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The toxic milk mouse is a murine model of Wilson disease

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Wilson disease (WD) is an autosomal recessive defect of copper transport characterized by massive accumulation of copper in the liver, which can lead to liver failure. Mutations in a copper transporting ATPase (WND or ATP7B) have been shown to cause the disease.The toxic milk mouse mutant (*tx)* accumulates copper in the liver in a manner similar to that observed in patients with WD. However, some physiological differences between *tx* mice and human WD patients have cast doubts on whether this mutant mouse is a valid model for WD. In this paper we report the isolation of cDNA clones encoding the murine homologue of WND. The predicted amino acid sequence is 1462 amino acids and contains the same functional domains identified in human and rat WND. As in the rat, the fourth metal binding domain is apparently non-functional. Similar levels of a 7.5 kb WND mRNA were detected in liver and kidney from normal and *tx* mice, indicating that transcription of this gene was unaffected in the mutant mice. The coding sequence of WND cDNA from the tx mouse liver identified a single nucleotide difference between the normal DL mouse and the *tx* which is predicted to change methionine1356 in the eighth transmembrane domain to valine. This methionine is conserved in all copper ATPases including those from bacteria and yeast. The conclusion that this is the causative mutation is supported by the recent mapping of *tx* and WND to the same region of mouse chromosome 8. Thus the *tx* mouse is presented as a valid model for studies of the role of WND in copper transport and for investigation of different treatment strategies for WD.

INTRODUCTION

Copper is an essential element required for a number of important enzymes. Due to the capacity of copper to cause oxidative damage to cells, efficient regulatory mechanisms exist to maintain tissue levels within the critical range. Copper homeostasis in mammals depends on a balance between intestinal absorption and biliary excretion [reviewed in (1)]. In Wilson disease (WD), an autosomal recessive inherited disorder, an impaired ability to excrete copper into the bile leads to an accumulation in the liver and extensive damage to this organ. WD can present either as a liver disease or a neurological disorder, the latter condition thought to be due to aberrant release of copper from the liver depositing in the central nervous system. As well as the phenotypic variation the age of onset of the disease can be from childhood to adult, but the reason for the variable clinical picture is not well understood.

The defective gene in WD has recently been cloned and encodes a copper transporting P-type ATPase (2,3). The WD gene (officially designated *ATP7B*, referred to here as *WND* for clarity) is expressed primarily in the liver, where it is thought to facilitate removal of excess copper from hepatocytes to the bile for excretion. The mRNA is found in other tissues such as kidney, brain and placenta, but the role of the protein in copper transport in these tissues is not understood. The predicted protein closely resembles the protein product of the Menkes disease gene (officially designated *ATP7A*, referred to here as *MNK*) (4). The products of *MNK* and *WND*, which will be referred to as MNK and WND, respectively, are transmembrane proteins and have the characteristic features of P-type ATPases, such as an ATP binding domain, and a conserved aspartic acid residue, which is phosphorylated during the transduction of copper across the membrane (5). An interesting feature of MNK and WND is the presence of six putative metal binding sites in the N-terminal half of the protein; copper ATPases from bacteria (6) and yeast (7) have only one or two of these putative copper binding sites. Analysis of patients with WD has identified 23 mutations of *WND* including 11 small insertions and deletions, seven missense mutations, two nonsense mutations and three splice site mutations. The missense mutations have been localized to mainly the transmembrane domain or the ATP binding region (8). The correlation of mutation with phenotype is complicated by the fact that most patients are compound heterozygotes.

Toxic milk (*tx*) is an autosomal recessive mutation in mice which causes hepatic accumulation of copper which commences in the third postnatal week. By 6 months of age the copper concentration can be 100 fold that of the normal adult. This

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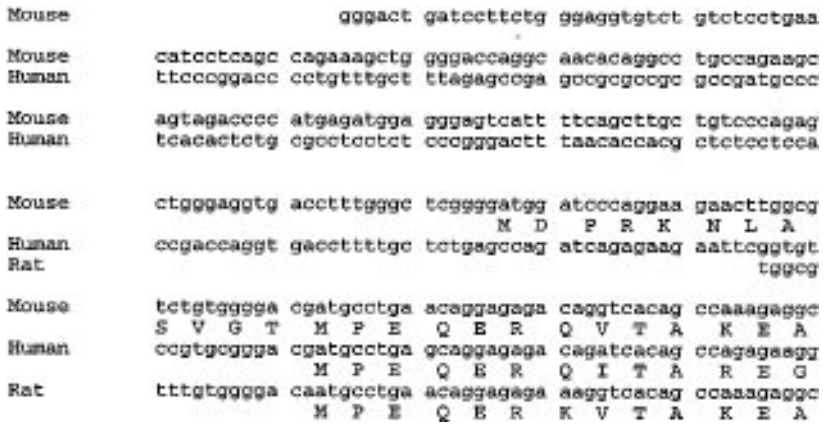

Figure 1. Comparison of the 5′ regions of WND cDNA from mouse, rat (13) and human (14). The mouse coding sequence is shown commencing from the first upstream methionine, which is not present in the human sequence, and no rat sequence is available for this region. The 5′-untranslated region is 162 nucleotides in both human and mouse.

gradual accumulation of copper in the liver resembles that seen in patients with WD. In addition, however, pups are born copper deficient and the milk produced by mutant mothers is low in copper, resulting in death of pups (9). Analysis of the morphology of livers from adult *tx* mice has shown significant differences from the liver damage seen in patients with WD. This fact, together with the absence of reports of copper deficiency in infants of mothers with WD, has raised doubts about whether the *tx* mouse is a valid model for WD (10). Recent studies, however, have mapped the toxic milk mutation to the same region of chromosome 8 as the murine homologue of *WND*, consistent with a mutation in this gene causing the *tx* phenotype (11,12).

Here we report the cloning and sequence of the murine homologue of the WD gene (GenBank submission number U38477). We demonstrate a point mutation in the *tx* mouse sequence which results in the conversion of a highly conserved methionine to valine in the eighth transmembrane channel, strongly suggesting that the *tx* mouse is indeed a valid model for WD.

RESULTS

A mouse liver cDNA library from a BALB/c mouse (Clonetech #ML3001b) was initially screened using a rat *WND* cDNA clone which included nucleotides encoding the C-terminal region of the protein (13). This screen identified three positive clones which were plaque purified, subcloned and sequenced as described in the Methods. The sequences of these clones showed a high level of identity to the corresponding rat sequence. The library was screened again with these clones and the procedure repeated until sequence covering the entire coding region was obtained. Translation of the sequence identified an open reading frame with two potential ATG start codons, one which corresponded to the start codon identified in rat and human *WND* (13,14), and the other 36 nucleotides upstream which also conformed to the Kozak consensus for initiation codons (15). Including this region, the open reading frame for mouse *WND* mRNA consists of 4386 bases, predicted to encode a protein of 1462 amino acids. Figure 1 shows the alignment of the 5′-regions of the WND cDNAs from mouse, human and rat. The upstream methionine is not present in human (the codon in this position is CCA), but sequence is not available for this region in the rat; it remains to be determined whether the mouse WND has an additional 18 amino acids at the N-terminus.

Figure 2 shows the predicted amino acid sequence compared with rat and human WND (13,14). There is a high level of amino acid identity (82% to human and 91% to rat) between all three sequences, and in particular all predicted proteins have the functional domains previously noted for the copper transporting ATPases: e.g. putative copper binding domains, transmembrane regions, ATP binding sites, and phosphorylation domain. Eight transmembrane domains are indicated, based on the model proposed for human and mouse MNK (16,17) and rat and human WND (13,14). Interestingly, both the mouse and rat sequences have apparently lost the fourth putative copper binding site (M4) in the N-terminal region, since the highly conserved motif, GMTCXXC has been altered to GITCSASS in mouse and GIPRDSS in rat (13). The sequence divergence from human WND extends over a region of about 190 amino acids, including the M4 region and almost to the start of M5, which is fully conserved.

To determine whether the expression of the mouse *WND* homologue was altered in the *tx* mouse, a 2 kb mouse WND clone was hybridized to a northern blot prepared from total RNA from the liver and kidney of *tx* mice, and the strain from which they were derived, the inbred DL line. As can be seen from Figure 3a, a 7.0 kb mRNA was detected in the liver RNA tracks from both normal and *tx* mice, a similar size to the WND mRNA from human (2,3). As found in humans, the gene was also expressed in the kidney, but at a lower level than in the liver. The size and quantity of the mRNA in the mutant appeared identical to normal (compare lanes 1 and 2), indicating that there are no large deletions in the promoter and coding regions, or major structural changes in *WND* in the *tx* mouse. RNA from the liver of a copper loaded mouse (lane 4) appeared to have lower amounts of WND mRNA, but this was likely to be due to loading differences, based on the rRNA staining (Fig. 3b, lane 4). Since the amount of WND was the same in normal and *tx*, it is unlikely that the mRNA is down-regulated by high copper levels. Copper loading did not significantly alter the amount of WND mRNA in the kidney (lanes 5 and 6). WND mRNA was also detected in brain, lung and small intestine (not shown).

Using primers based on the normal mouse WND cDNA sequence, RT–PCR products covering the coding sequence were

generated from RNA isolated from the livers of *tx* and DL mice. The RT–PCR products were subcloned and sequenced to obtain the predicted amino acid sequence. The coding sequence of normal DL and BALB/c differed at three residues but these only altered the third base of the codon and hence did not result in any predicted amino acid changes.

The coding sequence from the *tx WND* was identical to the sequence from the DL mouse except for a single A to G base change in the mutant sequence at position 4066. RT–PCR was performed in three separate experiments to ensure that this change was not due to an error introduced during the amplification and cloning procedures; all clones showed the same alteration in the *tx* mouse. This base change brings about a met1356val amino acid substitution within the proposed eighth transmembrane domain (TM8), shown in bold and underlined in Figure 4 in comparison with this region of copper transporting ATPases from other organisms. This comparison shows the highly conserved nature of this transmembrane domain and the affected methionine. This conservation, in organisms as evolutionarily divergent as mammals, yeast and bacteria, suggests that the methionine residue has an important function in copper transport, and hence its alteration is considered to be the causative mutation.

DISCUSSION

The sequence of the coding region of the WND homologue in the *tx* mouse has shown a single base difference between the normal (DL) and *tx* mutant mouse. We conclude that this is the causative mutation in the *tx* mouse, making it a true animal model of WD. This conclusion is supported by the following considerations. The *tx* mutation arose in an inbred strain (DL) and affected mice were subsequently back crossed to produce homozygous *tx* mutants (9). Therefore, any difference in sequence between the two is likely to be the result of a mutation rather than a polymorphism. The mutation results in the alteration of a highly conserved methionine to valine in the eighth transmembrane region of the predicted protein. The conservation of this methionine in all copper ATPases so far sequenced, including those from yeast and bacteria, suggests that it plays an important role in the copper transport process. Strong supporting evidence is also provided by the coincident mapping of both *tx* (11) and *WND* (12) to a site near the centromere of chromosome 8 in mouse. Although collectively these data provide compelling evidence that the methionine to valine change causes the defect in the *tx* mouse, there is still a remote possibility that the change is a polymorphism. Future work will directly test this possibility by determining whether a normal WND construct will correct the *tx* defect.

The mutation found in the *tx* mouse has not been reported in any patients with WD. However, a similar methionine to valine change in transmembrane domain number four (TM4) has been found in one patient with WD (8). This change was initially classified as a possible polymorphism. This methionine, how-

Figure 2. Predicted amino acid sequence of mouse WND from the DL mouse (GenBank accession #U38477) with amino acid differences with rat and human WND noted below the sequence. The amino acid number of the mouse sequence is shown on the right. Predicted structural motifs are indicated: M1 to M6 the putative core copper binding motif, GMTCXXC, note that the M4 has been altered in both rat and mouse and is consequently shown in parentheses. TM1 to TM8 refers to the predicted transmembrane domains of the protein. Phosphatase site, phosphorylation and ATP binding domains are also indicated.

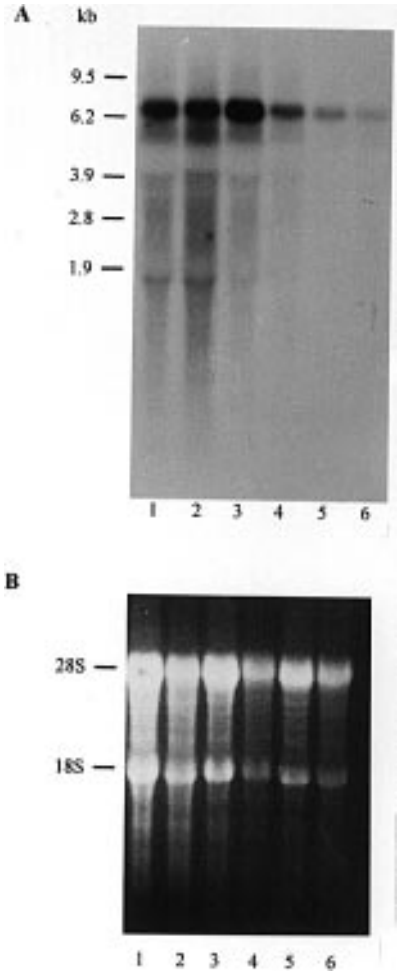

Figure 3. Northern blot analysis of RNA from normal and *tx* mice. Total RNA was isolated from the indicated mouse tissues and 10 µg electrophoresed on a 2.0 M formaldehyde 1% agarose gel, blotted on to Hybond N^+ membrane (Amersham) and probed with a 2 kb WND cDNA fragment as described in the Methods. Lane 1, liver, 150 day male DL; lane 2 liver 150 day male *tx*; lane 3, liver 60 day male BALB/c; lane 4, liver 60 day male Cu-induced BALB/c; lane 5, kidney 60 day male BALB/c; lane 6, kidney 60 day male Cu-induced BALB/c. (**A**) Autoradiogram; (**B**) ethidium bromide stained gel to demonstrate RNA loading.

Figure 4. Alignment of the eighth transmembrane region of copper transporting P-type ATPases from various species. The methionine to valine alteration in the *tx* mouse is shown in bold and underlined. The numbering above the sequence refers to mouse amino acid sequence (Fig. 2). Note that in the rat sequence published initially, the methionine in question and an adjacent alanine were shown as being absent in the normal rat. This has subsequently been shown to be the result of sequencing gel compression errors, and the corrected sequence is used here. The numbers in parentheses are the literature references.

ever, is also conserved in all copper transporting ATPases and it now appears likely, based on the *tx* mutation, that the TM4 mutation is the causative mutation in that WD family. In copper transporting proteins the metal ion is often bound to cysteine or methionine residues, thus the methionine in the transmembrane regions may form a transient complex with the copper ion as it passes through the membrane channel.

An interesting feature of the predicted structure of the mouse WND is the presumed loss of the putative copper binding site four in the N-terminal region. The rat and mouse sequences share some of these changes, suggesting that the alterations occurred in a common ancestor. The region of divergence with the human sequence includes a region of about 190 amino acids surrounding the M4 site, so the loss of function of the recognized motif may have been followed by subsequent drift of surrounding sequences. The loss of this site without inactivation of the copper transport function implies that not all copper binding sites are required for function, and raises the question of the role of the six metal binding sites which are conserved between WND and MNK in most species so far examined.

Animal models of WD are important for both clinical and scientific studies. Clinically the treatment of WD has been generally regarded as very successful with the advent of penicillamine therapy, yet significant complications sometimes develop (18). A convenient animal model allows a more complete assessment of various therapies, within the recognized limits of potential biological differences between an experimental animal and humans. Such models are also useful for basic studies of copper transport, which is still not well understood.

The only other confirmed animal model of WND is the LEC rat which has a deletion of the 3′ region of the WND homologue (13). Many of the physiological features of the LEC rat are also found in the *tx* mouse. The pattern of hepatic accumulation of copper is very similar: the accumulation of copper commences after weaning and most of the excess copper is bound to metallothionein (9,10,19). The histological appearance of the liver is also similar in both mutants, showing enlarged hepatocytes and abnormal nuclei (10,20–22). In both mutants, the amount of ceruloplasmin in plasma is decreased (9,23). The reduced ceruloplasmin has been shown to be a consequence of lower incorporation of copper into this protein in the LEC rat (23,24), which is also the case in most Wilson patients [reviewed by Danks (1)]. Thus the similarities between the rat and mouse models are in accordance with our finding of the mutation in the murine WND homologue.

The differences between the phenotype of patients with WD and the *tx* mouse have cast doubt on its validity as a model of that disease (10). Now it has been established that the *tx* mouse and the LEC rat have mutations of WND, it is worth considering the possible explanations for their differences. It should be noted that WD itself presents with quite a variable phenotype, and the reasons for this are not understood (1). An analysis of the specific phenotypes of animal models may elucidate the factors causing this clinical variability.

The most striking differences between the *tx* mouse and patients with WD as well as the LEC rat is the production of copper deficient milk by the *tx* mouse. Although pups of *tx* dams are always copper deficient, this does not invariably cause death. Indeed in some colonies of *tx* mice, with the same genetic background, death of pups is rarely observed (J. McC. Howell, pers. comm.), suggesting that environmental factors such as concentration of copper in the mother's diet can modify the

phenotype. It is also possible that some degree of copper deficiency may be present in the LEC rat pups, since the copper status of milk from LEC dams has not been reported. Copper deficiency in breast fed babies of mothers with WD has not been reported to the best of our knowledge, but it would be of interest to determine the copper concentration of milk from lactating mothers with WD. In any event the copper deficiency in the suckling mice raises the interesting possibility that WND may be involved in delivery of copper to milk. Another possible explanation for the low copper in milk is that WND is required for the incorporation of copper into ceruloplasmin in the liver, and ceruloplasmin is providing copper to the mammary gland and placenta (25).

Another possible reason for the differences in phenotype between the *tx* mouse and WD is that specific mutations may result in distinct phenotypes. Correlation of phenotype with genotype in WD is complicated by the fact that most patients are compound hetereozygotes, with two different mutations of WND. Interestingly, one patient with a very early onset WD proved to be homozygous for a null allele (8). The LEC rat having a partial deletion of WND may be a model of this class of patients. The effect of the *tx* mutation of the activity of WND has not been established, but it is possible that some residual activity may be maintained with a subsequent modification of the phenotype.

In conclusion, the evidence strongly suggests that the toxic milk mouse is a true model for WD. This is of importance for the study of WD and copper transport in general. The *tx* mouse may prove to be a better model for WD since the mutation is similar to some mutations found to cause WD, whereas no large deletions, such as found in the LEC rat, have been reported in WD patients. It is possible that the *tx* mouse retains some residual WND activity which could be of importance in the study of different therapeutic agents used for treatment of WD. A mouse model offers other advantages over the LEC rat, e.g. the possibility of transferring the mutation to many more defined genetic backgrounds, thus exploring the question of the effect of modifying genes on the expression of the mutation, which is of relevance to understanding the phenotypic variation seen in WD (8,26). The more advanced transgenic possibilities with mice will facilitate gene correction studies.

MATERIALS AND METHODS

Source of animals

The *tx* mutation first arose in 1974 in the DL strain which was at that time at F68 of brother sister mating. The *tx* mutation has been maintained in the DL strain subsequently and in 1983 was at F88 (9). Our colony was started from animals supplied by Dr H. Rauch.

Isolation and sequencing of cDNA clones

The rat WND clone 8/2, spanning nucleotides 1310–3742 of the rat coding sequence (13), was used to probe a BALB/c liver cDNA library in λgt11 (Clonetech #ML3001b). Inserts from positive λ bacteriophage clones were subcloned into pBluescript KS (Stratagene) for sequence analysis. Clones identified as being representative of the mouse WND homologue were used in subsequent screening of the library to obtain overlapping clones which covered the open reading frame. DNA sequencing was performed on double stranded plasmid DNA according to the protocols provided with the Sequenase sequencing kit (United States Biochemical).

Northern blot analysis

RNA was extracted from various mouse tissues using the RNeasy kit (QIAGEN) according to manufacturers' protocols. Total RNA (10 μg) from each tissue sample was run on a denaturing formaldehyde 1% agarose gel, and blotted on to Hybond N^+ nylon membranes as described previously (27). RNA was fixed using 0.05 M NaOH, and rinsed in 2× SSC prior to hybridization. DNA probes were inserts from λ library clones 3 and 4B, which comprised cDNA encoding the region from the second metal binding site to the fifth transmembrane domain. Probes were labeled with α-^{32}P-dCTP using a random priming kit (Boehringer Mannheim), hybridized overnight at 65°C in a solution containing 500 mM Na_2HPO_4, 5% SDS, 5 × Denhardt solution, 10 mM Tris pH 7.5, 5 mM EDTA and 100 μg/ml sheared and denatured salmon sperm DNA. Blots were washed to 0.5 × SSC, 0.1% SDS at the same temperature, and exposed at –70°C using intensifying screens.

RT–PCR and PCR cloning

Using the sequence derived from the mouse library clones, primers were designed which would allow reverse transcription and PCR amplification of the WND homologue from DL and *tx* liver RNA. Reverse transcription was performed at 42°C with the following reaction mixture: 5 μg total liver RNA, 500 ng primer (oligo $dT_{(30)}$ or internal primer), 300 μM dNTP, 1 unit RNase inhibitor (Promega), 50 mM Tris pH 8.5, 8 mM $MgCl_2$, 30 mM KCl, 1 mM DTT, 24 units AMV reverse transcriptase. A one-tenth volume of this reaction was used as template for PCR amplification, to which was added a reaction mixture containing: 750 ng each of forward and reverse primers, 200 μM dNTP, 50 mM KCl, 10 mM Tris pH 8.3, 1.5 mM $MgCl_2$ and 1.0 units *Taq* DNA polymerase (Boehringer Manneheim). Amplification was performed using 35 cycles of 95°C for 90 s, 55°C for 90 s, and 72°C for 120 s. PCR products were isolated by electrophoresis on agarose gels, bands were excised and purified using Qiaex DNA extraction kit (QIAGEN) and ligated into T-tailed pBluescript (29). Clones were sequenced according to Sequenase protocols for double-stranded plasmids. PCR primer pairs as follows (5′ to 3′) :

(i) TX011 AACATCCTCAGAAAGC and MW9B.2 GCTTAG GGAGACTTTGATTC;
(ii) TX531 CTCTATCGAGGGCAACATCC and MW4B.1 GA GATGGATGTTTGCTGCAC;
(iii) MW4B.2 GTGCAGCAAACATCCATCTC and TX2155 TA GAAGTACCACCCACCCAG;
(iv) MW3.1 CTCATCTTCTTCATCTTGTG and TX1734 TTGT GTGCCATCTCCAGAGG;
(v) MW1.1 CCCTAGCAAGCACATCTCGC and TX2181 ATC TCGTGATCTGTCATGGC;
(vi) TX3491 TAACCATCTCCAGTGACATC and TX4202 CC GCTGACTTGGGAGGCAC;
(vii) TX4265 CGACCTAGAGAGATGTGAGG and TX4703 TT CTGGAAGAGCAATCCTGC.

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