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Identification of Point Mutations in 41 Unrelated Patients Affected with Menkes Disease

Zeynep Tümer,[1] Connie Lund,[1] Jo Tolshave,[1] Burçak Vural,[2] Tønne Tønnesen,[1] and Nina Horn[1]

[1]The John F. Kennedy Institute, Copenhagen; and [2]Institute for Experimental Medical Research, Department of Genetics, Istanbul University, Istanbul

Summary

Genomic DNA of 41 unrelated patients affected with the classical severe form of Menkes disease was investigated for point mutations in the *ATP7A* gene (previously designated as the "*MNK*" gene). Using SSCP analysis and direct sequencing of the exons amplified by PCR, we identified 41 different mutations, including 19 insertions/deletions, 10 nonsense mutations, 4 missense mutations, and 8 splice-site alterations. Approximately 90% of the mutations were predicted to result in the truncation of the protein (ATP7A). In 20 patients the mutations were within exons 7–10, and half of these mutations affected exon 8. Furthermore, five alterations were observed within the 6-bp sequence at the splice-donor site of intron 8, which would be predicted to affect the efficiency of splicing of exon 8. Although a specific function has not been attributed to the protein region encoded by this exon, this region may be important in serving as a "stalk" joining the metal-binding domains and the ATPase core. The present findings not only help us in understanding the underlying genetic defect but are invaluable data especially for carrier detection and prenatal diagnosis of this lethal disorder.

Introduction

Menkes disease (MD) is a lethal disorder of copper metabolism and is inherited as an X-linked recessive trait (Menkes et al. 1962; Horn et al. 1992; Danks 1995; Tümer and Horn 1996). Progressive neurodegeneration and connective-tissue disturbances are the main manifestations, and most of the clinical features can be explained by malfunction of one or more important copper enzymes (Danks 1995). Although most MD patients (90%–95%) have a severe clinical course, variable forms of the disease exhibiting different degrees of nervous-system or connective-tissue involvement can be distinguished (Horn et al. 1995). The occipital horn syndrome (OHS), mainly characterized by connective-tissue manifestations, has been suggested to be a very mild allelic form of MD (Peltonen et al. 1983).

The disease locus has been mapped to Xq13.3 (Verga et al. 1991; Tümer et al. 1992*b*), and the gene (*MNK* or *ATP7A*) defective in MD has been isolated by positional cloning (Chelly et al. 1993; Mercer et al. 1993; Vulpe et al. 1993). The 8.5-kb mRNA transcript of *ATP7A* is expressed in all tissues analyzed, although in only trace amounts in liver. The protein product of 1,500 amino acids is predicted to be a copper-binding P-type ATPase (ATP7A) (Vulpe et al. 1993). Soon after the identification of *ATP7A*, the allelic relationship between MD and OHS was confirmed by mutation analyses (Kaler et al. 1994; Das et al. 1995). Furthermore, the gene defective in Wilson disease (WD) was isolated by use of sequences specific to *ATP7A*, and the predicted protein product (designated as "ATP7B") revealed high (57%) sequence homology to ATP7A (Bull et al. 1993; Tanzi et al. 1993; Yamaguchi et al. 1993). WD is an autosomal recessive disorder of copper metabolism and results from the toxic effects of copper, and it is mainly characterized by different degrees of liver disease and by neurological or psychiatric symptoms (Danks 1995).

Characterization of the exon-intron structure reveals that *ATP7A* is organized into 23 exons spanning a genomic region of ~150 kb (Dierick et al. 1995; Tümer et al. 1995). The exons of *ATP7A* are relatively large, ranging between 77 bp and 726 bp of coding sequence. The first exon is a leader exon containing only untranslated sequences, and the ATG translation-start codon is in the second exon. The last exon contains a 274-bp translated sequence, the TAA translation-termination site, the 3.8-kb 3′ UTR, and a polyadenylation site. The *ATP7B* gene (or *WND* gene) has 22 exons (Petrukhin et al. 1994; Thomas et al. 1995*a*), and its genomic organization shows remarkable similarity to that of *ATP7A* (Tümer et al. 1995). Starting from the exons coding for the fifth metal-binding domain (exon 5 in *ATP7A* and exon 3 in *ATP7B*), the coding regions of both genes are organized

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Address for correspondence and reprints: Dr. Zeynep Tümer, The John F. Kennedy Institute, Gl. Landevej 7, 2600 Glostrup, Copenhagen, Denmark. E-mail: zet@kennedy.dk

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into 19 exons, showing an almost identical structure (Tümer et al. 1995).

Mutations leading to MD show great variety, including cytogenetic abnormalities, partial deletions of *ATP7A*, and single-base-pair changes. In five MD patients, the underlying defect is a cytogenetically visible chromosome aberration involving the Xq13.3 region. One of the patients is a male with an intrachromosomal rearrangement (Tümer et al. 1992*b*), and four are females with a balanced X;autosomal translocation, including an X;2 translocation (Kapur et al. 1987), an X;1 translocation (Beck et al. 1994), and two X;21 translocations (Z. Tümer, N. Horn, D. Wattana, and M. Tsukahara, unpublished data). In three of the cases, the X-chromosome breakpoint was shown to be at the *ATP7A* (*MNK*) locus, by FISH analysis (Verga et al. 1991; Tümer et al. 1992*a*; Beck et al. 1994). In ~20% of >200 patients screened, the mutations were shown to be gross deletions or rearrangements, which could be detected by Southern blot analysis (Chelly et al. 1993; Tümer et al. 1994*a*, 1994*b*) or by PCR amplification of the individual exons with intron-specific primers (Z. Tümer, C. Lund, and N. Horn, unpublished data). The remaining 80% of the genetic defects are thus base-pair changes or very small rearrangements, and, so far, 12 point mutations have been reported in MD patients with the severe phenotype (Das et al. 1994; Tümer et al. 1996). In 10 patients the mutations were identified by analyzing the *ATP7A* cDNA by use of reverse transcriptase–PCR (RT-PCR) or the chemical cleavage mismatch–detection technique (Das et al. 1994). To screen for the alterations in the coding region of the gene as well as in the intronic sequences flanking the exons, we first identified the exon-intron structure of *ATP7A* (Tümer et al. 1995). Using SSCP analysis and direct sequencing of the exons amplified by PCR, we elsewhere had identified two frameshift mutations within *ATP7A* in two MD patients receiving early copper-histidine treatment (Tümer et al. 1996). In the present study, we report the genetic defects identified in 41 unrelated MD patients with the classical severe phenotype.

Patients, Material, and Methods

Patients

The patients ($n = 65$) investigated in this study were referred to The John F. Kennedy Institute during the period 1973–95, from several different countries, for the biochemical diagnosis of MD (table 1). The biochemical study was based on the intracellular accumulation of copper because of impaired efflux and was performed on cultured fibroblasts. Although a clear discrimination between affected and unaffected males is possible, milder phenotypes cannot be distinguished from the classical form (Horn et al. 1995). Classification of the phenotype therefore was based mainly on the clinical symptoms, and all the patients reported in this study had the severe MD phenotype. These patients were either <5 years old or had died at <6 years of age. The patients who had severe symptoms but who survived for more than the first 6 years of life were classified as the classical form with long survival (Horn et al. 1995) and were not included in this study.

Experimental Design

Each of the 23 *ATP7A* exons and the adjacent intronic sequences (Tümer et al. 1995) were analyzed with SSCP for each patient. For effective identification of SSCP mobility differences, exon 3 (490 bp) was divided into two overlapping fragments, and exon 4 (726 bp) was divided into three overlapping fragments. Oligonucleotide primers were designed by use of the OLIGO-Primer Analysis Program (Rychlik and Rhoads 1989), according to sequence data published elsewhere (Tümer et al. 1995). The PCR fragments included ≥25 bp of the flanking intron regions surrounding each exon. The nucleotide sequences of the primers used for amplification of the exons, as well as their product lengths, are given in table 2.

Amplification of Genomic DNA

Genomic DNA was isolated from either Epstein-Barr virus–transformed lymphocytes or cultured skin fibroblasts by use of the NaCl extraction method (Grimberg et al. 1989). PCR amplification (Saiki et al. 1988) of genomic DNA was performed in a total volume of 15 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 nM each dNTP, 0.1 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol), 0.5 U of *Taq* polymerase (Boehringer Mannheim), and 0.4 μM each primer (table 2). The initial denaturation was carried out for 7 min at 95°C and was followed by 40 cycles of denaturation at 94°C (1 min), annealing at 55°C (2 min), and extension at 72°C (3 min) and by final extension at 72°C (7 min).

Nondenaturing Gel Electrophoresis

The samples were diluted with four volumes of SSCP buffer (10 mM EDTA and 0.1% SDS) and one volume of formamide loading buffer (95% formamide, 20 mM EDTA, and 0.05% each of xylene cyanol FF and bromophenol blue), were heat denatured at 99°C (2 min), and were loaded onto 6% nondenaturing polyacrylamide gels. To achieve the best separation of fragments, glycerol concentration of the gels (0%, 5%, or 10%) and concentration of the electrophoresis buffer (0.5 or 1 × Tris-borate EDTA) were optimized for each exon. Once the conditions were settled, the same protocol was used throughout the study. Electrophoresis was performed at room temperature, with constant power of 4 W for 16

Table 1

Primers Used for SSCP Analysis of the *ATP7A* Gene in MD Patients

Exon[a]	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Product Size (bp)
1	GGGGGTGGGAAAAGAGAAGC	CATGACCTTCTCACCACACTCGT	262
2	GCATGGCTGAAATTAATGAATTT	CTTCAATGGCATTTGACTTCAGT	219
3A	TGTGATAGAATTTAATTAAACTGACTTTTG	AGCTCTTTTATCTGATTGGCATTC	352
3B	AAAATTTACCCTCAGAAAAGAACTGTA	AAACTCATGCTTAAGAAGAAAGCAA	349
4A	GTGGTTATTGATTTAGAAAACAGAATG	CAATGCATGCCATCAATGATG	336
4B	CAGAAGGGTCACAGCAAAGG	CAAGTCATGCCATCAATGTTTATC	376
4C	GAAGATTCCTTTGAATGTAGTTAGCC	TGCCTATGAATCATTTCTCATTGA	340
5	GATGCAATTGAATGATCAATACTGC	TGTAAGAGAACAAAAAAGATGGAGCT	331
6	TAATTATAACATTACTCTTTTTAAAAAGAA	CTTACCAAGTTTTTTTTCTGGAAGT	285
7	AGTGGTAACTCATGTTTAATGGTGGA	GATTCACGGGAGTGCTGATTAAA	290
8	GTTCTTAATGACAATACCATGGCTTAG	ACCATTTCATCCCGTATAAGGATG	272
9	TACCCATTAGCTATTTATGACCATGA	GCCTGCCAAATTTTGCTTATT	342
10	ATATATGTGAATTTCAGCATTTTTTAA	ATGTATTTCCAATGATTGGCC	376
11	AGACCTGAACTTTTTCTTCCTTAGC	TAAAAAAAGAGAAGAGGGAAGAGTG	182
12	GGAGAGGTCAGGGTAGGAAGC	CTCTCTGTTCATAATTTAGTTATTCATAGC	291
13	AACAAATAATGATATGTTTCATGATAGTG	GTGAAGATTTATACTGAGAAGGCTTTA	291
14	TACTCTGCATTCAGTATCAGAAAAGACTT	CCAATGAATTACTAATGCAAAAGCTC	280
15	ACAGCTCTAAATCAATAACCAAAAT	TTTGGTGGTACAGGTTTTAGTTCT	277
16	TTTCTGGAAAGGTGATGTGGA	TAGATTTGATCCGGGGAATTC	306
17	AATTAACTAGGGGTTTTTATCTTGC	CAGGGTTATAAAGTTAGCCATCTG	371
18	GAACTTGCTTCATGGGGTTT	AGAGGTCTCTCAAAAATAAATAAATTAAT	295
19	TATTCCAAGTTCTTTTATTTTGTGCTG	AAATTTCTTAGCATTTGAAGGCAAG	259
20	TATTGCTCAGTTATGTTTCACGTACT	GATTATCATTGACCACATAGGGC	332
21	AAGGTAGACGTAATCTTCAACATACACAG	ATAACTTTAGTGGAAAAAGCAGTACTAGT	258
22	ATGCTGAAGAAGTATCAAACAAAGAAA	CAAGAATAAAACCTACCAAGAATGAC	287
23	TCTCATTTACTTTTGGTTATTTGAAACT	AGTGCATGACAAGTTAAACTGGC	369

[a] Exon 3 is divided into two overlapping fragments, exon 4 into three overlapping fragments, because of the large size of these exons.

h. The fragments were visualized by a PhosphorImager 445 SI (Molecular Dynamics).

Sequence Analysis

Patient samples exhibiting shifts relative to normal samples on SSCP gels were subjected to direct sequencing, to identify the mutation. Genomic DNA was amplified under the same conditions as described above, except that the concentration of primers (100 nM) and nucleotides (80 nM) were reduced. Sequencing of PCR products was performed on both strands by the dideoxy method (Sanger et al. 1977), by use of the amplification primers and Sequenase version 2.0 (USB). Electrophoresis was performed on 6% or 8% denaturing polyacrylamide gels, and autoradiography was undertaken overnight at room temperature.

Results

The 65 unrelated male MD patients investigated in this study were all characterized by the classical severe MD phenotype. Elsewhere we had established the exon-intron structure of *ATP7A* (Tümer et al. 1995) and had constructed intron-specific primers to amplify each exon (table 1). The genomic DNA of 39 patients initially was analyzed either by Southern blot analysis or by PCR amplification of each exon, without detection of any gross DNA rearrangements (Z. Tümer, T. Tønnesen, and N. Horn, unpublished data). DNA of 26 patients was analyzed only with SSCP, and presence of the amplification product indicated that the exons analyzed had not been deleted.

All the exons were screened for each patient by SSCP using intron-specific primers (table 1). For the 4-kb last exon, only the 274-bp translated sequence and 21 bp of the untranslated sequence was analyzed. The promoter sequences upstream of the first exon, which contains only untranslated sequences, have not been analyzed either. With SSCP, an abnormal pattern was identified in a total of 44 patients. In three patients, the band shift observed in exon 10 was due to a polymorphism (Val767Leu) that had been reported elsewhere (Das et al. 1994). In 41 patients, we identified in *ATP7A* a sequence change predicted to be the disease-causing mutation (table 2). All these mutations would have an important effect on the structure/function of the protein product and include 19 insertions/deletions, 10 nonsense mutations, 4 missense mutations, and 8 splice-site alterations (table 2 and fig. 1).

Table 2

Mutations in the *ATP7A* Gene in Patients with Classical MD

Type of Mutation and Exon Number[a]	Mutation[b]	Predicted Effect[c]	Sample
Insertion/deletion:			
3	177 ins AAAG	Frameshift[d]	5349
4	379 del A	Frameshift	7158[e]
4	412 del C	Frameshift	24838[e]
7	590–591 del AG	Frameshift	24889
7	594 ins T	Frameshift	7325
7	606 del T	Frameshift (N)	14841
9	686 del A	Frameshift[d]	AM252
10	735 ins T	Frameshift	19925
10	762 del TT	Frameshift	AM267
10	779–784 del 14 bp	Frameshift (N)	20628
14	963 del A	Frameshift (N)	566[e]
15	980 del C	Frameshift	14463
16	1083 ins A	Frameshift	AM242
17	1164 del T	Frameshift (N)[d]	4734
19	1235 del A	Frameshift[d]	5610
20	1294 del A	Frameshift	16547
21	1344 ins TGCCA	Frameshift[d]	21448
22	1381–1384 del GGTTTGGTTT	Frameshift[d]	568
22	1400 del GT	Frameshift[d]	16268
Nonsense:			
4	Arg409Ter (CGA→TGA)	TP	9928
7	Cys578Ter (TGC→TGA)	TP	12902
8	Ser637Ter (TCA→TGA)	TP[d]	449[e]
8	Arg645Ter (CGA→TGA)	TP[d]	11138
9	Glu690Ter (GAA→TAA)	TP[d]	16111
10	Arg795Ter (CGA→TGA)	TP	15541
13	Gln896Ter (CAG→TAG)	TP[d]	23506[e]
13	Gln924Ter (CAA→TAA)	TP[d]	9927
14	Trp956Ter (TGG→TAG)	TP	9200
16	Glu1081Ter (GAA→TAA)	TP	15664
Missense:			
8	Ala629Pro (GCT→CCT)	DPS[d]	12098[e]
10	Gly727Arg (GGA→AGA)	DPS	2152
15	Leu1006Pro (CTG→CCG)	DPS	9855
15	Gly1019Asp (GGT→GAT)	DPS	AM247
Splice site:			
IVS7	AS (agAGC→acAGC)	Skips exon 8	AM351
IVS8	DS (ACAgta→ACActa)	Skips exon 8	14840
IVS8	DS (ACAgtaag→ACAgtaac)	Skips exon 8	4779
IVS8	DS (ACAgtaagt→ACAgtaagg)	Skips exon 8	15962
IVS8	DS (ACAgtaagt→ACAgagt)	Skips exon 8	20181
IVS8	DS (ACAgtaag→ACAgtaaa)	Skips exon 8	19769
IVS12	AS (agGGG→ggGGG)	Skips exon 13	6699
IVS22	DS (ACTgt→ACTgc)	Skips exon 22	AM254

[a] In each section, mutations are ordered according to exon numbers, in ascending order.

[b] Codon numbers are according to the *ATP7A* cDNA sequence published by Vulpe et al. (1993). For the deletions or insertions, the number of the first codon is indicated. AS = acceptor site; and DS = donor site. Intron and exon sequences are shown in lowercase and uppercase, respectively.

[c] N = premature-termination codon that occurs not in the mutation-bearing exon but in the next one. TP = truncates protein; and DPS = disrupts protein structure.

[d] An out-of-frame deletion of the transcript would occur if the mutation-bearing exon was skipped out.

[e] Families in which carrier diagnosis has been performed.

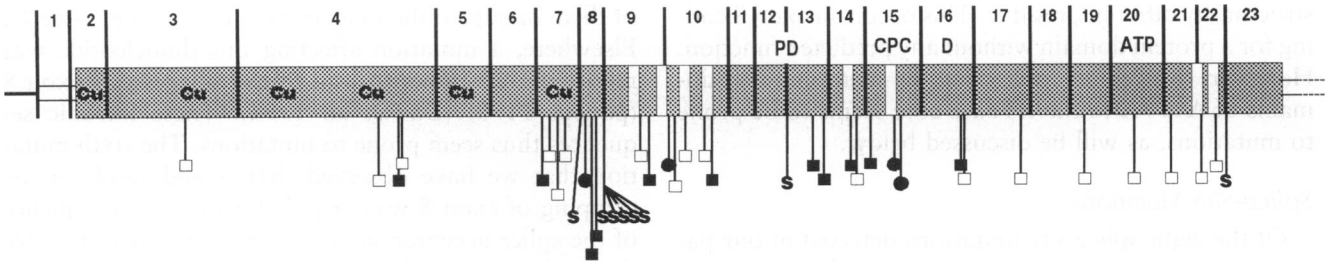

Figure 1 Location of the mutations within the coding region of *ATP7A* and the corresponding domains of ATP7A. The predicted copper-binding domains are indicated by "Cu," and the transmembrane domains are indicated by vertical white bars. PD = phosphatase domain; CPC = cation channel; D = phosphorylation domain; and ATP = ATP-binding domain. The coding region of *ATP7A* is represented by a gray horizontal box. The vertical lines indicate the positions of the introns, and the exons are indicated by numbers. The 5′- and 3′ UTRs are shown by a horizontal white box, and the flanking genomic sequences of the 5′ UTR are indicated by a horizontal line. Unblackened squares denote insertion/deletion mutations; blackened squares denote nonsense mutations; blackened circles denote missense mutations; and S = splice-site mutation.

Insertion/Deletion Mutations

We have identified a total of 19 insertion/deletion mutations occurring within the exons of *ATP7A*. Five of them are insertions of 1, 4, or 5 bp. Of the 14 deletions, the largest one is the deletion of 14 bp within exon 10. All these mutations are expected to alter the reading frame of the gene, leading to the introduction of a premature-termination codon; this would result in a truncated protein product. Alternatively, these mutations may lead to the skipping of the mutation-bearing exon (and of one or more of the subsequent exons), as has been reported elsewhere for a 2-bp deletion mutation in exon 7 of *ATP7A* (patient 1095) (Das et al. 1994). If skipping of one or more exons restores the open reading frame, an internally deleted but partially functioning protein still may be expected, depending on the importance of the protein domains missing. In six of the present mutations, skipping of the mutation-bearing exon would lead to a shift in reading frame, whereas in 13 of them the reading frame would be restored (table 2).

Nonsense Mutations

In 10 patients, base-pair substitutions within *ATP7A* introduce a termination codon, expected to result in the truncation of ATP7A after translation of 12%–94% of the coding region. In a case reported elsewhere (patient 1286) (Das et al. 1994), a nonsense mutation occurring in exon 8 of *ATP7A* led to the production of two transcripts: (1) a normal size transcript including the mutation and (2) a smaller size transcript in which exon 8 was spliced out, resulting in an out-of-frame deletion. Of the nonsense mutations presented in this study, five of them would lead to an in-frame deletion of the mRNA transcript if the mutation-bearing exon was skipped, and five of them would result in out-of-frame deletions due to codon phase differences (table 2).

Missense Mutations

A total of four missense mutations have been detected among the patients reported in this study. The possibility of a polymorphism has been excluded for each mutation, by analysis of 40 normal chromosomes by direct sequencing of the respective exons amplified by PCR. Three of the residues (Ala629, Gly727, and Leu1006) changed by the missense mutations were conserved in ATP7B, but mutations within these codons have not been reported for the WD gene.

The missense mutation, Leu1006Pro, occurs in exon 15 coding for the sixth transmembrane domain and the predicted cation channel (fig. 1). This mutation replaces the nonpolar leucine residue with the nonpolar but structurally different proline residue. Proline has a pyridolin ring containing part of the peptide backbone, which would cause a bend in the peptide. The missense mutation Gly1019Asp also is in exon 15, but Gly1019 is not conserved in ATP7B. This mutation replaces a nonpolar residue with a large polar residue. Both missense mutations occurring in exon 15 are expected to disrupt the function of the cation channel and/or the formation of the transmembrane domain. However, exon skipping may remove these mutations from the isoforms of the protein. Elsewhere a missense mutation in exon 20 had been reported as resulting in a normal size transcript and another transcript in which exon 20 was skipped out, leading to an in-frame deletion (patient 1645) (Das et al. 1994). Skipping of exon 15 in the present cases also would result in an in-frame deletion, but the protein product would be lacking the predicted cation channel.

The Gly727Arg change is within the second transmembrane domain, and a small nonpolar residue would be replaced by a large polar residue. This mutation would disrupt the formation of this domain. The same mutation has been observed elsewhere in another patient (patient 1407) and did not result in skipping of the mutation-bearing exon (Das et al. 1994).

The last missense mutation, Ala629Pro, replaces a small nonpolar residue with another nonpolar, but

structurally different, residue. This occurs in exon 8 coding for a protein domain without any predicted function. However, this region connecting the metal-binding domains of ATP7A to the ATPase core seems to be prone to mutations, as will be discussed below.

Splice-Site Mutations

Of the eight splice-site mutations detected in our patients, five occur at the splice-donor site of intron 8, and one occurs at the splice-acceptor site of intron 7. These mutations are predicted to affect the splicing efficiency of exon 8. One of the splice-site mutations is detected at the splice-acceptor site of intron 12, which would lead to the skipping of exon 13 coding for part of the phosphatase domain. The other splice-site mutation is at the splice-donor site of intron 22, which is expected to result in the skipping of exon 22 coding for both part of the putative transmembrane domain 7 and the last transmembrane domain. All these mutations are expected to disrupt the structure and the function of the protein product.

Discussion

We have identified 41 different point mutations in patients with the classical severe MD phenotype. All the mutations were unique except for two (patients 1286 and 1407), which were identical to mutations reported elsewhere (Das et al. 1994); one of these mutations is a nonsense mutation (Arg645Ter) occurring in exon 8. These patients belong to different families, but, since they are from the United States, it is worthwhile investigating these two families for kinship. The other mutation is a missense mutation within exon 10 (Gly727Arg), and, since these two patients originate from different countries (Holland and the United States), kinship seems unlikely.

Half (20/41) of the mutations observed in this study have occurred in exons 7–10, and half of these mutations affected exon 8. Similarly, 5 of the 12 point mutations reported elsewhere were within these exons (Das et al. 1994; Tümer et al. 1996). Furthermore, approximately half of the partial deletions detected by Southern blot analysis included at least one of these exons (Z. Tümer, T. Tønnesen, and N. Horn, unpublished data). It is thus relevant to screen mutations starting from these exons, regardless of the mutation-detection technique used. It is noteworthy that clustering of mutations in this region has not been observed for *ATP7B* (Figus et al. 1995; Houwen et al. 1995; Thomas et al. 1995*b*, 1995*c*; Chuang et al. 1996).

Of the eight splice-site mutations that we have identified, six were predicted to affect the splicing efficiency of exon 8. Five of these mutations were within the 6-bp sequence at the splice-donor site of intron 8, and two of them disrupted the obligatory GT sequence (table 1). Elsewhere, a mutation affecting this dinucleotide was reported and shown to lead to the skipping of exon 8 (patient 1190) (Das et al. 1994). These intronic sequences thus seem prone to mutations. The sixth mutation that we have observed that would result in the skipping of exon 8 was in the obligatory AG sequence of the splice-acceptor site of intron 7. Furthermore, we detected three other mutations (two nonsense mutations and one missense mutation) within exon 8 (table 1). The total number of mutations affecting this exon is thus 13, which comprises ~25% of the mutations observed in MD patients with the severe phenotype (Das et al. 1994; Tümer et al. 1996; present study). Exon 8 encodes a region between the last metal-binding domain and the first transmembrane domain of ATP7A (fig. 1). Although a specific function has not been attributed to this region, it may play an important role in the folding of the protein and may serve as a "stalk" joining the metal-binding domains and the ATPase core. The fact that all the patients with these mutations are severely affected supports the importance of this domain. In ATP7B this domain is encoded by exon 6 of the WD gene (*ATP7B*, or *WND*), and only two mutations—a missense mutation and a 2-bp deletion—have been described for this exon (Figus et al. 1995).

Approximately 70% (28/41) of the mutations that we have identified were frameshift mutations or nonsense mutations, which would lead to premature termination of translation, resulting in the production of a truncated protein. Furthermore, all the splice-site mutations observed were predicted to lead to a frameshift after exon skipping. Together with the two frameshift mutations that we have identified elsewhere (Tümer et al. 1996), the total number of the "truncating" mutations would be >90% of all the mutations that we have detected; for the mutations reported by Das et al. (1994), this figure was 80%. The protein-truncation test (Roest et al. 1993), which detects translation-terminating mutations by a combination of RT-PCR and in vitro transcription/translation reaction, therefore may be a suitable method for screening genetic alterations in *ATP7A*.

With SSCP, the total number of base-pair substitutions, including the three polymorphisms, was 24 (55%), and the total number of insertion/deletion mutations was 20 (45%). Since insertion/deletion mutations are more likely to be recognized by SSCP, and since this technique has only a 67% detection rate in our hands, the present results may not give a true picture of the frequency of different types of mutations in *ATP7A*. However, if one takes into account the large number of the patients to be analyzed, SSCP is a rather simple and convenient method for initial screening of a large number of mutations in a large gene.

Exon skipping due to point mutations within the

exons has been observed in *ATP7A* (Das et al. 1994). These mutations included a 2-bp deletion in exon 7 (patient 1095), a nonsense mutation in exon 8 (patient 1286), and a missense mutation in exon 20 (patient 1645) (Das et al. 1994). In each case, besides exon skipping, a normal size mRNA transcript also was detected. However, in other mutations that were of similar types, exon skipping was not observed, suggesting that several different factors contributed to the exon-skipping process. Some of the mutations described in this study also may lead to exon skipping. Analyzing the mRNA transcripts of these patients may help us in understanding not only this process in *ATP7A* but also some of the clinical differences in the patients. Similarly, alternative splicing of exon 10 observed in several normal tissues (Dierick et al. 1995) also may affect the clinical phenotype in patients carrying a mutation within this exon. However, it should be noted that all the mutations described in this study are expected to have detrimental phenotypic effects, sinces all the patients have a severe clinical course, despite minor differences.

Elsewhere, 14 point mutations had been identified by analysis of patient RNA (Das et al. 1994, 1995; Kaler et al. 1994); 10 patients had the classical severe MD phenotype (Das et al. 1994), 1 had a milder phenotype (Kaler et al. 1994), and 3 had OHS (Kaler et al. 1994; Das et al. 1995). In the patients with the nonclassical phenotypes, only splice-site mutations leading to splicing defects were observed. In three cases, the mutation was within the intronic sequences (Kaler et al. 1994; Das et al. 1995), and a normal size transcript also was present in low amounts, suggesting that residual activity of a normal protein could result in a milder phenotype. In the fourth patient, the mutation was a base-pair substitution (Ser833Gly) in the splice-donor site of exon 11 (Kaler et al. 1994). Besides two abnormal transcripts, a normal size transcript bearing the missense mutation also was present, although in lesser amounts. Since the amino acid change was conservative, the protein product of this transcript still might be functioning, although partially, resulting in a milder phenotype (Kaler et al. 1994). However, more data on the genetic defects leading to the atypical forms of MD are needed in the search for a correlation between the genotype and the resulting phenotype.

For MD, a definitive biochemical diagnosis exists and is based on the intracellular accumulation of copper in cultured cells, which is due to impaired efflux (Tønnesen and Horn 1989). Prenatal diagnosis is performed by measuring radioactive copper accumulation in cultured amniotic fluid cells in the second trimester (Horn 1981) and by determining the total copper content in chorionic villi in the first trimester, a test very susceptible to exogenous copper contamination (Horn et al. 1985). These analyses demand expertise and are performed in only a few centers in the world (Horn et al. 1992; Horn 1983; Danks 1995). Demonstration of a defect in *ATP7A* will be the ultimate diagnostic proof and eventually may decentralize the diagnosis of MD. However, mutation detection in MD is a formidable task: the 8.5-kb *ATP7A* transcript is organized into 23 exons, the genetic defect shows a great variety, and each family has its own unique mutation. A prenatal molecular-genetic diagnosis thus will be possible only if the mutation in the family already has been identified. Mutation detection in WD is likewise challenging, because of the presence of a large number of mutations in a large coding region. So far, ~40 disease-causing mutations have been reported within the *ATP7B* gene, by analysis of patients' DNA (Figus et al. 1995; Houwen et al. 1995; Thomas et al. 1995*a*, 1995*b*, 1995*c*; Chuang et al. 1996). These mutations are small base-pair changes; and, in contrast to MD, large deletions detectable by Southern blot analysis have not been observed. The largest deletion observed in *ATP7B* was a 24-bp deletion within exon 18 (Figus et al. 1995). However, in WD, haplotype data have been invaluable in mutation detection, since each haplotype generally is associated with a specific mutation (Figus et al. 1995; Thomas et al. 1995*c*), whereas MD is a new mutation disorder (Rossiter and Caskey 1990).

In MD, carrier determination is also possible by measurement of ^{64}Cu uptake in cultured fibroblasts (Horn 1980, 1983; Horn et al. 1980). However, carrier identification of X-linked disorders by biochemical means is not reliable in the case of negative results, because of random inactivation of the X chromosomes. Mutation analyses therefore will provide the ultimate proof of heterozygosity (Tümer et al. 1994*b*). This also will have an impact on the number of prenatal diagnoses referred to our institute, since 80% of the male fetuses tested have been found to be unaffected, indicating that a substantial number of the mothers were indeed not carriers. Elsewhere we have performed carrier detection and prenatal diagnosis using Southern blot analysis (Tümer et al. 1994*a*, 1994*b*). Using the results of the mutation analyses reported here, we hitherto have performed carrier diagnosis in six families (table 1). In each case, the mutation-bearing exon was amplified by PCR and was sequenced directly, to search for the mutation. In half of the families the mother of the index patient was not carrying the mutation in her somatic cells. Although germ-line mosaicism cannot be excluded in these cases, the results are crucial for genetic counseling of other female members of such families.

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