

Short Communication

## Expression of the Human Menkes ATPase in *Xenopus laevis* Oocytes

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**Menkes disease is an X-linked disorder of copper metabolism that is usually fatal. The affected gene has recently been cloned and encodes one of the two human copper ATPases. If the Menkes ATPase is defective, copper is trapped in the intestinal mucosa, leading to systemic copper deficiency. In order to study copper transport by this ATPase and the effects of disease mutations on its function, we developed a *Xenopus laevis* oocyte expression system. Wild-type Menkes ATPase cDNA and a fusion of this gene with the green fluorescent protein (GFP) gene was transcribed *in vitro* and the mRNA injected into oocytes. Expression in oocytes was analyzed by Western blotting and fluorescence microscopy. The Menkes ATPase-GFP chimera appeared to localize primarily to the plasma membrane as assessed by confocal microscopy. This system should thus provide an interesting new tool to study the function of the Menkes ATPase.**

**Key words:** ATPase/Copper/Green fluorescent protein/Menkes/Oocytes.

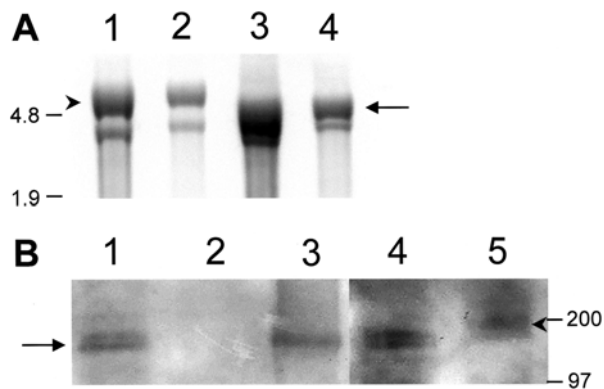
Menkes disease is a X-linked recessive disorder in copper metabolism that results in severe systemic copper deficiency. Copper is an essential cofactor in many enzymes, such as cytochrome c oxidase, superoxide dismutase, or lysyl oxidase (Linder and Hazegh Azam, 1996). The copper deficiency in patients with Menkes disease leads to connective tissue defects, severe neurological abnormalities, developmental delay, seizures, hypothermia and the characteristic depigmented kinky hair (pili torti). Menkes disease is clinically heterogeneous and can present in mild forms like the occipital horn syndrome; however, most patients (>90%) present with the classical severe form. These patients usually die before the age of 3 years.

The gene defective in Menkes disease was cloned in

1993 and encodes a copper ATPase (MNK, Menkes ATPase, or ATP7A) that is expressed in most tissues, including muscle, kidney, placenta, intestine, but not in the liver (Chelly *et al.*, 1993; Mercer *et al.*, 1993; Vulpe *et al.*, 1993). MNK is a protein of 1500 amino acids encoded by 23 exons (Dierick *et al.*, 1995), and its primary location is in the *trans*-Golgi network. When cells expressing Menkes ATPase are challenged by increased copper levels, the enzyme relocates to the plasma membrane by vesicle trafficking (Petris *et al.*, 1996, 1998). The significance of this trafficking in relation to copper transport is not fully understood.

If the Menkes ATPase is defective, copper is still absorbed by intestinal cells, but cannot be distributed to other tissues. More than 150 mutations causing Menkes disease have so far been identified (Tümer *et al.*, 1999; Moller *et al.*, 2000). There is ample evidence, albeit indirect, that Menkes ATPase is a copper pump. A role of the Menkes gene in copper extrusion was initially suggested by the observation that fibroblasts of Menkes patients accumulated more copper than wild-type cells (Goka *et al.*, 1976; Horn, 1976). More recently, it was shown that overexpression of the Menkes ATPase gene in Chinese hamster ovary cells renders them resistant to normally toxic copper levels (Camakaris *et al.*, 1995). Furthermore, it has been shown that membrane vesicles of such cells accumulate more copper than vesicles from normal CHO cells (Voskoboinik *et al.*, 1998). Finally, the Menkes gene can functionally complement yeast mutants defective in the orthologous CCC2 copper ATPase (Payne and Gitlin, 1998). Up to now, there have been few direct biochemical studies of the Menkes ATPase (Voskoboinik *et al.*, 1999).

We thus developed a *Xenopus laevis* oocyte expression system for biochemical analysis of the ATPase. To localize the enzyme in oocytes, we constructed a C-terminal fusion of the Menkes protein with green fluorescent protein (Menkes-GFP). The wild-type Menkes cDNA and the Menkes-GFP gene were transcribed *in vitro* with a 5'-cap structure followed by a polyadenylation step to generate stable, highly translatable RNA (Drummond *et al.*, 1985). Full-length transcription was verified by gel electrophoresis of the radiolabeled mRNA (Figure 1A). The mRNA exhibited a major band of approximately 5.1 kb, corresponding to the full-length transcript, and a minor band slightly below 5 kb. The minor band could result from transcription initiation from a cryptic promoter or premature termination of transcription. As expected, a shift toward larger transcript size by 0.7 kb was observed



**Fig. 1** *In vitro* RNA Synthesis and Expression of Menkes ATPase in Oocytes.

(A) Autoradiography of a 1.2% agarose gel. Lane 1, 1 µg of Menkes-GFP mRNA, not polyadenylated; lane 2, 0.3 µg of Menkes-GFP mRNA, polyadenylated; lane 3, 2.5 µg of Menkes mRNA, not polyadenylated; lane 4, 0.6 µg of Menkes mRNA, polyadenylated. The arrow indicates the full-size Menkes mRNA band, the arrow head the Menkes-GFP mRNA band, and the scale on the left side the migration of rRNA markers of the corresponding sizes in kilobases. Methods: the wild-type Menkes ATPase gene was cloned into the low copy *Escherichia coli* vector pWSK29 (Wang and Kushner, 1991). The GFP-Menkes fusion was obtained by ligating the GFP gene from pEGFP-N (Clontech, Palo Alto, CA, USA) to the C-terminus of the Menkes gene, which changed the C-terminal Menkes sequence from ...DDDTAL to ...DEDPPPVATM, with M being the start of GFP in plasmid pWM29. For *in vitro* transcription, 1 µg of pJFM19 or pWM29, linearized with restriction enzyme *Xho*I, was transcribed with T3 RNA polymerase using the mCap mRNA capping kit (Stratagene, La Jolla, CA, USA) in the presence of 10 µM [ $\alpha$ - $^{35}$ S]ATP. The mRNA was polyadenylated for 20 min at 30 °C with yeast poly(A) polymerase (Amersham Pharmacia Biotech, Dübendorf, Switzerland) as suggested by the manufacturer, phenol/chloroform extracted, precipitated, and the pellets resuspended in diethylpyrocarbonate-treated water and stored at -20 °C.

(B) Western blot of oocytes expressing Menkes ATPase. Single oocytes were analyzed as described below. Oocytes had been injected with the following template: Lane 1, Menkes RNA; lane 2, water; lane 4, Menkes RNA; lane 5, Menkes-GFP. Lane 3, 3 µg of total membranes from CHO cells overexpressing Menkes protein. The arrow indicates the Menkes ATPase bands, the arrow head the Menkes-GFP bands, and the scale on the right side the migration of standards with the corresponding molecular masses in kDa.

Methods: oocytes from mature female *Xenopus laevis* animals were collected by partial ovariectomy of anaesthetized frogs as described (Colman *et al.*, 1984). Immediately after surgery oocytes were incubated for 50 min in 10 ml of 5 mM HEPES, 1 mM CaCl<sub>2</sub>, 83 mM NaCl, 2.5 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM NaP<sub>i</sub>, pH 7.8, 2 mg/ml collagenase. Oocytes were then thoroughly washed and transferred to Barth's solution (15 mM HEPES, 88 mM NaCl, 2.3 mM NaHCO<sub>3</sub>, 1 mM KCl, 0.3 mM CaNO<sub>3</sub>, 0.4 mM CaCl<sub>2</sub>, 0.8 mM MgSO<sub>4</sub>, 10 µg/ml gentamycin, pH 7.6). Oocytes of stage V and VI were selected and incubated overnight at 18°C in modified Barth's solution. Healthy oocytes were then injected with 20 ng of RNA in 50 nl of water and incubated in modified Barth's solution at 18°C for 30 hours. Individual oocytes were lysed in 200 µl of sample buffer and resolved by sodium dodecyl sulfate gel electrophoresis (Laemmli and Favre, 1973). Western blotting was performed as described (Towbin *et al.*, 1979), and the signals were visualized using the ECL kit (Amersham Pharmacia Biotech).

for the mRNA of the Menkes-GFP fusion, and a small shift to larger size could also be observed in all bands upon polyadenylation.

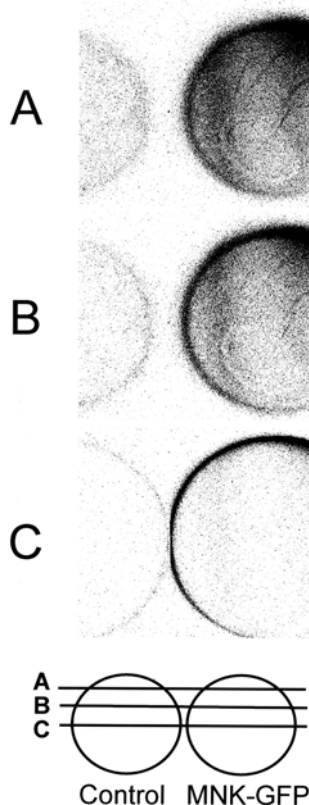
For expression, decollagenized stage V to VI oocytes were injected with *in vitro* transcribed wild-type Menkes mRNA and Menkes-GFP mRNA. Approximately 30 hours later, oocytes were lysed and protein expression analyzed by Western blotting. A double band reacting with anti-Menkes antibodies was observed in oocytes injected with Menkes mRNA (Figure 1B, lanes 1 and 4). The lower band of the doublets co-migrated with the Menkes ATPase band from control CHO cells (lane 3). The nature of the double bands remains unexplained at present, but could be due to posttranscriptional modification. Expression was observed in 80 to 100% of injected eggs and depended on the oocyte batch, but was similar for both mRNA types. Stage III to IV oocytes, which are less pigmented and would allow for easier GFP detection, were also injected but yielded lower expression levels (data not shown). Figure 2 shows an oocyte 30 hours after injection of Menkes-GFP mRNA together with water-injected controls. In order to visualize GFP fluorescence, the strong autofluorescence of the oocytes had to be reduced by preexposing them for 30 min to the exciting light source. The autofluorescence was clearly more susceptible to photobleaching than GFP. The fluorescence intensity and thus Menkes-GFP expression levels were similar from 24 hours to 3 days after mRNA injection. On the fourth day the intensity started to decline.

The Menkes-GFP fusion was localized by confocal microscopy of eggs 30 hours after injection of Menkes-GFP mRNA (Figure 3). The cell membrane was found to be the major (if not exclusive) site of localization. No loci of enhanced fluorescence could be detected within oocytes. The fluorescence was evenly distributed among the vegetal and animal hemisphere. This plasma membrane localization of the Menkes-GFP fusion protein under low copper conditions contrasts with the *trans*-Golgi localization observed in other cell types, including yeast. Conceivably, the C-terminal fusion with GFP conceals a signal required for a *trans*-Golgi localization. Mutation of the C-terminal di-leucine motif L1487-L1488 has been shown to result in a plasma membrane localization, presumably by prevented recycling of the ATPase from the plasma membrane back to the *trans*-Golgi network (Petris *et al.*, 1998). This mutation did, however, not affect the copper transport function. Also, none of over 150 identified Menkes disease mutations is localized in the last 65 amino acids of the protein (Tümer *et al.*, 1999), suggesting that the C-terminal domain is not critical for ATPase function. Fusing the C-terminus to GFP is thus unlikely to inactivate the ATPase. The plasma membrane localization of the Menkes-GFP fusion observed in our system is in fact advantageous for the study of Menkes ATPase function. Unfortunately, attempts to localize the wild-type Menkes protein through oocyte membrane fractionation or immunological staining of thin sections proved unsuccessful.



**Fig. 2** Fluorescence Microscopy of an Oocyte Expressing Menkes-GFP.

Oocytes from the incubation were directly put on glass slides without fixing. Endogenous fluorescence was bleached by exposing the oocytes to a mercury lamp for 30 min. Micrographs were recorded on Ektachrome 64T film (Eastman Kodak Co., Rochester, NY, USA) using the appropriate filters for GFP. The oocyte that is completely visible on the photography was injected with Menkes-GFP mRNA 30 h prior to photography. The other oocytes are controls that were injected with water only.



**Fig. 3** Confocal Microscopy of Oocytes Expressing Menkes-GFP.

Water (left) and Menkes-GFP mRNA (right) injected oocytes were photographed at the different section levels A, B, and C. The relative positions of these levels are shown schematically in the drawing. Photographs were taken with a Zeiss LSM 410 confocal microscope (Carl Zeiss, Göttingen, Germany) using an excitation wavelength of 488 nm (argon laser) and an emission filter set at  $540 \pm 30$  nm.

In summary, we have shown here the expression of the Menkes copper ATPase and of a Menkes-GFP fusion in *Xenopus laevis* oocytes. Menkes-GFP protein was localized in the plasma membrane, thus facilitating copper transport studies with such oocytes. It should now be possible to inject radioactive copper and to measure copper efflux from a single egg expressing Menkes ATPase (Sigel, 1990). This would allow the analysis of the effect of putative disease mutations on ATPase function. Such work is currently in progress.

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