Multiple transcripts coding for the Menkes gene: evidence for alternative splicing of Menkes mRNA

Manchi C. M. REDDY and Edward D. HARRIS[1]

Department of Biochemistry and Biophysics and Faculty of Nutrition, Texas A&M University, College Station, TX 77843-2128, U.S.A.

We isolated cDNA fragments from four human cell lines that had sequences for the Menkes Cu-transporting ATPase (ATP7A). Primers designed to generate a 4.8 kb cDNA with the complete open reading frame generated a 1.9 kb cDNA in addition to the expected 4.8 kb product. Sequence analysis revealed that the 1.9 kb cDNA encoded one of the six Cu-binding sites and two of the eight transmembrane domains of ATP7A. Stop and start codons were also present. More striking, however, was an unusual union between exons 2 and 16 that retained an in-frame reference to exon 23. The 1.9 kb cDNA thus appeared to be a truncated Menkes mRNA that coded for an ATP7A variant that lacked exons 3–15. A 530 bp probe specific for exon 23 that avoided sequences in the exon 3–15 region hybridized to a 5.5 kb band on Northern blot analysis. Western blotting provided immunochemical evidence for the presence of both a 170 kDa and a 57 kDa protein with ATP7A sequences in detergent extracts of Caco-2 and induced BeWo cells. Extracts from non-induced BeWo cells, which lack the capacity to express the Menkes gene (*MNK*), showed neither protein. In a cell-free reticulocyte lysate, a plasmid containing the 1.9 kb cDNA insert directed the synthesis of a 59 kDa protein with antigenic properties of ATP7A. These studies provide evidence that non-Menkes cells have the capacity to synthesize more than one *MNK* mRNA. The one characterized in this report codes for a 57–59 kDa protein that lacks the core structure of the ATP7A protein. The smaller variant could be an alternative spliced form of *MNK* mRNA.

INTRODUCTION

The transport of Cu ions across endothelial cell barriers such as in the intestinal mucosa and brain capillaries is an ATP-dependent process requiring a membrane-bound ATPase selective for Cu ions. A failure to pass Cu ions completely across the intestinal mucosa leads to a Cu deficiency in peripheral organs, severely impairing neurological, catalytic and connective tissue functions and giving rise to Menkes disease [1,2]. Cloning and sequencing the Menkes gene (*MNK*) has revealed that *MNK* codes for a P-type Cu-ATPase [3,4], one of a family of ion-transporting ATPases whose structural and functional properties resemble the bacterial Cu-ATPases [5]. The Menkes ATPase (ATP7A) transports Cu ions specifically [6,7]. The mRNA for Menkes ATP7A is 8.3–8.5 kb with a single open reading frame (ORF) that encompasses 23 exons for a protein of exactly 1500 amino acids. Strong expression of *MNK* mRNA is seen in muscle, kidney, lung and brain; weaker expression has been associated with placenta and pancreas, and liver shows only traces [3,4,8].

The biological function of the Menkes ATPase is still a matter of debate. Most studies, however, support Cu export as the dominant function, with release occurring at the plasma membrane. The ATPase thus preserves a favourable internal Cu environment. Studies from this laboratory have shown that thiol reagents have the capacity to block the release of Cu from C6 rat glioma cells, presumably by inhibiting the Cu-ATPase controlling release [9]. Others have shown that cultures of Chinese hamster ovary cells selected for over-expression of the Menkes Cu-ATPase are better able to tolerate toxic amounts of Cu in the extracellular environment, possibly by a forced expulsion of Cu ions from the cell [10]. Both observations have helped us understand the likely function of ATP7A in normal Cu homoeostasis.

Screening RNAs from a number of pure human cell lines has led to the discovery of multiple transcripts with ATP7A sequences. Especially prominent was a 1.9 kb cDNA that appeared with the full-length *MNK* mRNA. The nature of this smaller RNA is unknown and its biological significance has not been clarified. Since the 1.9 kb cDNA is seen in cells that do not display a Menkes phenotype, it cannot be a transcript from a mutant allele. Its persistent occurrence in practically every cell we examined has tended to minimize the likelihood that the 1.9 kb cDNA is an artifact of the PCR procedure. Here, we report that the 1.9 kb cDNA has an ORF for a protein that lacks exons 3–15 while maintaining an in-frame link between exons 2 and 16. The evidence suggests that a 1.9 kb cDNA codes for a non-membrane-bound form of ATP7A.

MATERIALS AND METHODS

Cells

BeWo cells were obtained from the American Type Culture Collection and cultivated in 15% fetal calf serum as described previously [9]. To induce *MNK* gene expression [11], the cells were grown on plastic transwell microporous filter surfaces (0.45 μm; Corning, NY, U.S.A.). Cell harvesting and preparation of total RNA have been described previously [12]. Normal fibroblasts were cultured in Dulbecco's minimal essential medium (Sigma, St. Louis, MO, U.S.A.) supplemented with 10% cosmic calf serum (Hyclone, Logan, UT, U.S.A.) to a final density of 1×10^5 cells/dish. Both Caco-2 and HepG2 cells were cultured in

Abbreviations used: ORF, open reading frame; RT, reverse transcription; *MNK*, gene for Menkes disease; ATP7A, Menkes ATPase.

[1] To whom correspondence should be addressed (e-mail eharris@bioch.tamu.edu).

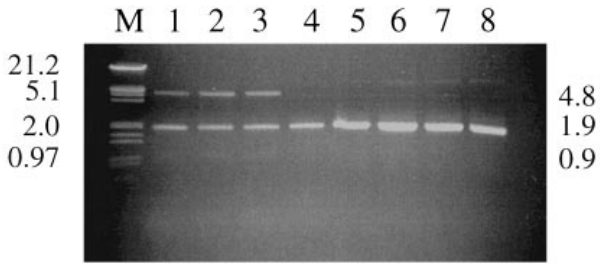

Figure 1 RT-PCR amplification of RNA from human cells

Total RNA from three human cell lines was amplified with primers complementing nucleotides in exons 1 and 23. Lanes 1–4 are cDNAs generated by RT-PCR amplification. Lanes 5–8 show products amplified from cloned cDNAs inserted in pCR3.1 plasmids. RNA was obtained from BeWo cells (lanes 1 and 5), Caco-2 cells (lanes 2 and 6), normal fibroblasts (lanes 3 and 7) and HepG2 cells (lanes 4 and 8). M, marker DNAs. Sizes are shown in kb.

Eagle's minimal essential medium with non-essential amino acids/sodium pyruvate/Earles balanced salt solution/10 % fetal bovine serum, to a final density of 1×10^5 cells/dish.

Reverse transcription (RT)-PCR

Total RNA was extracted from 4–8-day-old confluent BeWo cells, Caco-2 cells, normal fibroblasts and HepG2 cells using a modified guanidinium thiocyanate method [12]. A 5 μg aliquot of total RNA was reverse transcribed to generate first-strand cDNA with the Superscript Preamplification System (murine Moloney leukaemia virus; Gibco-BRL, Grand Island, NY, U.S.A.), slightly modified to include an increased incubation time of up to 2 h for the enrichment of longer species. PCR was accomplished with the eLONGase enzyme (Life Technologies, MD, U.S.A.) for amplification of longer fragments, and Promega Taq DNA Polymerase Amplification System (Madison, WI, U.S.A.) for shorter DNA templates. Primers were designed from the full cDNA sequence as reported by Vulpe et al. [4]. For generating 4.8 kb and 1.9 kb fragments, we used as a forward primer a 20mer that complemented sequences in exon 1 (22–41; 5′-ACTTCTCCGATTGTGTGAGC-3′), and as a reverse primer a 20mer that complemented sequences in exon 23 (4901–4882; 5′-CCTCACTGCTCTACCTTATC-3′). In addition, the following forward primers were utilized to amplify the fusion points between exons 2 and 16, 23mer (CATCACATTAAGGTAA-AGGTAGT), and between exons 2 and 3, 23mer (TCACAT-TAAGGTATCACTGGAAG), both with the same reverse primer (exon 23), as mentioned above. All primers were synthesized by Genosys Biotechnologies, Inc. (Houston, TX, U.S.A.). Amplification of cDNA was performed using 2 μl of reverse-transcribed oligonucleotides and *MNK*-specific primers, 2 units of eLONGase enzyme and a buffer provided by the manufacturer that contained 1.8 mM Mg^{2+}. PCR was carried out in a Perkin-Elmer Cetus Geneamp PCR System 2400 at 94 °C for 1 min (preamplification denaturation); for 25–30 cycles, 94 °C for 15 s, 58 – 65 °C for 30 s, 68 °C for 45–60 s per kb of fragment. The extension step of cycle 25 included the addition of 2 units of Expand Long Template Taq (Boehringer Mannheim, Indianapolis, IN, U.S.A.) and a 12 min incubation at 72 °C to allow TA cloning of the longer templates generated by eLongase. The amplified product was applied to agarose Tris/Borate/EDTA (FisherBioTech, NJ, U.S.A.) gel with Lambda-phage genomic DNA marker cut with *Eco*RI and *Hin*dIII (Promega). Gels were visualized with ethidium bromide.

Cloning of the 4.8 kb and 1.9 kb fragments

Purified PCR fragments of 4.8 kb and 1.9 kb were cloned into pCR 3.1 vector (Eukaryotic TA Cloning Kit, Invitrogen). A ligation reaction was prepared containing 2 μl (60 ng) of pCR3.1 vector, 1 μl of 10 × ligation buffer, 1 μl of T4 DNA ligase, 5 μl of purified PCR product (approx. 120 ng) and sterile water to a volume of 10 μl. The ligation reactions were incubated at 14 °C overnight. 0.5 M 2-mercaptoethanol (2 ml) was added to 250 ml of competent TOP 10F′ cells and mixed by stirring gently with the pipette tip. The ligation reaction mixture (2 ml) was added directly to the competent cells, mixed gently and incubated on ice for 30 min. The reaction mixture was then subjected to heat shock for exactly for 30 s in a 42 °C water bath and immediately placed on ice for 2 min. A volume of 250 μl of SOC medium was added to each vial and incubated at 37 °C for 1 h on a rotary shaking incubator. From each vial, 50 ml was spread on Luria–Bertani (LB) agar plates containing 50 μg/ml kanamycin. Colonies were randomly selected and screened by PCR.

DNA sequencing

RT-PCR products, purified from agarose gels with QIAEX Gel Extraction kit (QIAGEN, Valencia, CA, U.S.A.), were cloned into a eukaryotic expression vector and applied to cycle fluorescent sequencing using the ABI Dye Terminator Kit with AmpliTaq DNA polymerase and primers designed for the individual fragments. Extension products were purified on Sephadex G-50, suspended in sequencing buffer and applied to an Applied Biosystems 377 DNA Sequencer. Sequence data were analysed with GCG Wisconsin Package Version 8.1.

Northern-blot hybridization

Total RNA was isolated from Caco-2 cells, BeWo cells and normal fibroblasts. The partially purified RNA (10 μg) was applied to 1 % agarose gels containing 1 × Mops running buffer. The RNA was denatured using formamide and formaldehyde according to the basic protocol [13]. A probe was prepared by amplifying a 530 bp cDNA fragment from exon 23 of ATP7A using *MNK*-specific primers: forward primer 20mer (4372–4391, 5′-TTACAGGAAACCAACTTACG-3′) and reverse primer 20mer (4901–4882; 5′-CCTCACTGCTCTACCTTATC-3′). The cDNA probe was radiolabelled [α-^{32}P]dCTP (800 Ci/mmol) following a nick-translation procedure (Gibco-BRL). The RNA was transferred on to NYTRAN (Schleicher & Schuell, Keene, NH, U.S.A.) immobilization membrane using the Turboblotter system. Prehybridization, hybridization and filter washing were done using Expresshyb Solution protocol (Clontech, Palo Alto, CA, U.S.A.). The filter was exposed to X-ray film (Kodak, Rochester, NY, U.S.A.) for 3 days.

In vitro transcription/translation of 1.9 kb fragment

A cell-free translation system was used to estimate the size of the protein encoded by the 1.9 kb cDNA cloned transcript. Amplified PCR product was purified and subcloned into a pCR.3.1 eukaryotic expression vector. Purified plasmid DNA was obtained by QIAprep Plasmid Minipreparation (QIAGEN). Plasmid DNA (2 μg) was utilized as template in a coupled TNT reticulocyte lysate system (Promega). The plasmid DNA was added to a mixture consisting of 14 μl of rabbit reticulocyte lysate, 1 μl of TNT reaction buffer, 0.5 μl of RNA polymerase, 0.5 μl of amino acid mixture minus methionine, 0.5 μl of Rnasin, 4 μl (approx. 2 μg) of DNA template in a total suspension of 25 μl with nuclease-free water. Following incubation at 30 °C for

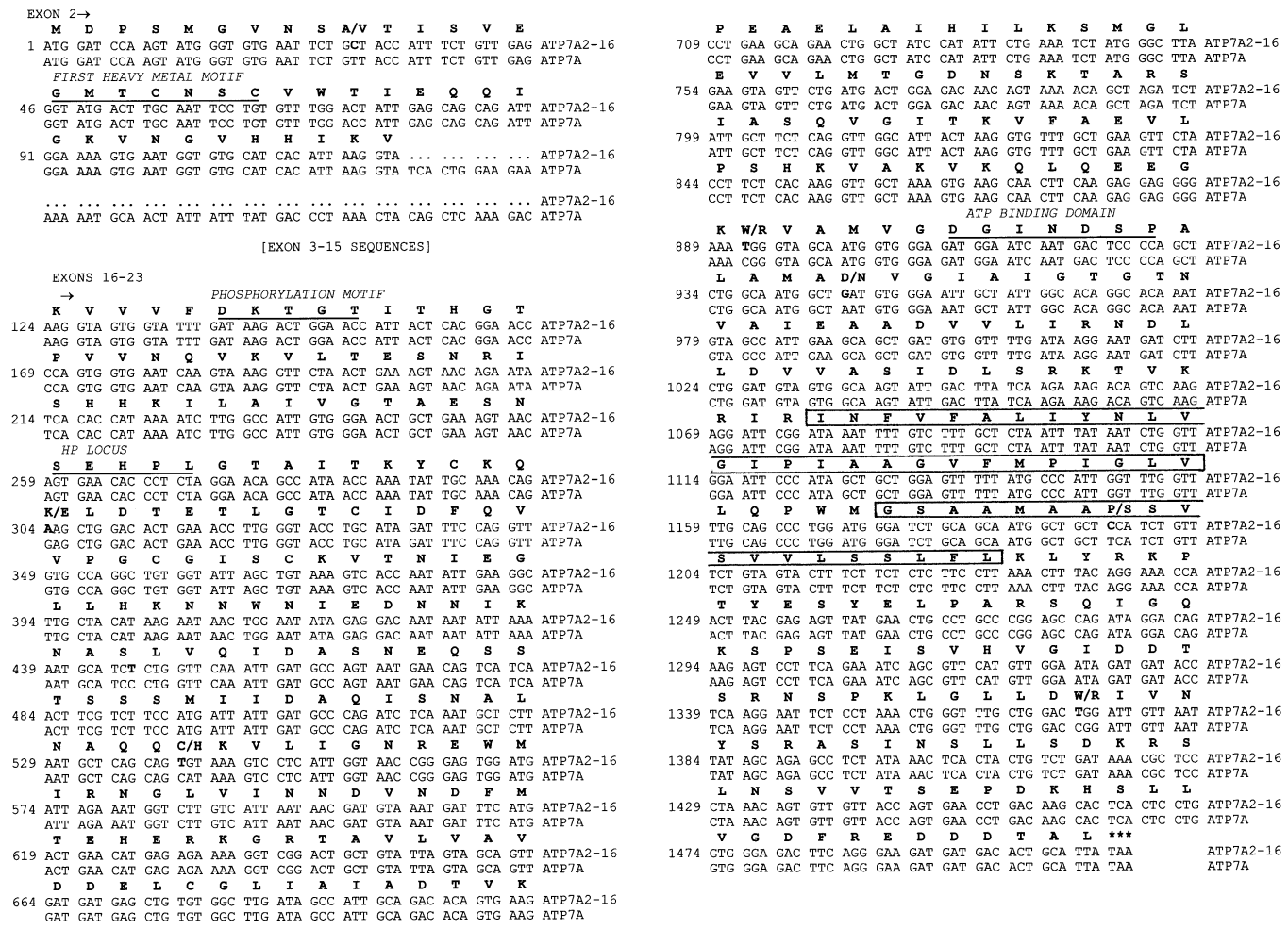

Figure 2 Nucleotide sequence of 1.9 kb cDNA from human fibroblasts

Alignment with ATP7A cDNA. 1.9 kb cDNA product is designated ATP7A2–16. ATP7A sequences were obtained from Vulpe et al. [4]. Structural motifs are underlined. The sequences of transmembrane domains 7 and 8 are in boxes. Bold letters in the sequences are bases in ATP7A2–16 that do not agree with ATP7A. Bases not found in ATP7A2–16 are shown as dots. The amino acid residues for each codon are shown as bold letters above the respective codons. Substitutions that would result in amino acid changes appear above the altered codons. For brevity, the complete exon 3–15 sequence is not shown. Numbers refer to amino acid positions in ATP7A2–16 with the A in the ATG start codon in ATP7A assigned position 1.

2 h, 5 μl was mixed with 20 μl of SDS sample buffer [2.0 ml of glycerol/2.0 ml of 10 % SDS/0.25 mg of Bromophenol Blue/2.5 ml of stacking gel/4 × buffer (6.06 g Tris base/4 ml of 10 % SDS, pH 6.8, in 100 ml of distilled water)/0.5 ml of 2-mercaptoethanol; final volume 10 ml] and heated at 100 °C for 2 min to denature the proteins. Proteins were resolved on a 5–12.5 % gradient SDS polyacylamide gel using Coomassie Blue stain to visualize the proteins. Transblots of translation proteins *in vitro* were resolved by SDS/PAGE and transferred to Immobilon PVDF membrane (Millipore, MA, U.S.A.) utilizing a buffer of 39 mM glycine/48 mM Tris base/0.037 % SDS/20 % methanol in an electroblotting apparatus at 100 mA for 3 h.

Western-blot analysis

Whole-cell lysates were prepared from about 10^8 BeWo and Caco-2 cells. Lysate buffer (50 mM Tris/HCl, pH 8/150 mM NaCl/0.02 % sodium azide) was supplemented with 2 μg/ml leupeptin/1 mM PMSF (prepared fresh)/1 % Triton X-100. Cells were suspended in the 4 °C buffer for 20 min and then sonicated for 15 s at half intensity. After sonication, the suspension was centrifuged at 25000 ***g*** for 30 min at 4 °C. Proteins in the supernatant were precipitated by 4 vols. of acetone and resuspended in 50 μl of Laemmli sample buffer. Approx. 100 μg were applied to a 5–12.5 % gradient SDS/PAGE gel. The resolved proteins were transferred to an Immobilon PVDF membrane. The membrane was immersed in blocking solution [5 % non-fat dry milk in PBS (pH 7.0)/0.02 % sodium azide/0.2 % Tween-20] for 2 h at room temperature and then incubated in 10 ml of blocking solution overnight at 4 °C with the primary antibody (*MNK* rabbit anti-human, kindly provided by Dr. J. Mercer). The antibody was developed from the heavy-metal-binding domains 1–4 in a region of ATP7A that had the least sequence similarity to ATP7B (*WND*, the gene for Wilson disease). The membrane was then washed three times with sodium azide-free PBS and once with Tris/NaCl buffer (150 mM NaCl/50 mM Tris/HCl, pH 7.5). The membrane was incubated with the secondary antibody, goat anti-rabbit alkaline phosphatase conjugate in Tris/NaCl buffer containing 5 % non-fat dried milk for 1 h at room temperature with gentle agitation on a platform shaker. After three washes with Tris/NaCl, the membrane was developed with the substrate bromochloroindolyl phosphate/Nitro Blue Tetrazolium (Bio-

Rad, Hercules, CA, U.S.A.), which generates an intense black–purple colour at the site of enzyme-conjugated antibody binding in approx. 15–20 min. The reaction was stopped with 200 ml of 0.5 M EDTA in 50 ml of PBS.

RESULTS

Oligonucleotides designed to generate a 4.8 kb RT-PCR fragment encompassing exons 1–23 of ATP7A generated at least two strong cDNA bands, the expected 4.8 kb and a more intense

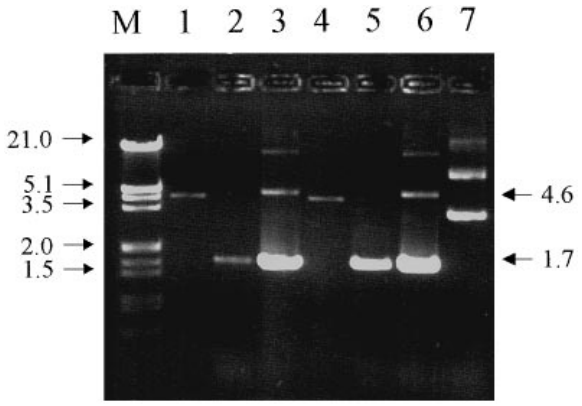

Figure 3 RT-PCR analysis and cloning of *MNK* products generated using exons 2–3 and exons 2–16 junction primers

Lane M, marker, Lambda digested with *Eco*RI-*Hin*dIII; lanes 1 and 4, 4.6 kb PCR product generated using 2–3 exon primers from Caco-2 cells and normal fibroblasts, respectively; lanes 2 and 5, 1.7 kb PCR product generated using 2–16 exon primers from Caco-2 cells and normal fibroblast cDNA, respectively. Lanes 3 and 6, 1.7 kb PCR product amplified with 2–16 primers when cloned plasmids used as templates from Caco-2 cells and normal fibroblasts, respectively. The high-molecular-mass bands seen in lanes 3 and 6 may have originated from the plasmid. Lane 7, fragments derived from empty plasmid alone. See Materials and methods RT-PCR section for more details.

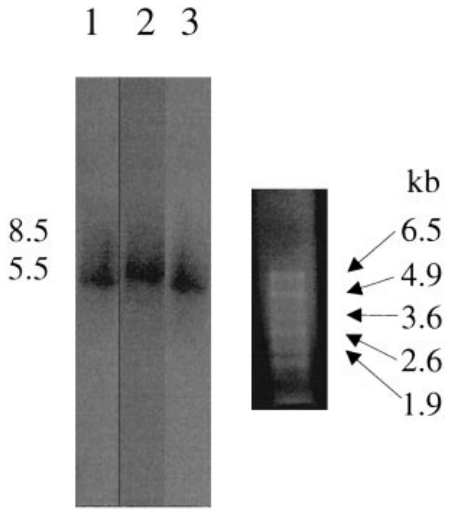

Figure 4 Northern-blot analysis of RNA extracted from human cells

RNA was extracted from BeWo cells (lane 1), Caco-2 cells (lane 2) and human fibroblasts (lane 3). BeWo cells were grown on filters to induce expression of ATP7A. Blots were probed with ^{32}P-labelled cDNA (530 bp) complementing nucleotide sequences in exon 23. Position of cDNA markers is shown on the right.

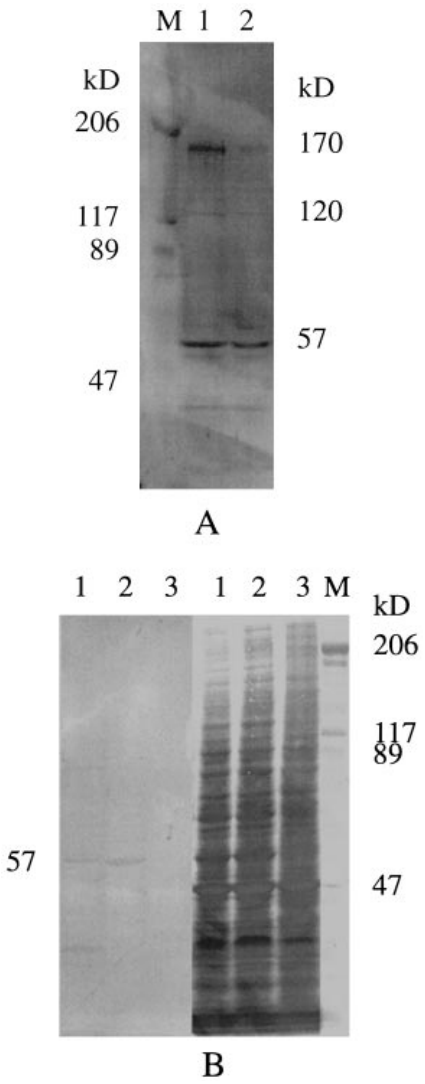

Figure 5 Western-blot analysis of proteins extracted from human cells

Proteins were detected using antibody to the heavy-metal binding domain. (**A**) Whole-cell proteins obtained from BeWo cells treated with 5.0 mM sodium butyrate (lane 1) or cells grown on filter surfaces (lane 2) to induce expression of ATP7A. (**B**) Whole-cell proteins extracted from Caco-2 cells (lane 1), filter-grown BeWo cells (lane 2) and dish-grown BeWo cells (lane 3). The three lanes on the right show the gel pattern when stained with Coomassie Blue. M, marker proteins.

1.9 kb cDNA (Figure 1). On some occasions the 1.9 kb cDNA was the only product seen. The same multi-band pattern was generated from RNAs from normal fibroblasts, Caco-2 cells and filter-grown BeWo cells; human liver cells (HepG2) showed only a 1.9 kb cDNA (Figure 1). Both the 4.8 and 1.9 kb fragments were subsequently cloned into separate TA cloning vectors (pCR 3.1) and sequenced. Figure 1 (lanes 4–6) confirms that 1.9 kb cDNAs from each of the four cell lines have been incorporated into the plasmids. Lanes 5–8 in Figure 1 show bands generated when plasmids with the 1.9 kb insert were used as a template in the PCR reaction. The higher-molecular-mass bands (> 5 kb) in lanes 5–8 correspond to plasmids without inserts.

Plasmid and *MNK*-specific primers were used to obtain the complete sequence data of the 1.9 kb cDNA from human fibroblasts (Figure 2). A comparison was made between the

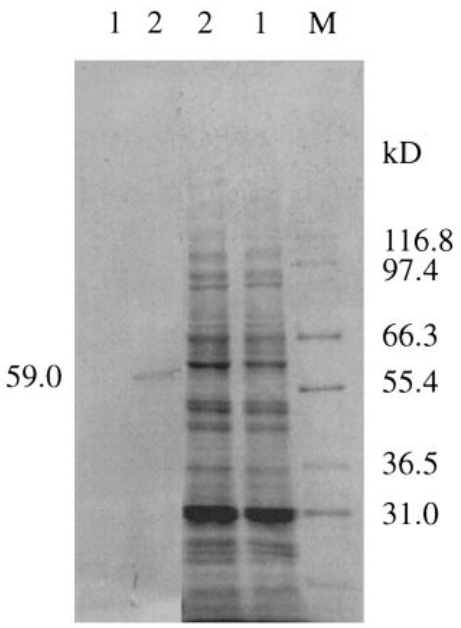

Figure 6 ***In vitro*** **translation product generated by 1.9 kb cDNA insert**

Plasmid alone (lane 1), plasmid plus 1.9 kb insert (lane 2). Detection was by antibody to heavy-metal binding domain of ATP7A. Coomassie Blue-stained proteins are shown in the two lanes on the right. M, marker proteins.

1.9 kb sequences and a full-length *MNK* mRNA from human fibroblasts reported by Vulpe et al. [4]. In Figure 2, the base A in the start codon ATG has been assigned position 1. Based on sequence data, the 1.9 kb fragment had both start and stop codons defining a putative ORF of 1509 bp, which encoded a protein of 503 amino acid residues (theoretical molecular mass 55400 Da). Key domains such as the DKTGT of the phosphorylation site, the SEHPL motif in the large flexible loop, and a DGINDSP of the ATP binding site were present in the 1.9 kb fragment. The TAA at position 1510–1512 matched the position of the stop codon ascertained by Vulpe et al. [4]. The 1.9 kb cDNA, however, had seven single-base substitutions in the coding region and two single base pairs (T-C) in the non-coding region at the 5′ end. The seven substitutions in the coding region would result in a change in the amino acid assignments. Also apparent, the 1.9 kb cDNA had a GMTCNSC motif at position 46–52, which matched the first heavy metal-binding site in ATP7A. This is the only heavy metal site coded by exon 2. Most striking, however, was that the 1.9 kb fragment had a direct link between exons 2 and 16. Thus, a stretch of 2331 bases incorporating exons 3–15 was missing. Despite skipping exons 3–15, the 1.9 kb cDNA maintained ATP7A coded sequences from exon 16 through to exon 23 (Figure 2). The novel splice site at the 2–16 junction obeyed Chambon's basic GT–AG rule [14] with an uninterrupted valine residue at residue position 101.

To determine if the 1.9 kb was an independent mRNA species, we designed oligonucleotides that would pair with bases at the 2–3 and 2–16 fusion points. In the design, 12 nucleotides base-paired with exon 2, 3 with the GTA common to both, and either 8 or 10 were unique to the sequences in exons 16 and 3, respectively. A lower primer complemented exon 23. The theoretical distance was about 4.6 kb between exons 2 and 23 and 1.7 kb from the 5′ end of exon 16. PCR analysis was performed for 25 cycles. Primers to the 2–3 junction generated a band of the expected 4.6 kb cDNA, whereas the 2–16 junction primers generated a much smaller cDNA of about 1.7 kb (Figure 3), the size predicted if exons 3–15 were not present. Lanes 3 and 6 in Figure 3 show fragments of about 1.7 kb generated by PCR amplification with 2–16 junction primers on a plasmid template. The 1.7 kb fragment was obtained when cloned fragments were used as templates from Caco-2 and normal fibroblasts, respectively. Lane 7 shows the empty plasmids, suggesting that the higher-molecular-mass bands in lanes 3 and 6 represent coiled and uncoiled plasmids.

Northern-blot analysis of *MNK* mRNA

To determine if the 2–16 fusion reflected the presence of a unique mRNA for ATP7A, we used PCR to prepare a 530mer oligonucleotide probe with sequences complementary to exon 23. The probe thus lacked the exon 3–15 sequences. A Northern-blot analysis was performed on RNAs extracted from Caco-2 cells, filter-grown BeWo cells and human fibroblasts. As seen in Figure 4, RNA from each cell type displayed a band that migrated at about 5.5–5.7 kb. This appeared to be the major band. There was also evidence for weaker bands representing larger transcripts that hybridized with the probe.

Western-blot analysis of cellular proteins

An important question is whether the ATP7A2–16 transcript coded for an abridged polypeptide with ATP7A sequences. The putative peptide would be expect to have a single binding site for Cu (coded in exon 2), which would allow a reaction with ATP7A antibody that targets this region of ATP7A. Figure 5(a) shows a Western-blot analysis of whole-cell lysates from BeWo cells. Sodium butyrate was used to induce expression of *MNK* mRNA in this cell line, which, unless induced to differentiate, will not express the *MNK* transcript [11]. Several bands were evident, but the greatest stain was to an immunoreactive protein migrating at about 170 kDa. A sharp band of about equal intensity was also seen in the 57–59 kDa region on the gel. In some extracts (lane 2, Figure 5a) the 57 kDa band was the major band and only traces of a 170 kDa band were visible. We also noted that a 57 kDa band without an accompanying 170 kDa band was present when buffers without detergents were used to prepare the extracts of whole cells (results not shown). Figure 5(b) shows that an immunoreactive 57–59 kDa protein was seen in extracts of Caco-2 cells (lane 1) and filter-grown BeWo cells (lane 2), but was not seen when non-induced (dish-grown with no butyrate) BeWo cells served as a source of the protein (lane 3). Although it is conceivable that the lower-molecular-mass proteins are degradation products of a full-length ATP7A, the possibility that the 57 kDa band is an independent polypeptide entity cannot be dismissed. The polyclonal antibody used in the study had been shown to react specifically with ATP7A sequences and will not cross-react with ATP7B, the protein for Wilson disease [15].

Cell-free synthesis

Using a reticulocyte cell-free lysate, we wanted to determine if the 1.9 cDNA coded for a polypeptide with structural properties predicted by its cDNA. A 54 kDa protein would be expected if the cDNA code was in-frame throughout the full 1.9 kb length. If the 2–16 junction was out of frame there is the potential to generate a termination signal that would stop synthesis before 55 kDa. Because it possesses a heavy-metal binding site, the peptide should recognize the ATP7A antibody. Using a plasmid

with the 1.9 kb cDNA incorporated into its structure to direct peptide synthesis, we found only one band that was immunoreactive to the antibody. That band corresponded to a peptide of about 59 kDa (Figure 6). Thus the 1.9 kb cDNA is capable of synthesizing a polypeptide of the approximate size and immunogenic properties of the peptide found in the cells.

DISCUSSION

RT-PCR-generated cDNAs from extracts of non-Menkes human cell lines has provided evidence for at least two cDNAs with ATP7A sequences. A 1.9 kb cDNA has the characteristics of having a single ORF encoding a smaller ATP7A variant. The variant would be expected to be missing exons 3–15, but to have exons 1, 2 and 16–23 intact and in frame. We have tentatively referred to the putative protein product as ATP7A2–16. When compared with ATP7A, we have noticed seven single base-pair substitutions in the coding region of ATP7A2–16 from each of the four cell lines tested. Further sequence analysis of the cloned 1.7 kb fragment confirmed six single base-pair substitutions downstream of the 2–16 junction at identical positions, as shown in the 1.9 kb fragment. At this juncture we have not been able to attribute the significance of substitutions in ATP7A2–16; further investigations will be necessary to clarify its importance.

With exons 3–15 missing, compared with ATP7A, ATP7A2–16 would retain only one of six Cu-binding sites and two of the eight transmembrane (Tm) domains (Figure 7). It would lack a phosphatase domain and the CPC sequence in the channel of Tm6 that moves Cu through the membrane [6]. With these essential domains gone, it is unlikely that ATP7A2–16 could export Cu from cells. However, with its lone GMTCNSC metal-binding motif, it is conceivable that ATP7A2–16 is capable of binding and transporting intracellular Cu. Moreover, the absence of six transmembrane domains suggests that ATP7A2–16 is likely to have the properties of a water-soluble protein and thus have the capability of moving freely in the cytosol, as opposed to having a fixed position in the membrane.

Several points become germane to the existence of an alternative *MNK* mRNA and ATP7A protein. First, Northern-blot analyses that used probes that incorporated a segment for hybridizing to the 3–15 sequences (a 3 kb core in the message) of *MNK* mRNA would have a lower recognition and binding affinity for *MNK* mRNAs lacking these sequences. Binding stringency would also be compromised. When we analysed RNAs from cells using a probe with exon 23 sequences, we found evidence for smaller transcripts. Chelly et al. reported a moderately strong band on Northern blotting that deviated from the expected 8.5 kb band by about 3.0 kb [8]. They further observed that the band was not seen in RNA extracted from all tissues. The 5 kb would be the predicted size for a Menkes transcript that lacked exons 3–15 (total size of exons 3–15 = 2.99 kb [15].

The possibility that the protein product ATP7A2–16 binds and transports Cu cannot be excluded. A 1.9 kb band was seen in every cell line we examined and appeared to exceed the intensity of the full-length transcript. Drawing parallels between ATP7A2–16, with its single Cu-binding site, and HAH1, a recently described human analogue of the yeast Cu-binding protein Atx1a [16], it is conceivable that ATP7A2–16 may be a member of the class of small Cu-binding proteins referred to as Cu chaperones. HAH1 has a single MTCXGC binding motif that is structurally similar to the heavy-metal motif in membrane-bound Cu-ATPases. The 8 kDa HAH1 protein and similar variants are postulated to donate Cu to enzymes, including the Cu-ATPases, by a simple ligand-exchange reaction [5].

Data from this study give evidence for a well-defined 57 kDa band on Western blots that reacts with antibody to a metal-binding region of ATP7A. Moreover, on some occasions, the 57 kDa band appeared without the 170 kDa band and, as noted earlier, there are times when the 1.9 kb fragment is the only product obtained by the RT-PCR analysis. Although a firm correlation has not been established at this time, there is reason to suspect that the occurrence of a 1.9 kb transcript may be coincident with the expression of the ATP7A2–16 protein. This could mean that, depending on the conditions imposed on the cells, ATP7A2–16 may be the only product synthesized.

The implication from the present study is that cells have the capacity to express more than one *MNK* mRNA and hence express more than one form of the Menkes protein, ATP7A. If

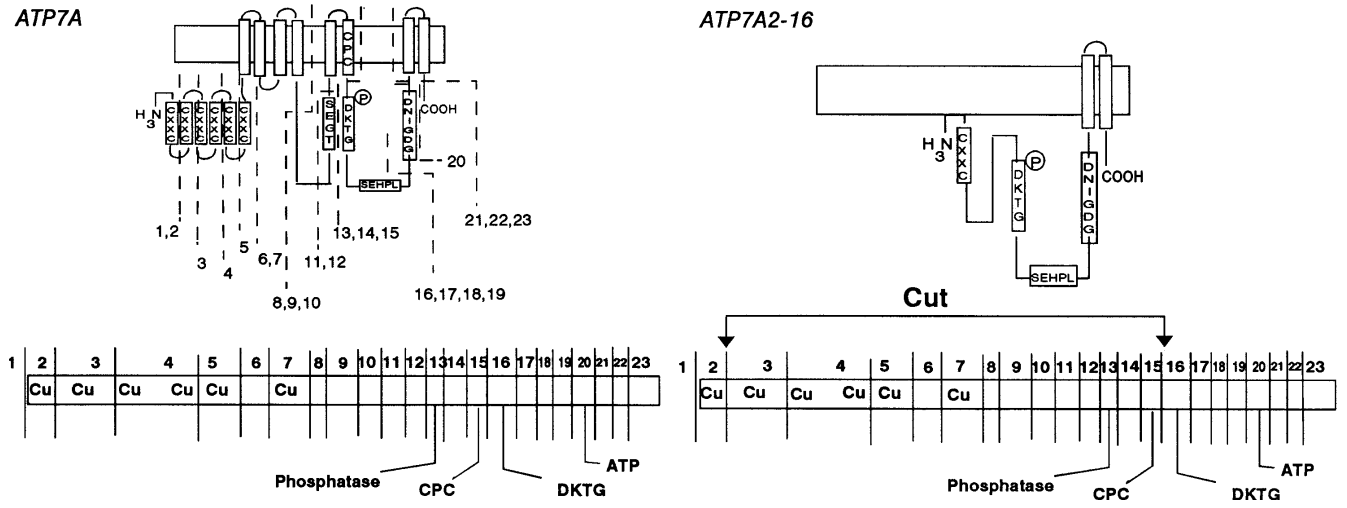

Figure 7 Exon map of ATP7A2–16 compared with ATP7A

Coding sequences for ATP7A are contained in 23 exons. Predicted structure of ATP7A2–16 is shown on the right. Corresponding structural features of ATP7A and ATPA2–A16 are given above each exon map.

multiple forms appear to arise from a single full-length transcript, there is reason to believe that what is referred to as the Menkes gene could be a copper locus that has the capacity to synthesize more than one protein that takes part in Cu transport. Conceivably, the relative quantity of each transcript is governed by the alternative splicing machinery of the cells. That alternative splicing can lead to smaller proteins bearing a GMTCXSC structural motif suggests a single genetic locus codes for cytosolic transport proteins designed to deliver Cu to the membrane-bound ATP7A. Additional research is needed to clarify if specific splicing factors lead to multi-transcripts and if splicing specificity is regulated by the Cu status of the cell as part of a mechanism controlling Cu homoeostasis. Another possibility is a second *MNK* gene which, at this stage in our understanding, cannot be ruled out. It is noteworthy that a 1.9 kb cDNA fragment was found in extracts of RNA from HepG2 cells. Liver from adult humans, as opposed to fetal liver, does not express a Menkes mRNA, but instead expresses ATP7B, the Cu-ATPase associated with Wilson disease [3,17]. Recently, ATP7B mRNA has been shown to code for both a membrane-bound and a soluble protein; whichever is synthesized is determined by alternative splicing of the primary transcript [18]. The data in the present study are compatible with this idea and suggest that *MNK* mRNA is likewise subject to post-transcriptional processing that gives rise to an ATP7A variant that is a soluble cytosolic protein with a specific transport function.

The research was funded in part by the National Institute of Child Health and Human Development Grant HD-29952 and USDA Grant 94–37200–0375. We thank Mr. S. Majumdar and Mr. J. Nelson for lending technical assistance. We also are grateful to Dr. Y. Qian for valuable discussions and suggestions. We would like to express our gratitude to Dr. J. Mercer for providing the antibody.

Received 27 January 1998/11 May 1998; accepted 21 May 1998

REFERENCES

1 Danks, D. M., Campbell, P. E., Walker-Smith, J., Stevens, B. J., Gillespie, J. M., Blomfield, J. and Turner, B. (1972) Lancet **i**, 1100–1103
2 Danks, D. M. (1988) Annu. Rev. Nutr. **8**, 235–257
3 Mercer, J. F. B., Livingston, J., Hall, B., Paynter, J. A., Begy, C., Chandrasekharappa, S., Lockhart, P., Grimes, A., Bhave, M., Siemieniak, D. and Glover, T. W. (1993) Nat. Genet. **3**, 20–25
4 Vulpe, C., Levinson, B., Whitney, S., Packman, S. and Gitschier, J. (1993) Nat. Genet. **3**, 7–13
5 Pufahl, R. A., Singer, C. P., Peariso, K. L., Lin, S.-J., Schmidt, P. J., Fahrni, C. J., Culotta, V. C., Penner-Hahn, J. E. and O'Halloran, T. V. (1997) Science **278**, 853–856
6 Solioz, M. and Vulpe, C. (1996) Trends Biochem. Sci. **21**, 237–241
7 Lutsenko, S., Petrukhin, K., Cooper, M. J., Gilliam, C. T. and Kaplan, J. H. (1997) J. Biol. Chem. **272**, 18939–18944
8 Chelly, J., Tümer, Z., Tonnesen, T., Petterson, A., Ishikawa-Brush, Y., Tommerup, N., Horn, N. and Monaco, A. P. (1993) Nat. Genet. **3**, 14–19
9 Qian, Y. C., Tiffany-Castiglioni, E. and Harris, E. D. (1995) Am. J. Physiol. **269**, C892–C898
10 Camakaris, J., Petris, M. J., Bailey, L., Shen, P. Y., Lockhart, P., Glover, T. W., Barcroft, C. L., Patton, J. and Mercer, J. F. B. (1995) Hum. Mol. Genet. **4**, 2117–2123
11 Qian, Y. C., Majumdar, S., Reddy, M. C. M. and Harris, E. D. (1996) Am. J. Physiol. **270**, C1880–C1884
12 Qian, Y. C., Tiffany-Castiglioni, E. and Harris, E. D. (1996) Am. J. Physiol. **271**, C378–C384
13 Sambrook, J., Fritsch, E. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor
14 Breatnach, R., Benoish, C., O'Hare, K., Gannon, F. and Chambon, P. (1978) Proc. Nat. Acad. Sci. U.S.A. **75**, 4853–4857
15 Dierick, H., Ambrosini, L., Spencer, J., Glover, T. W. and Mercer, J. F. B. (1995) Genomics **28**, 462–469
16 Klomp, L. W., Lin, S. J., Yuan, D. S., Klausner, R. D., Culotta, V. C. and Gitlin, J. D. (1997) J. Biol. Chem. **272**, 9221–9226
17 Kuo, Y., Gitschier, J. and Packman, S. (1997) Hum. Mol. Genet. **6**, 1043–1049
18 Yang, X., Miura, N., Kawarda, Y., Terada, K., Petrukhin, K., Gilliam, C. and Sugiyama, T. (1997) Biochem. J. **326**, 897–902