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Diverse Mutations in Patients with Menkes Disease Often Lead to Exon Skipping

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Summary

Fibroblast cultures from 12 unrelated patients with classical Menkes disease were analyzed for mutations in the *MNK* gene, by reverse transcription–PCR (RT-PCR) and chemical cleavage mismatch detection. Mutations were observed in 10 patients, and in each case a different mutation was present. All of the mutations would be predicted to have adverse effects on protein expression. Mutations that resulted in splicing abnormalities, detected by RT-PCR alone, were observed in six patients and included two splice-site changes, a nonsense mutation, a missense mutation, a small duplication, and a small deletion. Chemical cleavage analysis of the remaining six patients revealed the presence of one nonsense mutation, two adjacent 5-bp deletions, and one missense mutation. A valine/leucine polymorphism was also observed. These findings, combined with the prior observation of deletions in 15%–20% of Menkes patients, suggest that Southern blot hybridization and RT-PCR will identify mutations in the majority of patients.

Introduction

Menkes disease is an X-linked disorder of copper metabolism, characterized by progressive neurologic degeneration, hypothermia, connective-tissue defects, hypopigmentation, distinctive steely hair, and death in early childhood. The complex clinical phenotype can be attributed to a deficiency of copper-containing enzymes, resulting from a defect in copper transport (Danks 1989).

A gene for the Menkes disease locus (*MNK*), which was isolated by positional cloning (Chelly et al. 1993; Mercer et al. 1993; Vulpe et al. 1993), codes for a copper-transporting ATPase, on the basis of the homology of its derived amino acid sequence to bacterial copper-transporting ATPases (Odermatt et al. 1992, 1993). The pattern of expression of the gene is consistent with the findings of tissue-specific copper sequestration defects and the biochemical phenotype of Menkes disease. Partial gene deletions have been observed in 15%–20% of patients with Menkes disease, and qualitative and quantitative changes in the mRNA have been seen in many patients (Chelly et al. 1993; Mercer et al. 1993; Vulpe et al. 1993; S. Das, unpublished data).

Although the majority of patients with Menkes disease present with the severe, classical symptoms, individuals with milder symptoms or longer survival have been described (Haas et al. 1981; Gerdes et al. 1988) and are likely to have defects in the *MNK* locus. Two patients with X-linked cutis laxa, a copper-transport disorder primarily affecting connective tissue, show diminished levels of *MNK* mRNA (Levinson et al. 1993), implicating the *MNK* gene in this disorder as well.

To begin to understand the relationship between disease severity and the underlying mutations, we studied 12 Menkes patients, of uniformly severe phenotype, who exhibited no abnormalities by Southern analysis. We screened for mutations in *MNK* cDNA, using RT-PCR and chemical cleavage mismatch (CCM) detection (Cotton et al. 1988; Montandon et al. 1989). Alterations were found in 10 of 12 patients, and in 6 cases the mutations led to aberrant splicing. These findings have implications for the molecular diagnosis of Menkes disease and provide a framework for future studies on patients with X-linked cutis laxa and mild or atypical Menkes disease.

Material and Methods

Patient Samples

Sample 1981 is the GM1981 fibroblast cell line obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). All other patient samples are fibroblast cell lines prepared from skin biopsies of severely affected Menkes patients and were obtained following informed consent.

Preparation of mRNA and cDNA

Cultured skin fibroblasts from patients with Menkes disease and from one unaffected individual were used for poly A^+ RNA isolation. Cells were cultured as described

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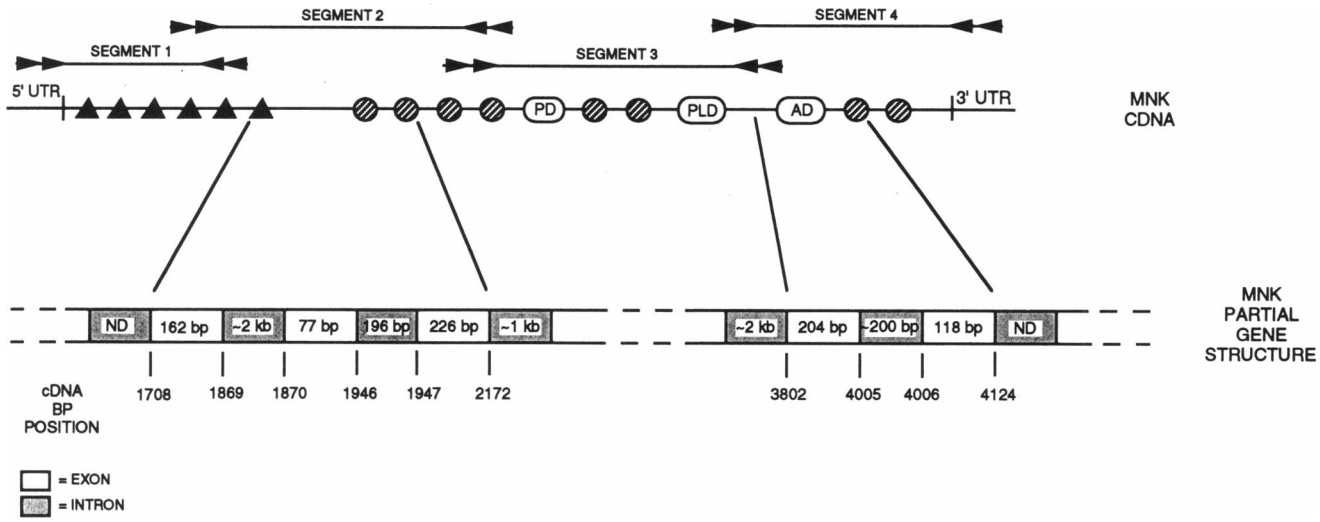

Figure 1 Diagrammatic representation of the *MNK* cDNA and a corresponding partial gene structure. The four overlapping segments amplified by nested PCR are indicated above the cDNA structure. Each segment was 1,200–1,300 bp, a size convenient for mutation analysis by CCM. Segment 1 represents the 5′ end, from nucleotide positions 100–1389, and codes for the first four metal-binding domains (*blackened triangles*). Segment 2, from nucleotide positions 1184–2535, codes for the remaining metal-binding domains and the first four transmembrane domains (*hatched circles*). Segment 3, from nucleotide positions 2323–3562, codes for the phosphatase (PD) and phosphorylation (PLD) domains. Segment 4, from nucleotide positions 3398–4764, codes for the ATP-binding domain (AD) and the 3′ end. The partial *MNK* gene structure is presented below, and the exons are localized relative to the cDNA. The exon/intron boundaries were obtained by PCR of genomic DNA, as explained in the text. The approximate sizes of introns are based on sizing of amplified fragments on agarose gels. ND = not determined.

elsewhere (Packman et al. 1987), and 1×10^7 cells were used for poly A^+ RNA isolation (Pharmacia QuickPrep Micro RNA Purification kit). A 0.5-μg aliquot of mRNA was used for the synthesis of single-stranded cDNA (Invitrogen cDNA cycle kit for RT-PCR).

PCR Amplification

Approximately 30 ng of single-stranded cDNA was subjected to PCR amplification. Nested or heminested PCRs were performed. The primary PCR was carried out in a 50-μl volume, with a 2-min extension time, while the secondary PCR was performed in a 100-μl volume, with a 1.5-min extension time.

The *MNK* gene–specific primers were designed to amplify the coding region in four overlapping segments (fig. 1). Segment 1, from nucleotide positions 100–1389, was amplified using primers 5′-CCACGGTCTGCGTAGTTCCA-3′ and 5′-GATCATACTCAACAGTCCCA-3′, followed by nested amplification with 5′-GGTTTAACCATAGGATAGAG-3′ and 5′-CTATTTGCAAGGGAGACTCG-3′. Segment 2, from nucleotide positions 1184–2535, was amplified with primers 5′-CCAATATGTAAGCAGCATAGTAG-3′ and 5′-AGCCATCGGCCTAGTGCAAT-3′, followed by nested amplification with 5′-ATCACAAGTCAAGTTGAGAG-3′ and 5′-AGCCATCGGCCTAGTGCAAT-3′. Segment 3, from nucleotide positions 2323–3562, was amplified with primers 5′-TATTGTGTGTACCTGTACAG-3′ and 5′-CCAATGAGGACTTTATGCTGC-3′, followed by nested amplification with 5′-CGGAGGCTGGTACTTCTACA-3′ and 5′-GTCCTCTATATTCCAGTTATTC-3′. Segment 4, from nucleotide positions 3398–4764, was amplified with primers 5′-TGTGGGAACTGCTGAAAGTA-3′ and 5′-CCCTAATATAAGAGCATGAG-3′, followed by nested amplification with 5′-GAACACCCTCTAGGAACAGC-3′ and 5′-CCCTAATATAAGAGCATGAG-3′. Amplification was also performed on the genomic DNA of patients and the normal control by using various cDNA-specific PCR primers. PCR products were analyzed by electrophoresis on agarose gels with concentrations of 1%–3%.

CCM Detection Analysis

Chemical cleavage was performed as described elsewhere (Grompe et al. 1989; Montandon et al. 1989), with a few modifications described below. Gel-purified PCR products were treated with agarase, and 100 ng of amplified product from an unaffected individual was end-labeled with [γ^{33}P]-ATP. For heteroduplex formation, 10 ng of labeled DNA (amplified fragment from the unaffected individual) was combined with 200 ng of target DNA (amplified fragment from a Menkes patient). The mixture was boiled and incubated at 65°C for 1 h. Half of the heteroduplex mixture was treated with 0.025% osmium tetroxide at 37°C for 2 h, and the other half was treated with 2.5 M hydroxylamine at 37°C for 45 min. The products were precipitated with 5 μg of tRNA and were cleaved with 1 M piperidine. Products were denatured and run on a denaturing 5% polyacrylamide gel, along with ^{32}P–end-la-

beled *Hae*III-digested φx174 markers. Film was exposed using intensifying screens at −70°C for 16–24 h.

Cloning and Sequencing of PCR Products

For reactions yielding more than one amplification product, 100-μl PCR mixtures were precipitated with 4 M NH_4OAC to remove excess primers prior to cloning. Cloning was performed using the pCR-Script SK(+) Cloning Kit from Stratagene. Sequencing was performed using Sequenase version 2.0 (United States Biochemical) and [α-^{35}S]-dATP.

Direct Sequencing of PCR Products

For reactions yielding a single fragment, PCR was performed using one phosphorylated and one unphosphorylated primer. The PCR product was purified and treated with λ exonuclease to create a single-stranded fragment, according to the procedure described in the PCR Template Prep for ssDNA Sequencing Kit from Pharmacia. The single-stranded product was used as template for the sequencing reaction.

Results

Twelve unrelated Menkes patients, in whom no deletions were previously detected in the *MNK* gene by Southern blot analysis, were tested for mutations in the copper-transporting ATPase gene. Randomly primed cDNA was prepared from fibroblast cell lines, and four overlapping ~1.2-kb segments of the 4.5-kb coding region were independently amplified by PCR (fig. 1).

PCR products of an abnormal size were observed in 6 of the 12 patients. The region of each abnormality was narrowed to 300–400 bp by additional PCR experiments. PCR primers were also used for the amplification of genomic DNA in the six patients. This resulted in the identification of introns (as these were mostly small enough to be amplified) and intron/exon boundaries for the regions examined (fig. 1). Sequencing of all the RT-PCR products and of the corresponding amplified genomic DNA revealed that the altered bands in all six patients were a result of aberrant RNA splicing. None of the aberrant mRNAs were observed by conventional northern analysis, presumably because the changes in size were too small to be detectable, or else because the levels of the aberrant RNA products were too low to be detected (data not shown). The RT-PCR findings, described below, are presented in figure 2 and are summarized in table 1.

For patient 1095, amplification of segment 2 yielded three PCR products. By further PCR amplifications, the altered region was narrowed to exons coding for the last metal-binding domain, through to the second putative transmembrane domain. Sequencing of both the cDNA and genomic DNA revealed a deletion of two bases (AG) in a 162-bp exon. Three alternatively spliced products result: one including the 2-bp deletion, another that skips only the 162-bp exon, and a third that is missing three contiguous exons (162-bp, 77-bp, and 226-bp exons). The 2-bp deletion would lead to a shift in reading frame, while both alternatively spliced products would result in larger, in-frame deletions.

Three additional patients had RT-PCR abnormalities in segment 2, resulting from a skipping of the 77-bp exon. Patient 1286 had a $C_{2078}\rightarrow T$ change resulting in the creation of a termination codon. This change leads to the production of two transcripts, one associated with a fragment of normal size and one with a smaller fragment lacking the 77-bp exon, causing a reading frameshift. (The largest band seen on RT-PCR in this patient represents a heteroduplex formed between the two products; fig. 2). A splice donor mutation (gt→gc), adjacent to the 3′ end of the 77-bp exon, was found in patient 1190 and is associated with a single, smaller RT-PCR product lacking the exon. RT-PCR of mRNA from patient 1433 resulted in two products, one of normal size and one smaller (as well as a larger heteroduplex). The same pattern was observed in an affected cousin of patient 1433 (data not shown). Sequencing of the patient's genomic DNA revealed a direct duplication of five bases (ATAAG) at the splice acceptor site preceding a 226-bp exon. Both products, one that includes the 77-bp exon and one that does not, contain the additional five bases at the 5′ end of the 226-bp exon. The combined deletion of 77 bp and the addition of 5 bp restores the reading frame in this mRNA.

Mutations that result in aberrant splicing were also detected in two patients by RT-PCR of the fourth segment. In patient 1645, a G→A missense mutation ($G_{1302}\rightarrow R$ in the ATP-binding domain) led to two fragments, one of normal size and the other deleted for the 204-bp exon containing this mutation. The deleted product does not change the reading frame. Sequence analysis of genomic DNA from patient 1488 revealed a splice acceptor mutation, ag→gg, in the 5′ flanking intron of a 118-bp exon. A closer examination of the two RT-PCR products indicated that the larger transcript employs the first available cryptic splice acceptor site within the 118-bp exon, while the other transcript skips the exon entirely. Both products lead to a shift in reading frame and a premature stop codon.

CCM was used to screen for mutations in the six patients for whom RT-PCR products were of normal size. Cleaved bands in one of the four segments tested (indicative of base change) were observed in five of the six patients (fig. 3). No change was detected in patient 1107, for whom only full-length bands were apparent (data not shown). The location of the mutations could be estimated from the size of the cleaved fragments in each case. Thus, primers that flank the mutation were used to sequence the amplified fragment of the patient.

The results, as summarized in table 2, are as follows: A

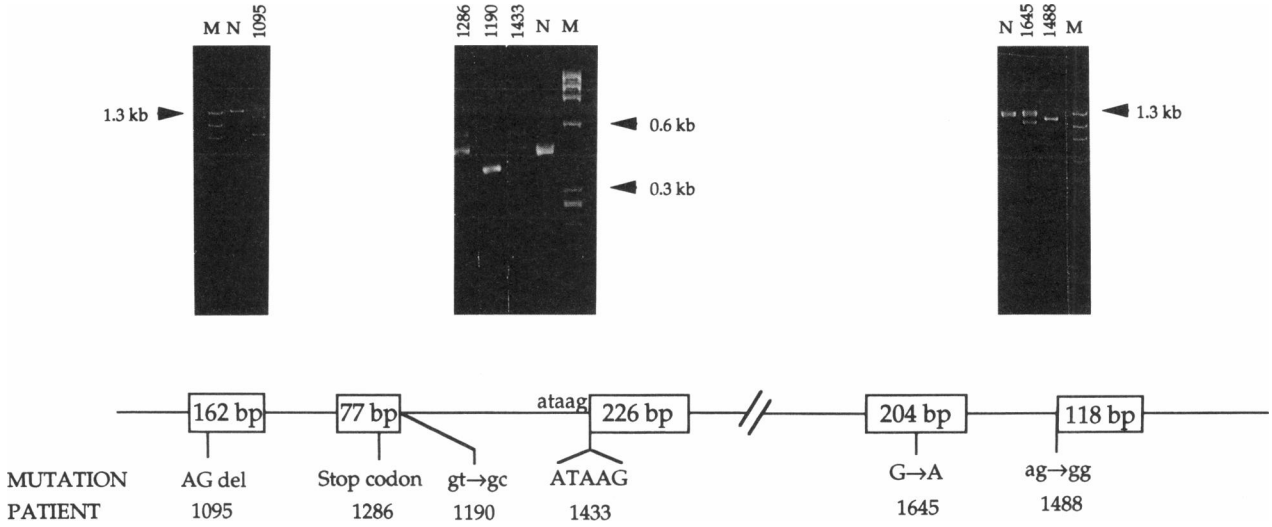

Figure 2 Analysis of mutations in patients 1095, 1286, 1190, 1433, 1645, and 1488, by RT-PCR and sequencing. *Top,* RT-PCR results for the six patients. N = normal control; and M = ϕx174/*Hae*III marker. Patients 1095, 1286, 1190, and 1433 showed abnormalities in segment 2, and patients 1645 and 1488 showed abnormalities in segment 4. Internal primers in segment 2 were used for amplification in patients 1286, 1190, and 1433, as this led to better separation of the different products. The larger bands seen in amplification of DNA from patients 1286 and 1433 are heteroduplexes of the smaller two amplification products. *Bottom,* Intron/exon structure of a portion of the *MNK* gene (not drawn to scale), with the gene oriented 5′ to 3′, from left to right. The exons are depicted as boxes, and the sizes are indicated. The lines between the exons denote introns, and the sizes as observed by PCR of genomic DNA are as follows: ~2 kb for the intron between the 162-bp and 77-bp exons, 196 bp for the intron between the 77-bp and 226-bp exons, ~1 kb for the intron 3′ of the 226-bp exon, ~2 kb for the intron 5′ of the 204-bp exon, and ~200 bp for the intron between the 204-bp and 118-bp exons. The base change observed by sequencing is indicated for each patient.

Table 1

Summary of Mutations Detected by RT-PCR

Patient	Nucleotide Change[a]	Size of RT-PCR Products	Consequence	mRNA by Northern Blot[b]
1095	AG deletion from 1893–1894 in 162-bp exon	Normal	Frameshift after Ile_{582}	No mRNA detectable
		Smaller	Skipping of 162-bp exon—no frameshift	
		Smallest	Skipping of 162-bp, 77-bp and 226-bp exons—no frameshift	
1286	C_{2078} → T in 77-bp exon	Normal	R_{645} → X	Decreased level—one normal and one larger
		Smaller	Skipping of 77-bp exon—frameshift	
1190	CAgt → CAgc junction 2091/2092	Smaller	Skipping of 77-bp exon—frameshift	Decreased level
1433	ataagAT → ataagATAAGAT junction 2091/2092	Normal	Frameshift after Gln_{649}	Decreased level
		Smaller	Skipping of 77-bp exon—restoration of frame	
1645	G_{4049} → A in 204 bp exon	Normal	G_{1302} → R	Normal level
		Smaller	Skipping of 204-bp exon—no frameshift	
1488	agAA → ggAA junction 4150/4151	Normal	Cryptic splice site—frameshift	Normal level
		Smaller	Skipping of 118-bp exon—frameshift	

[a] Lowercase letters indicate sequences in introns, whereas uppercase letters indicate sequences in exons. Numbering of bases is as described by Vulpe et al. (1993).

[b] Data not shown.

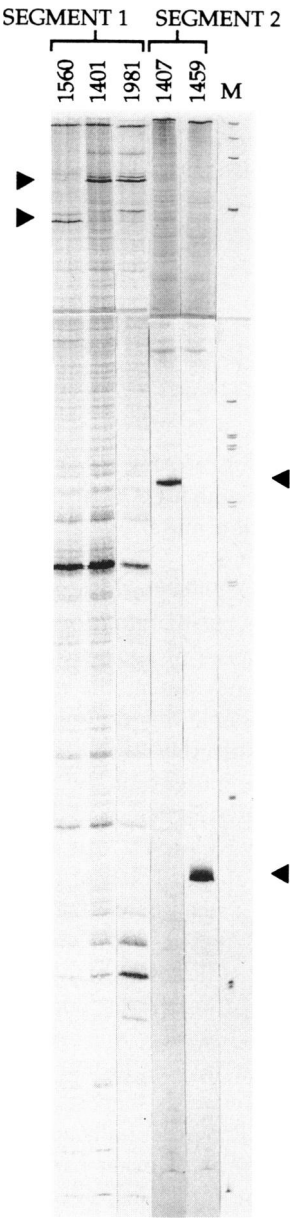

Figure 3 Aberrant cleavage products observed in patients 1560, 1401, 1981, 1407, and 1459, by CCM. Changes were observed in segments 1 and 2. Only the hydroxylamine reactions are presented, as this reaction revealed all of the base changes. The full-length uncleaved fragment can be seen at the top of the gel, and the cleaved fragments are indicated (*arrowheads*). The marker is ϕX174/*Hae*III.

very premature stop codon was discovered in patient 1560, resulting from a C→T change between the sequences coding for the first and second metal-binding domains. Two patients were found to have tandem deletions of 5 bp (ATCTT and ATCTC in patients 1401 and 1981, respectively) following the sequence encoding the second metal-binding domain. Patient 1407 had a G→A change resulting in replacement of glycine$_{727}$ by arginine in the second transmembrane domain. Finally, a G→C change resulting in a valine$_{767}$→leucine conversion in the third transmembrane domain was observed in patient 1459. This conservative, hydrophobic replacement suggested a possible polymorphism. DNA samples from an additional 23 individuals were amplified and assayed, revealing the presence of the C (leucine) in 10 and confirming that this change is a polymorphism. Based on all samples, the predicted frequencies of the valine and leucine alleles are .71 and .29, respectively. Thus, no mutation responsible for Menkes disease in this patient was uncovered.

Discussion

Mutations in the copper-transporting ATPase gene were detected in 10 of 12 patients with severe Menkes disease, and these mutations were all different. These results clearly demonstrate that independently occurring mutations in the *MNK* gene are responsible for the vast majority of cases of Menkes disease. All of the mutations, including nonsense, frameshift, splice site, and nonconservative amino acid replacements, as well as the small deletions and frameshifts generated by alternative splicing, would be predicted to adversely affect the copper-transporting ATPase.

Half of the mutations associated with Menkes disease in this study lead to splicing abnormalities detectable by RT-PCR. Two of the six mutations (in patients 1190 and 1488) alter splice donor or acceptor sites directly. In both cases the mutation results in a skipped exon and a shift in reading frame, and in one case (1488) a cryptic splice site is also used. Three other mutations, a 2-bp deletion, a 5-bp duplication, and a nonsense mutation, are associated with exon skipping but also produced a normal product. The exon skipping keeps the reading frame intact in two of the three cases. Thus, either truncated or partially deleted proteins would result from these mutations. Finally, in patient 1645, a missense mutation resulting in a glycine$_{1302}$ →arginine change, located within the highly conserved ATP-binding site, would be predicted to destroy ATPase activity. A protein lacking 68 amino acids could also be produced from the alternatively spliced mRNA in this patient. We note that the only aberrant PCR products observed for any of the amplified patient samples were those associated with the mutations, suggesting that these are not artifacts, but, rather, that they reflect a consequence of the mutations themselves.

One-third of the patients studied were found to have mutations that resulted in the abnormal splicing of a 77-bp exon, which codes for a region between the sixth metal-binding domain and the first putative transmembrane domain. This finding is unexpected, and only with analysis of more Menkes patients will it become clear whether this region is particularly vulnerable to mutations.

The association of a nucleotide change and exon skipping has been described in a variety of disease genes. Non-

Table 2

Summary of Mutations Detected by CCM

Patient	Nucleotide Change	Consequence	mRNA by Northern Blot
1560	$C_{644} \rightarrow T$	$Q_{167} \rightarrow X$	Decreased level
1401	ATCTT deletion from 798–802	Frameshift after His_{218}	Decreased level
1981	ATCTC deletion from 803–807	Frameshift after Leu_{219}	Decreased level
1407	$G_{2324} \rightarrow A$	$G_{727} \rightarrow R$	Normal level
1459[a]	No change observed		Normal level
1107	No change observed		No mRNA detectable

[a] G_{2444}/C (Val_{767}/Leu) polymorphism was discovered and is described in the text.

sense mutations resulting in exon skipping have been observed in the factor VIII gene in hemophilia A (Naylor et al. 1992), the *FBN*1 gene in Marfan syndrome (Dietz et al. 1993), the *OAT* gene in gyrate atrophy (Dietz et al. 1993), and the *FACC* gene in Fanconi anemia (Gibson et al. 1993). Tumor cells with nonsense mutations in the RB gene (Yandell et al. 1989) and the p53 gene (Fukuda and Ogawa 1992) have also resulted in exon skipping. Missense mutations resulting in exon skipping have occurred in the *HEXB* gene in juvenile Sandhoff disease (Wakamatsu et al. 1992). Deletions within an exon have also resulted in exon skipping (Matsuo et al. 1991). In most of these cases, the loss of an exon(s) generated transcripts that were in-frame and therefore possibly produced a partially functional protein.

Several hypotheses have been put forth to explain exon skipping. It has been proposed that point mutations in exons can lead to an alteration of secondary structure, with consequent aberrations in RNA splicing (Ligtenberg et al. 1990; Steingrimsdottir et al. 1992). In addition, certain nucleotides in the exon are thought to be important for splicing and, when mutated, to affect the normal splicing process (Ligtenberg et al. 1990; Fukuda and Ogawa 1992). Another hypothesis, proposed by Dietz et al. (1993), is that deleterious mutations may be recognized before RNA splicing occurs and that the mutated exon is skipped in an attempt to produce at least a partially functional protein. Many mutations, however, do not result in exon skipping. A combination of the above-mentioned factors, as well as features such as exon length, structure, and the position and type of nucleotide change, may contribute to the exon-skipping process.

Nucleotide changes in 4 of the 12 Menkes patients do not result in a splicing aberration. These changes included a nonsense mutation (patient 1560) and a missense mutation converting a hydrophobic amino acid to a hydrophilic one ($glycine_{727} \rightarrow$arginine) in patient 1407. This missense mutation is probably quite deleterious, as it occurs in the second (hydrophobic) transmembrane domain. Two 5-bp deletions of adjacent and nearly identical sequence (patients 1401 and 1981) at the 5′ end of the *MNK* gene also had no effect on splicing. These deletions could have arisen by "slipped mispairing" during DNA replication of these similar sequences (Farabaugh et al. 1978), and thus we speculate that deletion mutations at this site will be observed in other patients.

No changes on CCM were observed in patient 1107, nor was the disease-causing mutation identified in patient 1459. The *MNK* transcript appeared to be normal in patient 1459 but was not detectable in patient 1107 (data not shown). It is possible that the mutations in these two patients occur outside the coding region of the *MNK* gene, either in the promoter or in the 5′ or 3′ UTR. It is also possible that the mutation occurs in the coding region but was not detected by CCM. CCM is a very sensitive technique and identifies almost 100% of mutations (Condie et al. 1993); however, a small percentage of GT mismatches are sometimes not detected.

Like many X-linked diseases, Menkes disease is characterized by a variety of mutations, complicating molecular diagnostic testing. However, the presence of mutations that lead to aberrant splicing in half of the patients with Menkes disease in this study suggests that rapid molecular diagnosis by RT-PCR is potentially applicable to many patients and their families. If other patient populations exhibit similar types of mutations, a combination of Southern blotting and RT-PCR could enable molecular genetic diagnosis in ~60% of families with severe Menkes disease. In the future it may be possible to use CCM as a routine diagnostic technique as well. In addition, we predict that ~40% of families should be informative for the G-versus-C polymorphism at position 2444, which affects a *Bfa*I restriction site. This polymorphism should augment diagnosis by linkage analysis (Tønnesen et al. 1992).

Almost all of the mutations are associated with a decreased level of mRNA as observed by northern blot analysis. This decrease is observed in patients with nonsense mutations, frameshift mutations, and, with one exception (patient 1488), splice-site mutations. A normal level of mRNA was observed in the two patients (1645 and 1407) with missense mutations. Thus, in this patient population, there is a suggestion of a correlation between the type of mutation and either the level of transcription or mRNA stability.

A recent study of family members affected with a mild form of Menkes disease revealed a +3 mutation at the splice donor site of the 118-bp exon described herein (Kaler et al. 1994). The splice acceptor mutation at the same exon, which leads to only nonfunctional transcripts, results in a severe, classical Menkes phenotype in our patient 1488. We speculate that the mild phenotype in the family studied by Kaler et al. (1994) could be due to the presence of a small amount of normally processed transcript. These and future comparative studies should help to clarify the role of mutations leading to mild and atypical Menkes disease, X-linked cutis laxa, and classical Menkes disease.

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