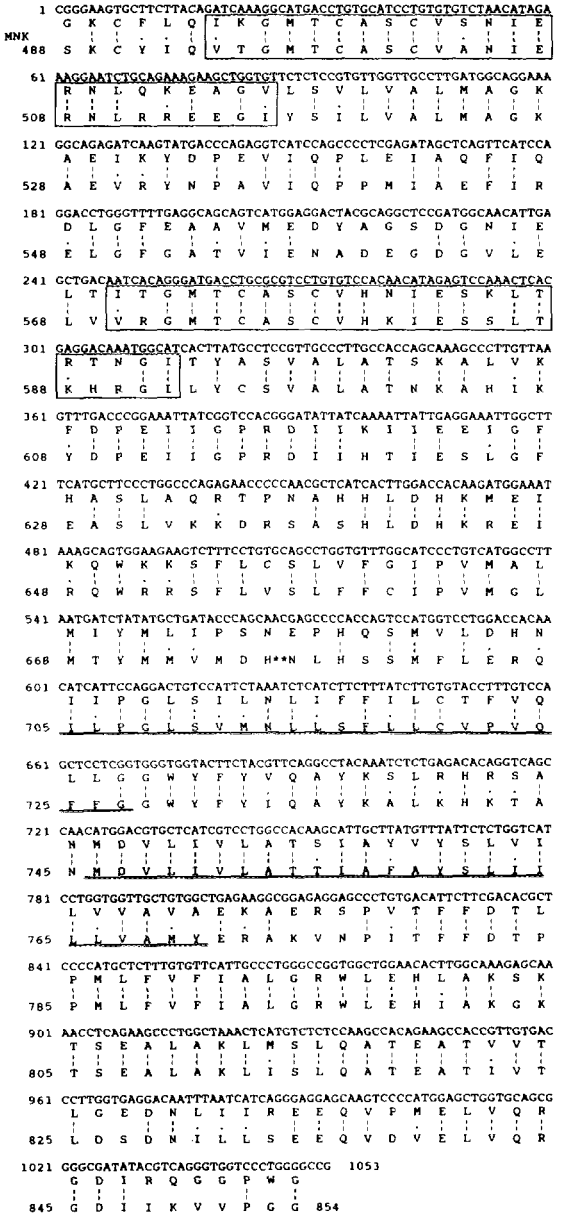

Figure 2. Nucleotide sequence of clone Y2 and deduced amino acid sequence aligned with Menkes protein sequence (MNK) as shown in Fig. 1. Conservative amino acid substitutions are indicated as well as a single region (**) interrupted in Menkes sequence (676-694) for homology alignment. The putative copper binding domains are boxed and the transmembrane segments are underlined.

RNA blot analysis using the Y2-specific probe revealed transcripts of 7.5 and 10 kB in human liver and peripheral blood cell lines (Fig. 4). Only the 7.5 kB transcript was observed in liver tissue and this was not found in RNA from human lung or HeLa cells (lanes 6-7). To determine transcript abundance in patients with Wilson disease, control RNA

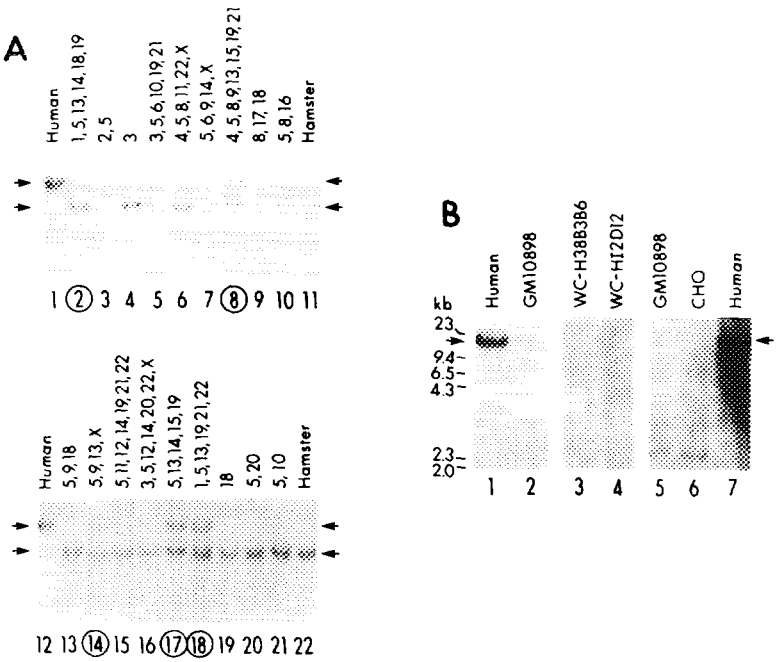

Figure 3. A. Southern blot of BamH1 digested genomic DNA from human/hamster somatic cell hybrids containing human chromosomes as indicated. Arrows indicate unique BamH1 fragment (upper arrow) detected in human DNA as well as single cross-reacting fragment detected in hamster DNA. **B.** Southern blot of EcoRI digested genomic DNA from human placenta (lanes 1,7), human/hamster somatic cell hybrids containing chromosome 13 (lanes 2,5) and chromosome 13 deletions (lanes 3,4) and CHO cells (lane 6) Arrows indicate a single chromosome 13 specific EcoR1 fragment in human DNA. Blots were hybridized with Y2 and analyzed as described in Materials and Methods.

samples were selected from patients with primary biliary cirrhosis who had a similar degree of hepatic cirrhosis and elevated hepatic copper content and a patient with oxalosis who had no histologic evidence of liver disease. The 7.5 kB liver-specific transcript was absent in one Wilson patient and greatly reduced in the second in comparison with controls (lanes 1-2 vs. 3-5). Similar analysis of peripheral blood cell lines revealed cell-specific expression of the two transcripts (lanes 8-11).

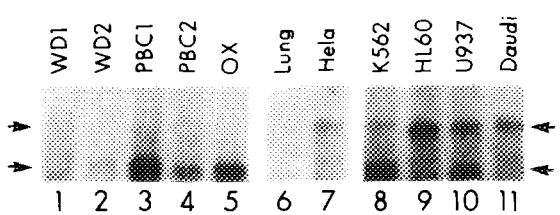

Figure 4. RNA blot of human liver (lanes 1-5), lung (lane 6) and cell lines (7-11) hybridized with an antisense Y2 specific cRNA probe analyzed as described in Material and Methods. Arrows identify 7.5 (lower) and 10 kB transcripts. WD, Wilson disease; PBC, primary biliary cirrhosis; Ox, oxalosis.

Discussion

These studies characterize the nucleotide sequence, expression pattern and chromosomal location of a 1 kB human liver cDNA. This cDNA encodes an open reading frame with 65% homology to the Menkes protein and identifies two putative copper binding domains very homologous to the fifth and sixth domains in the Menkes protein. This clone likely encodes a partial cDNA as there is no translation start site and the detected transcript size is much larger than 1 kB. The alignment with the Menkes protein is relative, based upon the established sequence similarity and may need to be modified as further sequence becomes available. Nevertheless, the presence of transcript in liver and the absence in lung and HeLa cells is opposite to that observed for the Menkes gene confirming that the results observed here are not due to cross hybridization to the Menkes transcript.

The chromosomal localization of this cDNA clone and the specific absence of transcript in liver tissue from one patient with Wilson disease strongly supports the hypothesis that this clone is a candidate gene for Wilson disease. The failure to detect transcript in this patient is specific and not the result of hepatic cirrhosis or copper content because abundant transcript was detected in control samples from patients with biliary cirrhosis. These differences were not the result of variable amounts of RNA analyzed because RNA blot analysis revealed equivalent amounts of 28S RNA in each lane (data not shown). Furthermore the results are specific for the transcript observed here since previous analysis has revealed similar abundance of other liver-specific mRNAs in these samples (22). The presence of transcript in the second patient suggests genetic heterogeneity at this locus which is consistent with the known phenotypic presentations of the disease (1). The origin of the second transcript observed in the cell-lines is unknown but may indicate the presence of additional copper transport proteins.

We conclude from this initial work that the Wilson disease locus encodes an hepatocyte-specific intracellular copper transporter which is likely to be a member of the P-type ATPase family based upon sequence homology with the Menkes protein. Although such a conclusion is entirely consistent with the known pathogenesis of this disorder future studies will be necessary to define the nucleotide sequence of this locus in normal individuals and patients with Wilson disease. Furthermore, functional studies will be needed to determine if any of these proteins are indeed capable of transporting copper and if so through which intracellular compartments.

Acknowledgments

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