Am. J. Hum. Genet. 56:570–576, 1995

Similar Splicing Mutations of the Menkes/Mottled Copper-Transporting ATPase Gene in Occipital Horn Syndrome and the Blotchy Mouse

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Summary

The connective-tissue disorder occipital horn syndrome (OHS) is hypothesized to be allelic to Menkes disease. The two diseases have different clinical presentations but have a similar abnormality of copper transport. Mice hemizygous for the blotchy allele of the X-linked mottled locus have similar connective-tissue defects as OHS and may represent a mouse model of this disease. We have analyzed the Menkes/mottled copper-transporting ATPase in these two potentially homologous disorders and have identified similar splicing mutations in both. Some expression of normal mRNA was detectable by reverse transcription–PCR in the mutant tissues. These findings contrast with the more debilitating mutations observed in Menkes disease and suggest that low amounts of an otherwise normal protein product could result in the relatively mild phenotype of OHS and of the blotchy mouse.

Introduction

Occipital horn syndrome (OHS), or X-linked cutis laxa, is a rare connective-tissue disorder characterized by hyperelastic and bruisable skin, hernias, bladder diverticulae, hyperextensible joints, varicosities, and multiple skeletal abnormalities (Lazoff et al. 1975). The disease is sometimes accompanied by mild neurological impairment, and bony abnormalities of the occiput are a common feature of this disorder, thus giving rise to the name. The clinical features are related to the deficient activity of lysyl oxidase, which initiates cross-linking of collagen and elastin (Byers et al. 1980). However, the assignment of lysyl oxidase to chromosome 5 (Hamalainen et al. 1991) excludes it as a candidate for this disease.

Several lines of evidence suggest that OHS may be an allelic variant of Menkes disease, a disorder of copper transport. The specific activity of lysyl oxidase, a cuproenzyme, is also reduced in Menkes disease. In both diseases, intestinal absorption of copper is deficient, serum copper and ceruloplasmin are low, and cultured fibroblasts accumulate copper, although in OHS, these abnormalities are less pronounced (Kuivaniemi et al. 1982). In contrast to the limited connective-tissue findings associated with OHS, patients with classic, severe Menkes disease suffer from a host of additional problems, including neurological degeneration with death in early childhood, hypothermia, hypopigmentation, and pili torti (Danks 1989). The pathology in Menkes disease has been ascribed to insufficiency of multiple cuproenzymes, not just that of lysyl oxidase (Danks 1989). Thus, in both OHS and Menkes disease, a comparable copper-transport defect appears to lead, on the one hand, to a relatively mild connective-tissue disorder and, on the other, to a lethal phenotype.

Further support for allelism of OHS and Menkes disease comes from analogy with the mottled (*Mo*) mouse, which is a proven model for Menkes disease (Levinson et al. 1994; Mercer et al. 1994) and which exhibits similar defects of copper transport (Miller 1990). Over 10 alleles at the mottled locus have been described, which range in severity from coat hypopigmentation to death in utero. One of the mild mottled mutants, blotchy (Mo^{blo}) (Russell 1960), exhibits connective-tissue abnormalities reminiscent of those seen in OHS patients, including weak skin and bony abnormalities. In blotchy males, hindlegs are occasionally deformed, vibrissae are kinked at birth, crosslinking of skin collagen and aortic elastin is defective, and death frequently results from aortic rupture (Green 1989; Miller 1990). Therefore, in the mouse homologue of Menkes disease, at least one mutant allele affords an example of a connective-tissue phenotype similar to OHS.

The recent identification of a copper-transporting ATPase gene (*MNK*) responsible for Menkes disease (Chelly et al. 1993; Vulpe et al. 1993*b*; Mercer et al. 1993), allows a direct test of allelism between Menkes and OHS. A preliminary analysis revealed that *MNK* mRNA is greatly reduced in fibroblasts from two unrelated patients with OHS (Levinson et al. 1993), a finding indistinguishable from that observed in many Menkes patients (Mercer et al. 1993; Vulpe et al. 1993*b*). Although this finding supports allelism,

Received September 14, 1995; accepted for publication November 21, 1994.

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0002-9297/95/5603-0003$02.00

it raises the question of how mutations in a single gene, with seemingly similar effects on mRNA expression, can give rise to such clinically disparate phenotypes.

In this report, we describe mutations in the *MNK* gene of two unrelated OHS patients and in the *Mo* gene of the Mo^{blo} mouse. Comparison of these mutations with the debilitating mutations found in classic severe Menkes patients (Chelly et al. 1993; Vulpe et al. 1993*b*; Das et al. 1994) provides a possible explanation for this paradox.

Patients and Methods

Patients

Patient T181 has not previously been reported, to our knowledge. He presented to a genetics clinic, at age 14 years, for evaluation of musculoskeletal abnormalities and recurrent bladder rupture. There was no family history of mental retardation or birth defects, save for the isolated occurrence of cleft lip, in a first cousin of the patient's mother. In the perinatal period, mild hypotonia and cranial contour abnormalities and wormian bones, on radiographs, were observed. His medical history has been marked by extensive orthopedic intervention, including osteotomy procedures for leg straightening and treatment for multiple compression fractures of his vertebrae. There is a history of recurrent bladder rupture, bladder diverticulae, vesicular calcium stones, and an atonic bladder requiring intermittent catheterization. Physical examination in the genetics clinic revealed dolicocephaly, prominent and simple ears, downslanting palpebral fissures with bilateral ptosis, dental crowding, pectus carinatum, cutis laxa, and muscle wasting. Neurological status, including cognition, was normal. Serum ceruloplasmin was 18 mg/dl (normal range is 20–60 mg/dl). Radiographs revealed osteopenia, dislocated radial heads, and the characteristic occipital horns. Radiocopper accumulation in fibroblasts (measured by Dr. Tonne Tonneson) was elevated, at 55 ng ^{64}Cu/mg protein/20 h. This was comparable to values for Menkes disease cells and was well above the upper normal value of 21 ng ^{64}Cu/mg protein/20 h.

Patient 1519 has not been reported previously as a clinical case report, though northern blot data on mRNA from his fibroblasts was included in a previous report (Levinson et al. 1993). He represents a singleton case who presented to a medical genetics service as a 15-year-old Caucasian male, wheelchair bound because of genu valgum deformities at the knees and coxa vara deformities at the hips. He is mentally retarded. His skin has a cobblestone appearance, with hyperelasticity at the elbows and without skin friability. There is laxity of the interphalangeal joints, with contractures at the elbows and knees. Serum ceruloplasmin and copper determinations were normal. Radiographs revealed bilateral occipital horns of the skull. Intravenous pyelography revealed bladder deformities with large diverticulae. Renal ultrasound was normal. X-rays of hands, wrists, and forearms revealed osteoporosis, fusion anomalies in the wrists, and dysplasia of the radius and ulna, with dislocation of the radius at the elbow. Copper concentrations in his cultured skin fibroblasts were measured by atomic absorption spectrophotometry (Packman et al. 1984). Copper content was elevated, at 0.26 μg Cu/mg protein, well above the control range of <0.05 μg Cu/mg protein and well within the range observed for Menkes disease patients.

Samples

Skin and blood samples were collected from patients T181 and 1519, respectively, after informed consent was obtained. Similar samples isolated from unaffected males served as controls. Fibroblast cells were cultured from skin samples, as described elsewhere (Packman et al. 1987). The lymphoblastoid line was prepared by infection with Epstein-Barr virus. Mo^{blo} animals were provided to us by Dr. Frank Verley and were purchased from Jackson laboratories, and fibroblast cultures were prepared according to the method of Packman et al. (1987).

cDNA Preparation

Ten million cells were used for polyA$^+$ RNA isolation (Pharmacia QuickPrep Micro RNA purification kit). One half microgram of mRNA was used for the synthesis of single-stranded cDNA (Invitrogen cDNA cycle kit for reverse-transcription [RT]–PCR).

PCR Analysis

Approximately 30 ng of randomly primed, single-stranded cDNA was subjected to PCR amplification. Initial amplification of the *MNK* cDNA in the OHS patients, in four overlapping segments, was performed as described elsewhere (Das et al. 1994). The PCR primers used to narrow down the alternatively spliced region of the human control sample were 5′-GCTTAGGTTTTGAAGCTTCT and 5′-GAAATTAACTTTGCAAGAGCCTCTG. cDNA from blotchy cells was amplified in four overlapping segments corresponding to the *Mo* cDNA as follows: segment 1 (nt 56–1326), segment 2 (nt 1049–2428), segment 3 (nt 2241–3547), and segment 4 (nt 3265–4662), according to the cDNA numbering of GenBank accession number U03434 (Levinson et al. 1994).

Sequence Analysis of RT-PCR Products

In cases in which RT-PCR yielded more than one amplification product, fragments were cloned using the TA cloning kit version 2.2 (Invitrogen) prior to sequencing. In reactions yielding a single fragment, direct sequencing of the PCR products was performed using the PCR Template Prep for ssDNA sequencing kit (Pharmacia). Sequencing was performed using Sequenase version 2.0 (United States Biochemical) and [α-^{35}S]-dATP.

Identification of Introns by Genomic DNA Amplification and Vectorette PCR

Some intron sequences were obtained by PCR of patient and normal genomic DNA by using cDNA-

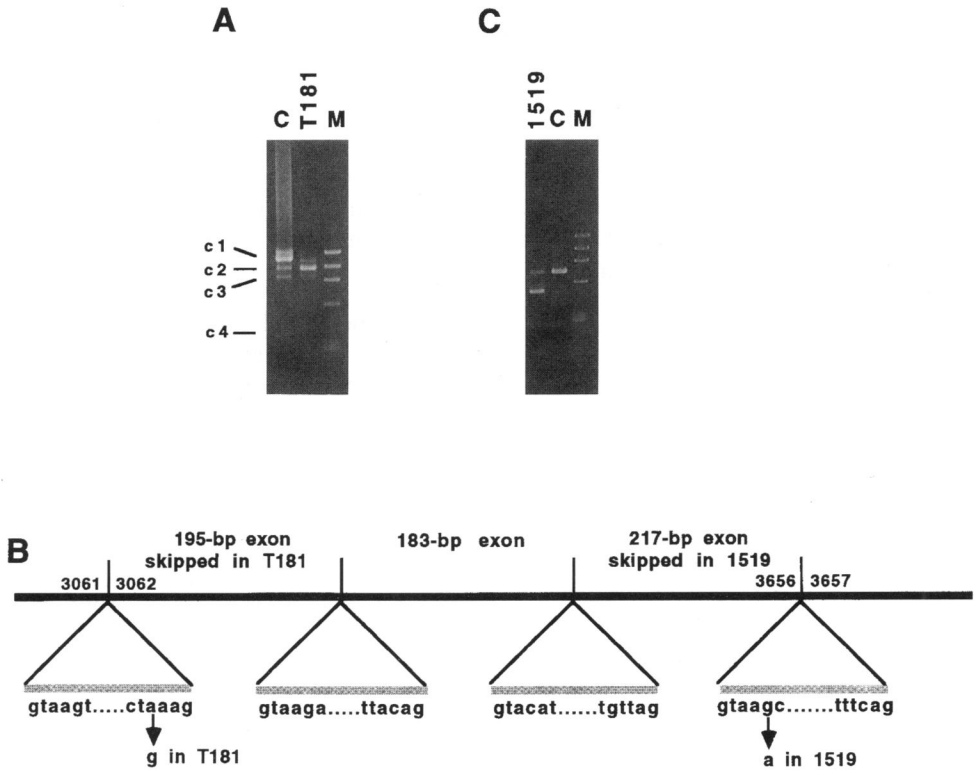

Figure 1 Illustration of splicing defects in two OHS patients. *A*, PCR amplification of segment 3 of the *MNK* cDNA, resulting in a smaller-sized product for patient T181 in addition to a small amount of normal product. A number of RT-PCR products were obtained in the control, where c1 is the normal transcript, c2–c4 are alternate transcript forms, and the larger band represents a heteroduplex formed between the smaller products. c2 is missing a 234-bp exon (nt 2318–2551), c3 is missing a 135-bp and 195-bp exon (nt 2927–3061 and nt 3062–3256, respectively), and c4 is missing a 375-bp region (number of exons not determined and corresponds to nt 2552–2926), the 135-bp and 195-bp exons, and a 183-bp exon (nt 3257–3439). *B*, Schematic diagram showing the mutations in patients T181 and 1519. The solid line represents the cDNA, and the stippled lines represent the introns. The intron positions are noted with cDNA numbering of Vulpe et al. (1993*b*). Approximately 200 bp of sequence at both the 5′ and 3′ ends of the introns were determined in both the control and patient samples, but only 6 nt at the splice-donor and -acceptor sites are shown, along with the corresponding changes in patient samples. The sizes of the introns in which the mutations were found appear to be large, as PCR amplification between the flanking exons in genomic DNA failed to produce a product, even with Klentaq1/*Pfu* (see Patients and Methods). The other two introns are slightly larger than 2 kb. *C*, PCR amplification of a portion of segment four of the *MNK* cDNA, revealing a shorter product for patient 1519, as well as a smaller amount of normal sized product. The PCR primers used for this amplification were 5′-TGTGGGAACTGCTGAAAGTA and 5′-CCTGTTCCGAATAGCAATTCC.

specific primers, with either Taq polymerase or a KlenTaq1/*Pfu* combination (Barnes 1994). For amplification of longer DNA fragments, a KlenTaq1/*Pfu* combination (Ab Peptides) was used in the presence of 10 mM of each dNTP and 15 pmol of each primer, under the following conditions: initial denaturation at 95°C for 10 min, followed by the addition of the enzyme mixture, then followed by 25 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 30 s, and extension at 68°C for 10 min.

To obtain the normal intron sequence for the two very large introns in figure 1*B*, vectorette PCR was performed on an *MNK* gene–containing YAC clone, 231F2 (ICI Laboratories). The YAC DNA was digested with a series of blunt-ended restriction enzymes and was ligated to the *Rsa*I blunt-ended "bubble" primer, essentially as described by Riley et al. (1990). Amplification was performed by using the "bubble"-specific primer 224 and *MNK* cDNA–specific primers. PCR fragments of suitable sizes were sequenced directly.

Testing of the Murine Strains for the +3-Splice-Donor Mutation

Murine samples were tested for the mutation in two ways. First, since the splice-donor mutation in the blotchy mouse generates an *Ava*II site, amplified genomic samples were assayed for digestion with *Ava*II. Mouse genomic DNA was amplified by using a cDNA-specific primer, 5′-GGGCAAAACCTC-CGAGGC, and an intron-derived primer, 5′-CTG-ACCTCCACACATGTGTCAT. The 195-bp PCR product was purified by using the Qiagen PCR purification kit, digested with *Ava*II under standard conditions, and was electrophoresed on a 10% polyacrylamide gel. Second, all samples were tested by using allele-specific hybridization (Wood et al. 1985) using wild-type–specific (5′-TCCTGA-

GGTACTTCATTTG) and Mo^{blo}-specific (5′-TCCTGAGGTCCTTCATTTG) primers.

Results

Mutations in the MNK Gene Detected in Two OHS Patients

Two approaches were taken to find mutations in the copper-transporting ATPase gene of the OHS patients. First, DNA samples from the patients were screened with cDNA probes, by Southern blotting. Normal hybridization patterns were observed in DNA from patients T181 and 1519 (data not shown). In a second approach, mutations were sought by RT-PCR and chemical cleavage–mismatch detection. RT-PCR of the *MNK* cDNA was performed in four overlapping segments that span the coding region, as previously described for analysis of Menkes patients (Das et al. 1994).

For patient T181, RT-PCR of the *MNK* cDNA in the third segment (nt 2323–3562) (Vulpe et al. 1993*b;* Das et al. 1994) resulted in a product shortened by ~200 bp, compared with the normal product (c1) in the control (fig. 1*A*). In addition, a much smaller quantity of a normal sized RT-PCR product was occasionally observed, which was sequenced and confirmed to be normal. Chemical cleavage–mismatch analysis on the other segments showed no changes. Sequence of the smaller RT-PCR product revealed an in-frame deletion of 195 bp, coding for the transduction domain, and analysis of genomic DNA showed that the 195 bp correspond to a single exon. Sequences of the flanking introns were obtained by genomic DNA amplification and by vectorette PCR (see Patients and Methods). Sequence comparison of ~200 bp at each junction revealed only an A-to-G transition at the −4 position of the splice-acceptor site, 5′ of the skipped exon (fig. 1*B*).

It is noteworthy that, in addition to the expected product, three smaller RT-PCR products (c2, c3, and c4) were also faintly observed in the control sample, as well as a heteroduplex formed between the smaller RT-PCR products (fig. 1*A*). Analysis of the smaller fragments showed that they correspond to alternative transcripts, as described in figure 1, legend. The smaller products, which all maintain the *MNK* cDNA reading frame, would produce proteins that lack the third and fourth transmembrane domains (c2); the fifth and sixth transmembrane domains and the critical CPC motif of the transduction domain (c3); and the fifth and sixth transmembrane domains, the CPC motif of the transduction domain, and the invariant DKTGT motif of the phosphorylation domain (c4) (Vulpe et al. 1993*a,* 1993*b*). The two smaller resulting proteins would not be expected to be functionally active copper-transporting ATPases. Interestingly, alternative splicing involving the same exons has been observed in the Wilson disease gene (Petrukhin et al. 1994), which codes for a P-type ATPase very similar to the Menkes protein. The smaller RT-PCR products seen in patient T181 for segment 3 (fig. 1*A*) probably correspond to other alternative transcript forms.

For patient 1519, RT-PCR of the *MNK* cDNA revealed a change between nt 3373–4108 (Vulpe et al. 1993*b;* Das et al. 1994), with normal chemical cleavage results on the remaining segments. Two RT-PCR products were observed, one of the normal size and a more abundant fragment of a smaller size (fig. 1*C*). Subsequent analysis of genomic DNA showed that the smaller-sized band reflects the skipping of a single 217-bp exon, resulting in an out-of-frame deletion of mRNA encoding a portion of the phosphorylation domain. Sequence analysis of the genomic DNA flanking the exon indicated only a G-to-A transition at the +5 splice-donor site, following the skipped exon (fig. 1*B*). The normal sized fragment was also sequenced, and no changes were found. None of 3 control or 12 Menkes samples previously studied showed either of the two splicing mutations seen in T181 or 1519, suggesting that they are unlikely to be polymorphisms.

Splice-Donor Mutation in the Mo^{blo} Mouse

Previously, no alterations were detected in genomic DNA of Mo^{blo}, by Southern analysis using murine cDNA probes (Levinson et al. 1994). Northern analysis showed the presence of three transcripts, one of normal size and two >8.4-kb *Mo* mRNA (Levinson et al. 1994; Mercer et al. 1994).

RT-PCR amplification of *Mo* cDNA in four overlapping segments (as described in Patients and Methods) yielded a smaller product for segment 3 in the blotchy mouse. The region of deleted cDNA was narrowed by a series of further RT-PCR amplifications, using pairs of PCR primers within segment 3. As shown in figure 2*A,* four products—the normal 270 bp, one smaller, and two larger than the normal sized product—were produced in the blotchy RT-PCR sample, with one such pair of primers (see fig. 2, legend). The small PCR product in the blotchy sample was cloned and sequenced. Comparison with genomic sequence from a wild-type animal revealed an out-of-frame deletion of a single 92-bp exon, corresponding to nt 2462–2553 (fig. 2*B*) (Levinson et al. 1994; Genbank accession number U03434), which codes for the region adjacent to the fourth transmembrane domain. A +3 splice-donor mutation (A to C) was detected by sequencing the introns adjacent to this 92-bp exon in the blotchy mouse. The normal sized fragment was also sequenced, and no changes were found.

Attempts to clone the larger two fragments yielded only clones of the smaller, 326-bp PCR product. The sequence of this clone revealed the inclusion of 56 bp of the downstream intron. It appears that a cryptic donor splice site, gtgagt, is activated and results in the larger transcript. The fourth product is likely to be a heteroduplex formed during PCR amplification between the 270-bp and the 326-bp species (Nagamine et al. 1989). This is based on our inability to recover clones of this ~350-bp product, even by using gel-purified fragment DNA. Also, a PCR reaction using purified 270-bp and 326-bp fragments as substrates yielded three products, the two input bands and one the same size as the largest band.

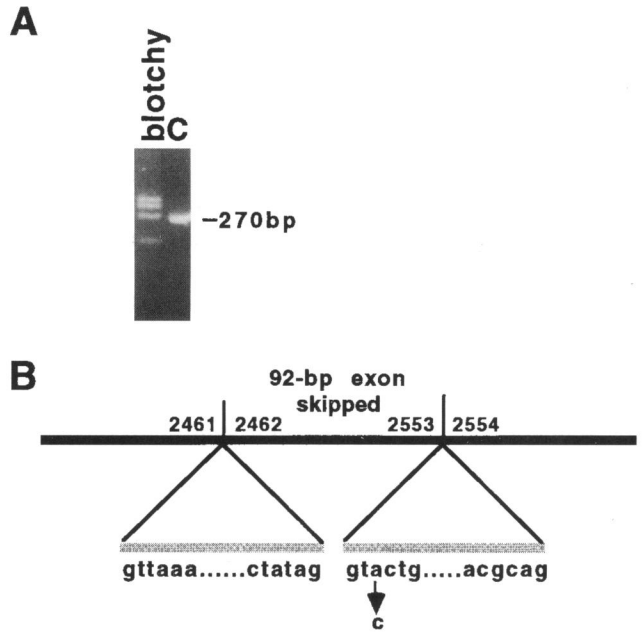

Figure 2 Analysis of a splicing mutation in the blotchy mouse. In *A*, in addition to the normal product, three other RT-PCR products were generated from cultured cells of the blotchy mouse by using primers 5′-GACGATGGCTGGAACATATAGCG and 5′-CTTAGCCACAGGCATTGCCTCC, corresponding to positions 2436 and 2704 of the murine cDNA, respectively (GenBank accession number U03434). The upper band represents a heteroduplex formed between the 326 bp and normal sized products. In *B*, a schematic diagram depicts the location of the splice-donor mutation (A to C) and the skipped exon in the blotchy mouse. The cDNA numbering is according to GenBank sequence U03434 (Levinson et al. 1994). The introns 5′ and 3′ of the skipped exon are ~1.4 kb and ~1.0 kb, respectively. Approximately 200 bp of intron sequence at each boundary was determined, with only the single change in blotchy DNA as shown.

To determine whether the splice-donor change is the causative mutation, rather than a polymorphism, in the blotchy mouse, genomic DNAs from other strains of mice were assayed for the A-to-C transversion. This was done by restriction digestion, as the mutation creates an *Ava*II restriction site, and by allele-specific hybridization, as described in Patients and Methods. The Mo^{blo} sample yielded a 95-bp fragment, after digestion, whereas the other tested mottled alleles (Mo^{dp}, Mo^{ml}, Mo^{br}, Mo^{vbr}, or Mo^{pew}), and wild-type mouse strains (C57Bl/6J, C3H, 101, CBA/J, or PT) remained undigested (data not shown). (The background strain for the Mo^{blo} mutation is unknown). Thus, we conclude that the splice-donor mutation is the cause of the defect in the blotchy mouse.

Discussion

We describe mutations in the splice-donor and -acceptor sites of the Menkes/mottled copper-transporting ATPase gene in two patients with OHS and in the blotchy allele of the mottled mouse. The mutations are not in the invariant GT or AG sequences at the intron boundaries, but rather in the flanking conserved residues, which together with the invariant dinucleotides define a splice-donor or -acceptor site (Senapathy et al. 1990). In each of these three cases, the mutation affects the efficiency of normal splicing and results in two RT-PCR products, one deleted for a single exon and a second of normal sequence. All three of the exon-deleted transcripts would produce a nonfunctional protein, as two are out-of-frame deletions and one (T181) would lack the highly conserved transduction domain. We suggest that these subtle splicing mutations underlie the phenotype in OHS and in the blotchy mouse.

The effect of this inefficient splicing on the final mRNA product appears to be surprisingly variable (table 1). By northern analysis, *MNK* mRNA from patient sample T181 appears grossly normal in size and amount (data not shown). The RT-PCR results for this patient suggest that the predominant mRNA is a 195-bp exon-deleted transcript, a size difference difficult to distinguish from the normal mRNA by northern blot analysis. In contrast, very little *MNK* mRNA, of approximately normal size, is observed in patient 1519 (Levinson et al. 1993). Three mRNA species, one of normal size and two of much higher molecular weight, are seen at reduced levels on northern analysis of mRNA from tissues of the blotchy mouse (Levinson et al. 1994; Mercer et al. 1994). Each species is produced at reduced levels, as compared to the wild type transcript levels. The identity of the higher molecular weight mRNA species is as yet unknown. By RT-PCR, one new transcript was detected in the blotchy mouse that included 56 bp of the adjacent intron, but this finding would not explain the two very large transcripts seen on the northern blot. These transcripts may retain large intron sequences that could not be detectable by RT-PCR, under standard conditions. We do not have an explanation for the contrasting northern findings in these three cases, but they suggest that the exact nature and location of the splicing mutations can affect either the level or the type of transcript. Thus, the data suggest that a reduced amount of normally processed mRNA is present in each of the three cases analyzed.

The mutations described above for OHS and for the blotchy mouse contrast with the debilitating mutations observed in Menkes disease. Partial deletions of the *MNK* copper-transporting ATPase gene were observed in ~15% of patients with classic Menkes disease (Chelly et al. 1993; Vulpe et al. 1993*b*). In addition, by screening patient samples for mutations by RT-PCR and by the chemical cleavage–mismatch technique, we recently identified 10 different mutations associated with classic Menkes disease (Das et al. 1994). These included nonsense mutations, small deletions and insertions resulting in frameshifts, and, of particular interest, splice-donor and -acceptor mutations that alter the invariant GT and AG sequences. Some of the mutations were associated with exon skipping but involved different exons from those found to be deleted in the two OHS patients described, and, in each case, the

Table 1

Summary of Mutations Observed in OHS Patients and Mo^{blo} Mouse

Sample	Location of mutation	Mutation	mRNA by Northern Analysis
Patient T181	Intron before nt 3062	−4, A to G	~Normal amount and size[a]
Patient 1519	Intron after nt 3656	+5, G to A	Very little mRNA, ~normal size[b]
Mo^{blo} mouse	Intron after nt 2553	+3, A to C	Decreased amount, 3 sizes[c]

[a] This paper, data not shown.
[b] Levinson et al. 1993.
[c] Levinson et al. 1994 and Mercer et al. 1994.

normal sized transcript contained a mutation. Thus, all such mutations would be predicted to have a severe effect on the structure or expression of the gene product. Furthermore, expression of the *Mo* gene in the prenatally lethal dappled allele of the mottled mouse is not detectable by northern analysis (Levinson et al. 1994; Mercer et al. 1994). We note that similar splicing defects have recently been observed in a patient with a mild variant of Menkes disease and in a patient with OHS (Kaler et al. 1994). Thus, we would postulate that, whereas no functional Menkes/mottled copper-transporting ATPase protein is produced in Menkes patients or the dappled mouse, a small amount of normal protein is made in patients with OHS and the blotchy mouse.

The question remains, however, of why the mild mutations observed in the OHS patients and in the blotchy mouse manifest primarily as a connective-tissue disorder. OHS patients and the blotchy mouse share the phenotype of connective-tissue defects with Menkes disease but do not manifest the other severe abnormalities. The connective-tissue disease in these three disorders has been ascribed to reduced production and activity of the cuproenzyme lysyl oxidase (Royce et al. 1980, 1982; Kuivaniemi et al. 1982). These considerations suggest that the cuproenzyme lysyl oxidase is particularly sensitive to defects in the activity of the Menkes/mottled copper-transporting ATPase.

Indeed, lysyl oxidase responds differently to copper supplementation than do other cuproenzymes in mutant cells. Copper supplementation restores the activity of the cuproenzymes tyrosinase (Hunt 1977) and cytochrome c oxidase (Meguro et al. 1991) in mottled mutants. In studies of blotchy cultured fibroblasts, copper is fully available for incorporation into superoxide dismutase (Packman et al. 1984), while copper supplementation has little effect on lysyl oxidase in blotchy extracts (Royce et al. 1982) or in the OHS cell lines (Kuivaniemi et al. 1985). These studies parallel observations on copper therapy in patients with Menkes disease. Copper supplementation of patients born prematurely or identified at birth ameliorated many symptoms of the disorder, with the notable exception of persistent connective-tissue defects (Christodoulou et al. 1994; Danks 1994). Copper treatment can similarly prevent the seizures and perinatal death of males carrying the brindled or macular mottled alleles but has no effect on the outcome or symptoms of the blotchy mouse (Hunt 1976; Mann et al. 1979; Royce et al. 1982; Meguro et al. 1991).

Lysyl oxidase differs from other cuproenzymes in that it is secreted, and this difference may explain the comparatively poor response of lysyl oxidase to copper supplementation. We suggest that the Menkes/mottled copper-transporting ATPase could be required for transfer of copper to the specific cell compartment in which holoenzyme is formed. In contrast, other cuproenzymes may be located in the cytoplasm or cellular compartments accessible to the available copper, without requiring an additional transport step mediated by the Menkes/mottled protein. Additional factors that may uniquely affect lysyl oxidase holoenzyme formation are possible apoenzyme instability (Byers et al. 1980; Kuivaniemi et al. 1985), as well as effects of the mutation on transcription or stability of lysyl oxidase mRNA (Gacheru et al. 1993). Therefore, the limited levels of intestinally absorbed copper in OHS patients and in the blotchy mouse ultimately may be insufficient for production or activity of lysyl oxidase yet sufficient for the formation of other cuproenzymes.

Acknowledgments

We thank Dr. Sam Yang, Dr. Louis Elsas II at the Division of Medical Genetics–Department of Pediatrics, Emory University School of Medicine, and Chris Cunniff at the Department of Pediatrics, Section of Genetics, University of Arkansas (currently at the University of Arizona), for providing patient materials. Drs. Herman Dierick and Tom Glover kindly provided sequence data. Dr. Tonne Tonneson kindly provided the results of the copper accumulation test in patient T181. We gratefully acknowledge the assistance of Drs. Bruce Elder and Frank Verley in the mouse work, Woldeselassie Omer for transformation of T181 cells, and Martha Gunthorpe in the preparation of oligonucleotides. This research was supported by grants from the NIH (DK47192) and the March of Dimes Birth Defects Foundation. J.G. is an assistant investigator with the Howard Hughes Medical Institute.

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