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Pretranslational control of Menkes disease gene expression

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Abstract

The gene for Menkes disease codes for a Cu-transporting ATPase that regulates Cu homeostasis in all tissues with the exception of adult liver. The basis for developmental or tissue-specific regulation at present is not understood. To learn if the regulation is associated with the promoter, we cloned and sequenced a 2.2 kb genomic DNA fragment flanking exon 1. When ligated into a pGL2 luciferase reporter gene construct, the 2.2 kb showed promoter activity, but not nearly to the extent of a 1.3 kb fragment previously reporter by Levinson et al. Sequence analysis of the nucleotides spanning the region between 1.3 kb and 2.2 kb revealed a 13-nucleotide motif ACACAAAAAAATA 2059 bp upstream from the start site that duplicated the 'hunchback' binding site, a key site controlling developmental gene expression in Drosophila. Eliminating 129 bp containing the hunchback site (Hb) from the 5′ end of the 2.2 kb stimulated promoter activity, suggesting the Hb site was basically suppressive. When ligated upstream of an SV40 and tested in SY5Y cells, however, the SV40 promoter activity was strongly stimulated, which conflicts with the site being suppressive. Mutating the site in the 2.2 kb weakened the promoter activity in SY5Y and HepG2 cells and a fragment with mutated sequence ligated upstream of the SV40 cancelled the activation of SV40 promoter activity. All data suggested the Hb site was a positive controlling site for Cu-ATPase expression. Nuclear extracts from SY5Y and HepG2 cells were observed to bind to a 106 bp probe with the Hb site in a gel-shift assay. Only SY5Y proteins, however, showed a slower moving shift band indicative of a secondary interaction. A probe with mutated sequences displayed the same shift pattern, suggesting other sites in the 106 bp DNA strand were also recognizing the nuclear proteins. A Southwestern analysis suggested that proteins of 125 kD, 70 kD, 50 kD and 42 kD bound to the wild type probe; a 60 kD and all with the exception of the 42 kD bound to the mutant probe. The data support the conclusion that the distal promoter of the Menkes disease gene contains elements that interact in combinatorial fashion to regulate Cu-ATPase expression and that tissue specificity may lie with the quantity or types of distinct DNA binding proteins in the nucleus.

Abbreviations: RAGE – rapid amplification of genomic DNA ends; kb – kilobase(s); PCR – polymerase chain reaction; Hb – hunchback protein DNA binding site; MRE – metal response element(s); TESS – transcription element search system.

The 2.2 kb sequence of the Menkes Disease Gene promoter mentioned in this paper is reported in GenBank, accession number AY039755

Introduction

Menkes disease is a relatively rare X-linked disorder of male infants that results from an irregularity in Cu metabolism (Danks 1995). Cu ions do not cross the intestine and blood brain barriers, which lead to a general disruption of Cu homeostasis in most tissues. The Menkes disease gene (*MNK*) was shown to code for a membrane-bound Cu-transporting ATPase whose structural homology extended back to Cu-transporting proteins in prokaryotes (Vulpe *et al.* 1993, Mercer *et al.* 1993; Chelly *et al.* 1993). The gene transcript encompasses 23 exons encoding a protein of exactly 1,500 amino acid residues. The translation start codon is on exon 2 and a terminating site is in exon 23. The Cu-ATPase product, given the systematic name ATP7A (Lutsenko & Kaplan 1995; Solioz & Vulpe 1996) controls the cellular Cu environment by an energy-driven mechanism that expels Cu ions form the cytosol (Qian *et al.* 1996b). A failure to express ATP7A has been linked to abnormal cellular Cu retention (Goka *et al.* 1976; Horn 1976), whereas overexpression empowers a cell with superior Cu tolerance (Camakaris *et al.* 1995).

Knowing what controls Menkes gene expression in different tissues and during development is one of the least understood aspects of the Menkes gene. Cell-specific regulation is seen by strong expression of ATP7A mRNA in muscle, lung and brain, much weaker expression in placenta, kidney, and pancreas and practically zero expression in mature liver tissue (Mercer *et al.* 1993; Vulpe *et al.* 1993). Mouse liver likewise shows practically no expression of the gene (Levinson *et al.* 1994). The control in development is seen by a failure of undifferentiated BeWo cells to express a full-length ATP7A mRNA, but achieving expression when the cells are induced to differentiate (Qian *et al.* 1996b). With differentiation BeWo gain the capacity to express ATP7A mRNA, which restores Cu efflux function to the cells (Qian *et al.* 1996a). The gene for Atp7a is expressed in all tissues of the mouse throughout embryogenesis, but with maturation, expression in liver is canceled, suggesting a normally active gene becomes inactive as the animal matures (Kuo *et al.* 1997). The basis for select repression of the *MNK* gene during development is not understood.

Multiple transcripts for the Menkes gene

An important clue to *MNK* regulation is that many cells including liver cells express spliced variants of the *MNK* transcript (Reddy & Harris 1998). This suggests that post-transcriptional processing at the level of splicing may be one method a cell uses to regulate ATP7A mRNA levels. Spliced variants, however, have been linked with the disease, as for instance the loss of exon 10 with Occipital Horn Syndrome (OHS), a mild form of Menkes (Qi & Byers 1998). Moreover, exon skipping is found in many patients with classical Menkes disease (Das *et al.* 1994; Tümer & Horn 1997) and the mottled mouse model (*Atp7a*^{mo}) of the disease (Cecchi *et al.* 1997), showing clearly the link between improper splicing and the pathology. Thus, the finding of alternative transcripts in normal, i.e., non-Menkes, cells has made the task of assigning splicing deviations as a hallmark of the pathology more difficult. Moreover, one must question whether the abridged transcripts themselves code for Cu-binding factors that have functional roles in Cu metabolism. That many of the spliced mRNAs retain coding sequences for the Cu-binding domain of ATP7A gives credence to the idea (Reddy & Harris 1998).

Figure 1 shows a gel pattern of RT-PCR products obtained by amplifying poly $(A)^+$ RNA from SH-SY5Y neuroblastoma cells, Caco-2 colonocarcinoma cells, and normal fibroblasts. The transcripts were generated using primers that flanked the exon 1–23 region. A full-length ATP7A mRNA, i.e., exons 1 through 23, appears as a 4.7 kb band (labeled 4.7a in Figure 1). Table 1 gives the composition of the PCR products generated from RNA obtained from SH-SY5Y cells. The stronger band designated 4.5b immediately beneath 4.5a is a full-length transcript that lacks exon 10. As noted above, exon 10 excision has been linked to transcripts from patients with OHS. Here, it is seen that a variant lacking the exon appears prominently in SY5Y cells. A similar observation has been noted earlier in lymphoblasts, fibroblasts and normal brain tissues (Dierick *et al.* 1995), which suggests exon 10 omission alone is not a pathogenic abnormality. The band labeled 1.8 was characterized as lacking exons 3–15, but retaining an in-frame fusion between exon 2 and 16 (Reddy & Harris 1998). The band labeled 0.8a has an insert of 45 bases positioned between exons 1 and 2 and is candidate for a mRNA that codes for an important component in Cu metabolism. The component is discussed in detail below.

NML45

NML45 mRNA (0.8a cDNA in Figure 1) was generated when RNA from cells representing numerous human tissues was primed with primers that amplified the exon 1-23 region of ATP7A mRNA. Thought at first to be an artifact of the PCR procedure, the transcript has been validated by an RNase protection assay and sequence analysis (Reddy *et al.* 2000). The transcript features an open reading frame (ORF) for

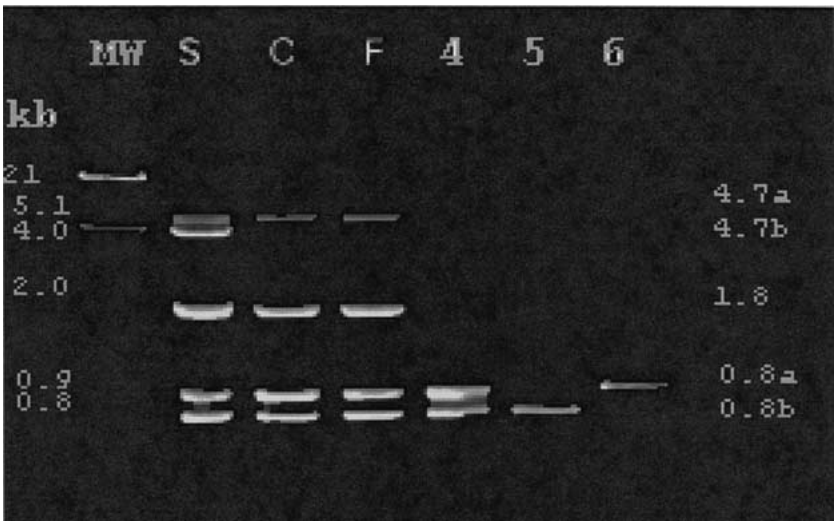

Fig. 1. RT-PCR products from Human cell lines. Lanes 1, SY5Y neuroblastoma cells; lane 2, Caco-2 cells; lane 3, normal fibroblasts; lane 4 purified fragments of 0.8a and 0.8b bands; lanes 5 and 6, 0.8b and 0.8a fragments amplified from clone. Band labeled 4.7a corresponds to full-length ATP7A; 4.7 b lacks exon 10. Note the presence of this band in neuroblastoma cells and its apparent absence from Caco-2 cells and normal fibroblasts.

a 103-residue peptide. The 5′ region of the transcript has Menkes-like sequences with the exception of a 45 bp nucleotide inserted between exon 1 and exon 2. An ATG on the 5′ end of the insert is in-frame with the downstream ATP7A start site. Because the ATP7A translation start site is 21 bases into exon 2 (Vulpe *et al.* 1993), the 66 (45 + 21) bases are positioned to code for a 22 amino acid N-terminal extension to the protein, i.e., assuming the upstream ATG is an alternative translation start site. Within the 22 amino acid extension is the sequence RK. . . RKRP, which features basic residues (arginine and lysine) set apart and flanked by a proline residue. This sequence motif has the features of a classical nuclear localization sequence (NLS), a structural feature that is necessary for cytosolic peptides to be transported between the cytosol and the nucleus of eukaryotic cells (Moore 1998; Weis 1998). Cytogenetic expression with a green fluorescent protein (GFP) constructs fused to NML45 cDNA gave evidence for a protein with nuclear penetrating properties. A similar construct without the NLS motif is incapable of nuclear translocation. These data have provided evidence that NML45 peptide is capable of penetrating the nucleus, but only when the putative NLS is positioned on the N-terminus (Reddy *et al.* 2000). The significance of this observation is unknown, but serving as a shuttle or nuclear chaperone for Cu, Zn, Mn or acting as a transcription activator/suppressor of Cu related genes are meaningful ideas to investigate. Because of its apparent nuclear targeting capability and 45 bp insert, we have named the peptide NML (nuclear-Menkes-like) 45. NML45 transcript is predicted to encode an 11.2 kDa protein that has an out-of-frame link between exon 3 to exon 22. A cysteine-rich region with the motif CxxxCxxCxC, which could represent a binding site for heavy metals, further distinguishes the C-terminal half. A diagrammatic representation of NML45 is shown in Figure 2.

The Menkes gene promoter

Regulating elements that control pretransciptional expression of the Menkes disease gene undoubtedly are within the promoter region of the gene. Surprisingly, the *MNK* promoter has received little attention. In one of the few published studies Levinson et al isolated a 3.5 kb fragment from an X-chromosomal library and subcloned a 1.3 kb sequence upstream of exon 1 (Levinson *et al.* 1996). Sequence analysis revealed a GC-rich region near the transcription start site at exon 1. Within the promoter region was a unique 98 bp tri-repeat, each segment of which contained one AP-1 site and sites for binding a cAMP responsive protein. The initial characterization also gave evidence for metal response elements (MREs) harbored within E-box sequences. When ligated upstream of a CAT reporter gene, the 1.3 kb sequence drove

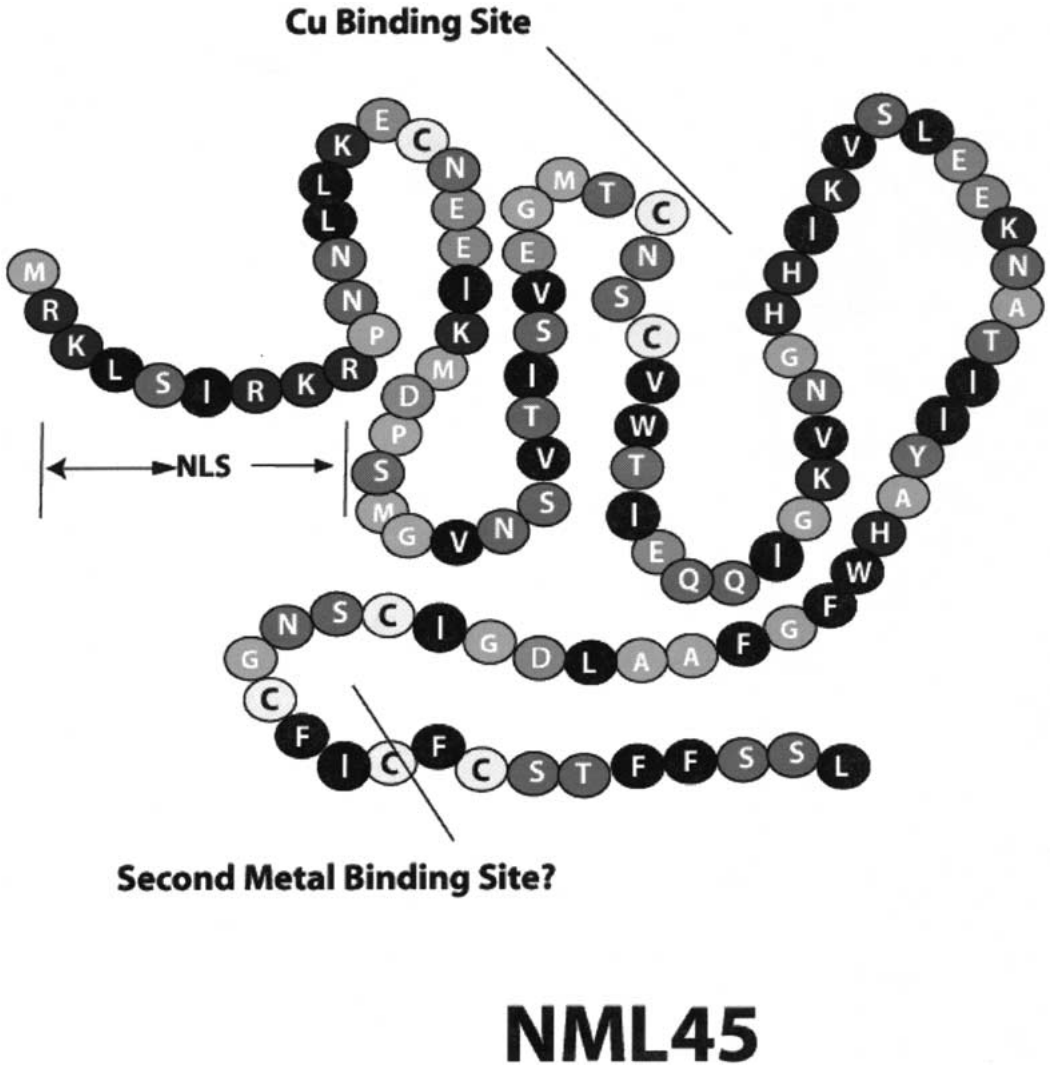

Fig. 2. Primary structure of NML45. Identified in the figure are the nuclear localization sequence in the N-terminal, the Cu-binding site and the cysteine-rich heavy metal binding sites. The protein has a predicted molecular mass of 11.2 kDa.

CAT gene expression. Importantly, a patient known to have occipital horn syndrome, a mild form of Menkes disease, had one of the 98 tri-repeat regions deleted from the transcript and this deletion reproduced in the constructs negated reporter gene expression (Levinson *et al.* 1996).

In our analysis of the 5′ flanking sequences of exon 1, we corroborated the existence of a tandem 98 bp tri-repeat upstream from exon 1. We also obtained evidence for a second metal-responsive site TTTCATGCTG that in the yeast *S. pombe* has been shown to respond to low Cu status (Beaudoin & Labbé 2001). This site is within the 98 tri-repeat region. On the assumption that distal elements in the promoter play an important role in the regulation, we used RAGE analysis to obtain a fragment that extended 2150 bp 5′ to exon 1. Approximately 2.1 kb from the start site is the sequence ACACAAAAAAATA (Figure 3). This sequence is the mammalian ortholog of the 13-bp consensus sequence ACNCAAAAAANTA known to be a sequence-specific DNA-binding site for a product of the Drosophila gap gene *hunchback* (*hb*). Operating through this site the gene governs early segmental development along the body axis (Treisman & Desplan 1989). By itself the hunch protein, a zinc-finger transcription factor, activates the transcription mechanism. In combination with two other proteins (*bicoid* and *Krüppel*), it forms a transcription repres-

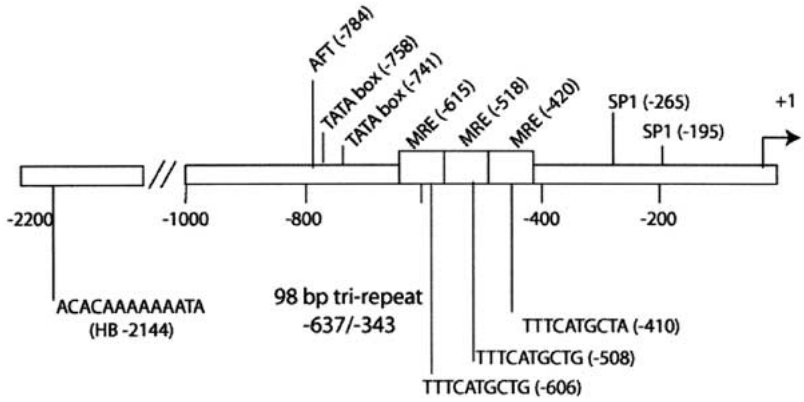

Fig. 3. Promoter region of the Menkes Disease Gene. The sequence extends 2.2 kb upstream from the transcription start site on exon 1. Identified are the 98 bp tri-repeat region with metal responsive elements (MREs) and Cu sensing motifs TTTCATGCTG/A. In the distal region is shown the putative hunchback protein-binding site. Arrow indicates transcription start site at exon 1.

Table 1. SY5Y cDNAs and potential protein products deduced from PAGE analysis.

Band	Exons Present	Feature	Estimated Mwt (kDa)
4.7a	1–23	Intact ATP7A	170
4.7b	1–9, 11–23[a]	lacks exon 10	145
1.8	1–2, 16–23[a]	lacks Exons 3–15 8 non-conforming bases	57
0.8a	1–3, 22[b]	45 bp insert at 5′ end	11.2
0.8b	unknown	192 bp insert at 5′ end	unknown

[a]In frame
[b]Out of frame stop codon

sor complex. An ortholog of the hunchback protein, *leech zinc finger II* (LZF2) controls CNS development in leech (Iwasa *et al.* 2000). In higher animals, the C-terminal zinc finger domain of 'Ikaros' is related to the hunchback protein of Drosophila. Ikaros one of a family of Zn-finger, DNA-binding protein forms a heterodimer with a second factor Aiolos to regulate the production of T and B lymphocytes and natural killer cells (Morgan *et al.* 1997), suggesting proteins with the hunchback binding motif have diverse regulatory roles in mammals. One is led to suspect the hunchback site could be a factor in tissue specific expression of *MNK*.

A related study from the same laboratory (Levinson *et al.* 1997) reported the sequence of the mouse promoter for the Menkes gene analog, Atp7a. (accession number U95086). Unlike the human, the mouse promoter has no tri-repeat, but is endowed with a GC-rich region and other binding sites for DNA factors that are found in the human promoter. An unusual feature of the mouse promoter not cited in the original paper is the base sequence TTTTTTAGTC in the distal 5′ region of the promoter. A TESS analysis of bases in the region identified the sequence as the reverse complement of GACTAAAAAA, which closely matches the 10-bp consensus sequence G/A C/C ATAAAAAA reported for Drosophila (Štanojević *et al.* 1989), suggesting that the Drosophila *hunchback* is an evolutionarily conserved site.

Functional tests of promoters

Levinson *et al.* studies revealed regions in the tri-repeat area, which when deleted, seriously impaired expression of the reporter gene. We have used luciferase reporter gene to basically repeat the analysis with the extended 2.2 kb promoter. An immediate focus was the role of the putative mammalian hunchback protein-binding site. Figure 4 shows a composite of a number of experiments that have led to the conclusion the Hb site is basically an activating site, but within the confines of the 2.2 kb promoter, the site represses the

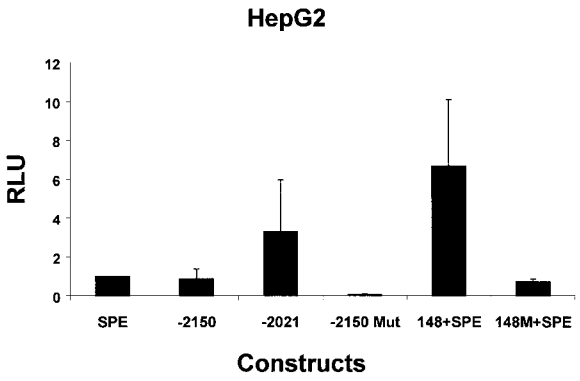

Fig. 4. Luciferase Reporter Gene Analysis of *MNK* Promoter HepG2 cells were used as hosts. Cells were transiently transfected with pGL2- plasmid containing test elements upstream of a luciferase reporter gene driven by the following: SPE, early SV40 promoter; −2150, *MNK* full promoter; −2021, full lacking 148 bp at 5′ end; −2150 Mut, full with mutated+ Hb sequences; 148 + SPE, wild type 148 bp ligated upstream of SV40; 148M + SPE , mutated 148 ligated upstream of SV40.

activity of the reporter gene. The conclusion was based on a paradox, which showed that removing 148 bases containing the Hb site from the 5′ end of the 2.2 kb promoter strengthened promoter activity. Mutating 4 of the 13 bases within the site, however, weakened promoter function, suggesting that the Hb site activated promoter responsiveness. These data would also suggest that repressive sites in close proximity to the Hb site were influencing expression. In a further study, Hb wild type and mutated fragments were ligated upstream of an early SV40 promoter that was used to drive a luciferase reporter gene. When tested in HepG2 cells, the wild type sequences stimulated the SV40 promoter activity, confirming the Hb's role as an enhancing element. The mutated sequences, however, failed to activate SV40 promoter function. The data, albeit preliminary, support the hypothesis that the Hb site (and neighboring distal sites) coordinate with elements in the proximal region to control expression of the *MNK* gene. The studies, therefore, suggest a hierarchy to promoter functions with newly discovered distal elements modulating elements further downstream.

Conclusion and future studies

Two basic mechanisms are likely candidates for controlling Menkes gene expression at the pretranslational level of cell function, promoter activation (or suppression) and alternative splicing. The proximal promoter region consists of a series of E-boxes, AP-1 sites, and MREs within the confines of a 98 bp tri-repeat. A distal region (2.2 kb upstream) has a sequence motif that matches a hunchback protein-binding sites (Hb) and which appears to be capable of regulating luciferase reporter gene constructs. The distance disparity of elements suggests promoter functions involve a complex coordination of distal and proximal sites with nuclear protein factors consistent with cell-specific regulation.

Yet to be understood is the biochemical basis for Menkes disease gene expression in tissues. Liver cells fail to express the gene, despite having the capacity to recognize and activate the Menkes gene promoter (Figure 4). Instead, adult liver express ATP7B, the Cu-ATPase analogue associated with Wilson's disease and, like ATP7A, controls movement of Cu into vesicles or exports Cu (as biliary Cu) from the cell (Petrukhin *et al.* 1994). This observation along with the appearance of abridged transcripts with ATP7A sequences seems to suggest that posttranscriptional as opposed to pretranscriptional regulation is a primary mode of controlling the expression of ATP7A protein levels in liver and other tissues. To assume, however, that the sole purpose of splicing is to regulate the full-length ATP7A mRNA is to overlook what may be uniquely structured transcript variants that code for other proteins that play Cu transport or regulatory roles. NML45 is one such transcript product that localizes to the cell nucleus. With its two binding sites for metals as sequence data would suggest, NML45 is poised to transport metals such as Cu, Zn, or Mn into the nucleus or serve as a reservoir for these and other essential heavy metals in the nuclei. It is imperative, therefore, to determine if the other transcripts and translation products mentioned in Table 1 have functional roles in Cu metabolism.

Finally, research has now reached the stage where the splicing mechanism that controls the processing of the *MNK* transcript needs to be characterized. The inclusion of a 45 bp nucleotide between exon 1 and exon 2 is governed by a spliceosome assembled within intron 1. The properties of the spliceosome and factors that control its assembly are not known and need to be understood to determine when a cell synthesizes the full length transcript or when a spliced variant that includes the 45 bp insert is produced. What controls the splicing could be critical to providing additional insights into the mechanism of Cu homeostasis in mammalian cells.

Acknowledgements

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