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Expression of the Menkes gene homologue in mouse tissues lack of effect of copper on the mRNA levels

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Abstract The expression of the homologue of the Menkes disease gene (Mnk) in mice was studied using RNA blots. The highest level of expression of the 8.0 kb mRNA was found in placenta, substantial expression was noted in lung, heart, brain, testis and kidney and gut mucosa, but very low levels were found in spleen and adult liver. In fetal liver, the amount of Mnk mRNA is similar to that found in kidney, however, it declines soon after birth. Results with copper-loaded normal mice and mutant mice with genetic defects in copper transport suggested that Mnk mRNA levels are not regulated by tissue copper concentrations.

Key words: Copper transport; Menkes disease; Mottled mouse; RNA blot; mRNA

1. Introduction

The gene involved in Menkes disease (MNK) has been recently identified and shown to be a member of the cation transporting P-type ATPases [1–3]. From the pattern of copper transport and the structure of the gene it is reasonable to propose that the role of this protein is in copper efflux [3]. Only limited information on the pattern of expression of the gene is available in humans [2,3] and mice [4] and nothing has been reported as to possible regulation by copper. We have recently isolated clones of the murine homologue of the MNK cDNA (Mnk) [5] and showed that the predicted protein product was very similar to the human MNK protein. Abnormalities in structure and expression of this gene in two of the mottled mice mutants, which have a genetic defect of copper transport were demonstrated [4,5]. Although it seems very likely that the gene product of the Mnk locus is involved in copper transport across cell membranes, no functional data has appeared and the reason for the diverse phenotypes of the mottled mutants has yet to receive a molecular explanation, although some progress in this area has been made [4,5]. To explore further the role of the Mnk gene in copper transport will require more basic information about patterns of expression during development and in different tissues. In particular it is of great interest to determine whether the gene is copper responsive.

2. Materials and Methods

Normal mice used for the copper treatment and tissue analysis were BALB/c. The mottled mutant mice, brindled (Mo^{br}) and blotchy (Mo^{blo}) were maintained as previously described [6,7]. The normal mice used for comparison with the mutants were litter mates carrying the normal X chromosome. The toxic milk mouse mutation arose on a DL background [8]. Mice were treated with copper by administration of daily i.p. injections of copper acetate in normal saline at a dose of 2 mg copper /kg for 2 days and livers were isolated on day 3. Total RNA was isolated from tissues using a guanidinium hydrochloride procedure [9]. For RNA blots, 10 μg RNA was denatured by heating for 10 min at 65°C in 50% formamide, 2.0 M formaldehyde, 40 mM MOPS (MOPS is 3-(*N*-morpholino)propansulphonic acid) and fractionated on a 1% agarose gel containing 0.66 M formaldehyde, 40 mM MOPS, 10 mM Na-acetate, 20 mM EDTA, pH 7.0. Gels were stained with ethidium bromide and photographed with UV light to visualize the 18 S and 28 S rRNA which provided an indication of the evenness of loading and the integrity of the RNA. The RNA was transferred to Hybond N^+ nylon membranes (Amersham) using 20× SSC (1× SSC is 0.15 M NaCl, 0.015 M Na-citrate). Mnk RNA was detected by hybridization with a 959 bp fragment 2C/2D of the mouse Mnk cDNA [5] which spans from nucleotide 3,166 to 4,105, based on the human sequence numbering [3]. A number of the blots were probed with cDNAs spanning other segments of the sequence and identical results were obtained (not shown). Probes were labelled with [^{32}P]dCTP to a specific activity of greater than 1.0×10^8 dpm/μg by random priming, using a kit supplied by Bochringer-Mannheim. Hybridization was carried out for 18 h at 65°C in a solution containing 2× Denhardt's reagent (4 mg each of polyvinylpyrrolidone, Ficoll and bovine serum albumin per ml), 100 μg/ml of sheared and denatured salmon sperm DNA, 5 mM EDTA, 10 mM Tris-HCl, 5% SDS and 0.5 M Na phosphate pH 7.0 (20°C). Filters were washed to 0.2× SSC, 0.1% SDS at 60°C. The gels were exposed to X-Omat XAR film (Kodak) at −70°C. The glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA clone [10] was used as a control for RNA loading but proved rather variable as reported previously [11], consequently we also show the ethidium stained gel as an indication of RNA loading and quality. Copper concentrations in tissues were determined using flame atomic absorption spectrophotometry using a Perkin-Elmer 5000 spectrophotometer.

3. Results and discussion

The probe detected an 8.0 kb mRNA in various tissues (labelled 'M' in the Figs.) which has been shown to be from the mouse Mnk gene [5] and is slightly smaller than the MNK mRNA in humans. The strongest signal was seen with RNA from placenta. This is the first report of expression of Mnk in mouse placenta and contrasts with the low expression in human placental RNA reported by two other groups using the same commercial RNA blot [2,3]. Relatively high expression of Mnk in placenta is not unexpected since this organ is involved in copper transport from maternal to fetal circulation and copper is found to accumulate in the mottled mutant placenta [6]. The difference between mouse and human might be explained by species differences or stage of development.

Mnk mRNA is clearly present in lung, heart, brain, and kidney and testis (Fig. 1A) and is also detectable in gut mucosa, but was somewhat degraded (note Fig. 1C, lane 9). There was very little Mnk mRNA in spleen and liver (Fig. 1A, lanes 2, and 6) and also low amounts of GAPD mRNA in these samples

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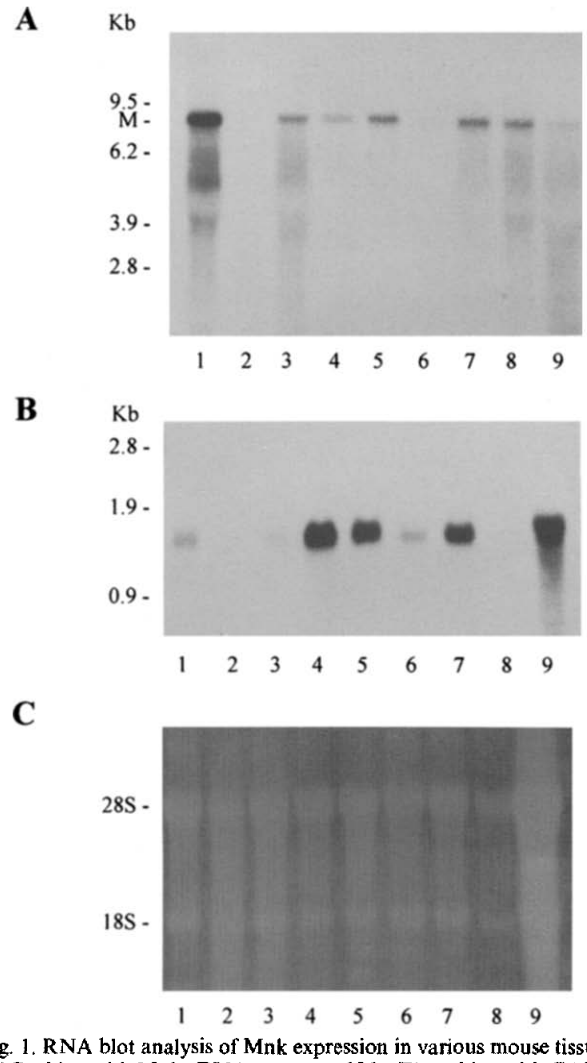

Fig. 1. RNA blot analysis of Mnk expression in various mouse tissues. (A) Probing with Mnk cDNA, exposure 18 h; (B) probing with GAPD, exposure 2 h; (C) ethidium bromide stained gel. Lane 1, placenta, gestation day 19; lane 2, spleen; lane 3, lung; lane 4, heart; lane 5, brain; lane 6, liver; lane 7, kidney; lane 8, testis; lane 9, gut mucosa. Tissues in lanes 2–9 are from an adult male.

(Fig. 1B), yet the rRNA staining (Fig. 1C) shows intact rRNA is present; some degradation is apparent, but not sufficient to explain the low signal. This contrasts with the recent report of similar levels of Mnk mRNA in mouse spleen to that in brain and testis [4]. In general these data support the observation of wide spread expression of this gene in human tissues [2,3] and mouse [4] with the exception of the liver as discussed below. The variable signals with GAPD with samples containing similar amounts of rRNA (compare Fig. 1B,C), demonstrates again the unreliability of relying only this probe as a guide to RNA loading especially when different tissues are being compared [11].

The pattern of expression of Mnk in the liver is of particular interest since this is the main organ of copper homeostasis. In patients with Menkes disease, most cells or tissues involved in copper transport are found to accumulate copper due to defective efflux [12]. Hepatic copper is very low, however, suggesting either that the Mnk gene is not expressed in liver, or that the additional mechanisms of copper efflux from liver (e.g. via caeruloplasmin and bile), prevent excess copper accumulation [12]. Preliminary data suggested that the expression of Mnk in the liver is low and variable [2–4], but the effect of variation in hepatic copper concentration or developmental regulation has not ben reported.

In adult mouse livers the hepatic expression of Mnk is always low (e.g. Fig. 1A, lane 6) and sometimes undetectable, e.g. in Fig. 2D are two livers with no Mnk mRNA detected (lanes 1 and 4). Where a signal was detected in liver it was not due to cross-hybridization with mRNA from the Wilosn disease gene homologue [13–15], since this 7.5 kb mRNA is clearly resolved from Mnk mRNA on our gels (unpublished data). In contrast to adult livers, the Mnk gene is well expressed in the liver from a gestation day 19 foetus (Fig. 2A, lane 4). The apparent second species of mRNA at about 4 kb is an artefact due to the presence of 28 S rRNA. The mRNA is also apparent in the liver of gestation day 17 animal (Fig. 2A, lane 2), although in this case the RNA is somewhat degraded and appears as a smear down the lane. Fetal kidney expressed Mnk mRNA to a similar degree to the fetal liver (Fig. 2A, lane 3). In early neonates (day 2 after birth), the expression in the liver is still detectable, but has decreased markedly (Fig. 2A, lanes 6–9). Fairly low expression was also noted in livers of day 10 and day 15 neonates (Fig. 3A, lanes 1 and 3).

These results suggest that the fetal liver expresses the Mnk gene to a similar extent as the fetal kidney but the hepatic expression declines soon after birth, and can disappear entirely in adults. An interesting question is whether this is a developmental process in the hepatocyte: the Mnk gene may, for example, be active in the hepatocyte prior to the expression of the Wilson disease gene, which may become active when the biliary excretion of copper commences after birth. To date there is no information about the pattern of expression of the Wilson disease gene during development. Alternatively, the expression we detected in the fetal liver may be due to cells other than hepatocytes. For example the fetal liver is active in hemopoesis and there is a rapid drop in hemopoeitic cells around the time of birth [16] which would fit with the decline in Mnk mRNA from the fetal to neonatal period. We have attempted to identify the cellular location of the Mnk mRNA using in situ hybridization to liver sections, but to date the mRNA has not been detected with this technique.

Brindled (Mo^{br}) and blotchy (Mo^{blo}) are allelic mutations of the mottled locus and micewith these mutations have defective copper transport, resulting in accumulation of copper in kidney and gut mucosa and deficiency in many tissues, most notably in the liver and brain [17]. Two abnormal forms of Mnk mRNA are found in Mo^{blo} kidney and brain but the mRNA from the brindled mouse appears normal on RNA blots [4,5]. The variable amounts of copper in the tissues of these mice allow an assessment of the effect of tissue copper concentrations on the expression of the Mnk gene. Since the gene is involved in the copper transport pathway, regulation by copper might be expected, but not necessarily at the level of transcription. In Fig. 3A, the signal from the kidney of the neonatal brindled mouse is the same as the normal (compare lanes 2 and 4) despite a 5-fold higher copper concentration in the mutant organ [6,18]. Also hepatic expression is similar in both (Fig. 3A, lanes 1 and 3), even though copper concentrations in the mutant liver are very low [6]. Tissues from Mo^{blo} (Fig. 3D) reveal a similar picture, the expression of the three forms of Mnk mRNA found

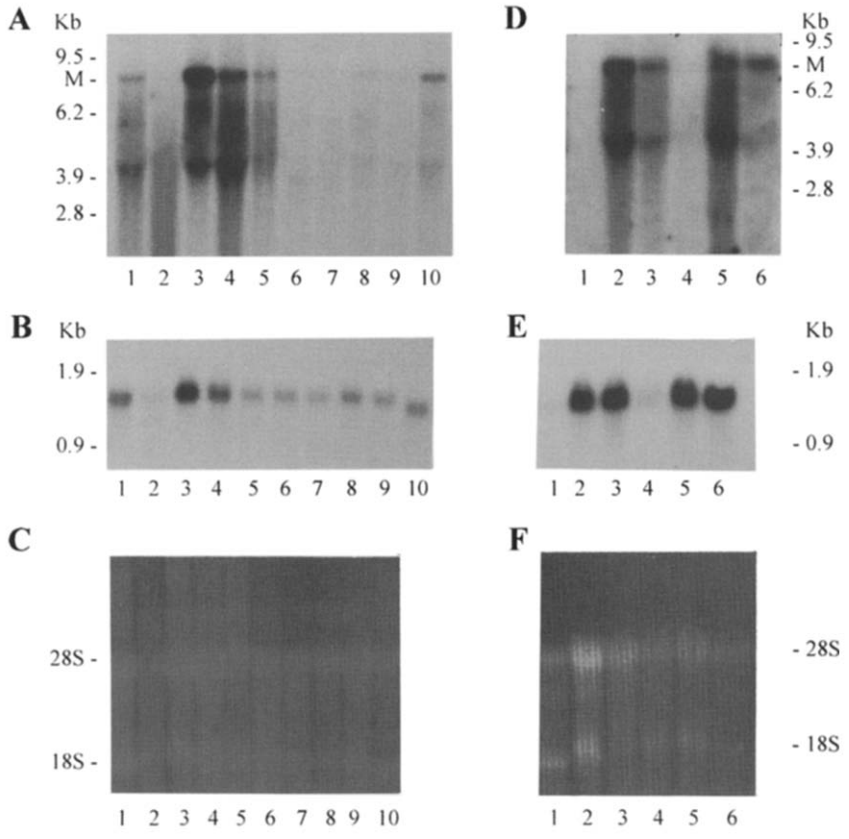

Fig. 2. Expression of Mnk mRNA in developing mice. (A) Probing with Mnk. Lane 1, gestation day 17 kidney; lane 2, gestation day 17 liver; lane 3, gestation day 19, kidney; lane 4, gestation day 19, liver; lane 5, day 2 kidney; lanes 6–9, day 2 liver; lane 10, day 10 kidney. (B) Same samples as (A) probed with GAPD. (C) Ethidium bromide stained gel used for (A) and (B). (D) Lanes 1,4, adult liver; lanes 2,5, adult kidney; lanes 3,6, adult brain. (E) Same blot as (D) probed with GAPD. (F) Ethidium bromide stained gel used for (D) and (E). Day 0 is the day of birth, each lane contains RNA from a single mouse.

in this mutant were not clearly responsive to the differences in tissue copper. Interpretation in this case is not as clear because of the presence of the aberrant mRNAs and the lower signals from the blotchy mutant used in lanes 7–9. The appearance of the two abnormal forms in the blotchy liver (lane 10) is further evidence that the hepatic mRNA being detected by the probe is Mnk and not a related gene.

The toxic milk mouse (*tx*) is a mouse model of Wilson disease, in the liver of the adult the hepatic copper concentrations are very high, e.g. mean of 716 μg/g compared with 13 μg/g in the normal liver [8,19], thus providing a useful system to examine the effect of extreme copper loading on hepatic Mnk mRNA. The liver of a normal adult litter mate (DL strain) contained little Mnk mRNA (Fig. 3A, lane 5), whereas the liver of the *tx* mutant litter mate was expressing a significant amount of the mRNA (Fig. 3A, lane 8). Increased Mnk mRNA has been found in 4 other *tx* livers (not shown), suggesting that the expression of Mnk may be consistently higher in the mutant liver, but this will require further confirmation. Increased hepatic expression in the *tx* liver could suggest that elevated hepatic copper induces Mnk expression. To investigate this possibility, normal mice were injected with 2 mg/kg of copper (as copper acetate) over a two day period, which increased hepatic copper from 12 μg/g to over 200 μg/g dry wt. The Mnk mRNA, however, was not increased in the liver of the treated animals: e.g. in Fig. 4A compare lanes 1 and 3 (Cu-loaded livers) with lane 5 (untreated liver) which has more Mnk RNA (but this lane also has more RNA). Similarly the Mnk mRNA in the copper-loaded kidney in lanes 2 does not differ from the control animals (lanes 6 and 8), despite an increase of copper from 18 to 70 μ/g dry wt. The weaker signal in the copper-loaded kidney in lane 4 is posibly due to lower RNA loading (see Fig. 4B).

Overall the evidence is consistent with a lack of a marked response of the Mnk gene in liver, kidney and brain to copper loading or deficiency. We consider that any effect of cellular copper concentration on efflux rates of copper will be mediated by modulation of the activity of the Mnk protein rather than by increase in transcription. Sporadic appearance of Mnk mRNA in the adult liver may be due to expression in non-hepatic cells, and this is most likely the explanation of the expression observed in the fetal liver, the Mnk mRNA in that case deriving from hemopoetic cells. The Mnk mRNA in the *tx* liver could also come from the mononuclear cells seen in large numbers in some *tx* livers [19]. It is possible that hepatocytes do not express Mnk at all. Since we have not identified the cellular location of Mnk mRNA in the fetal liver, however, it is still formally possible that fetal hepatocytes do express Mnk, perhaps prior to the establishment of the biliary excretion of copper which occurs after birth [20].

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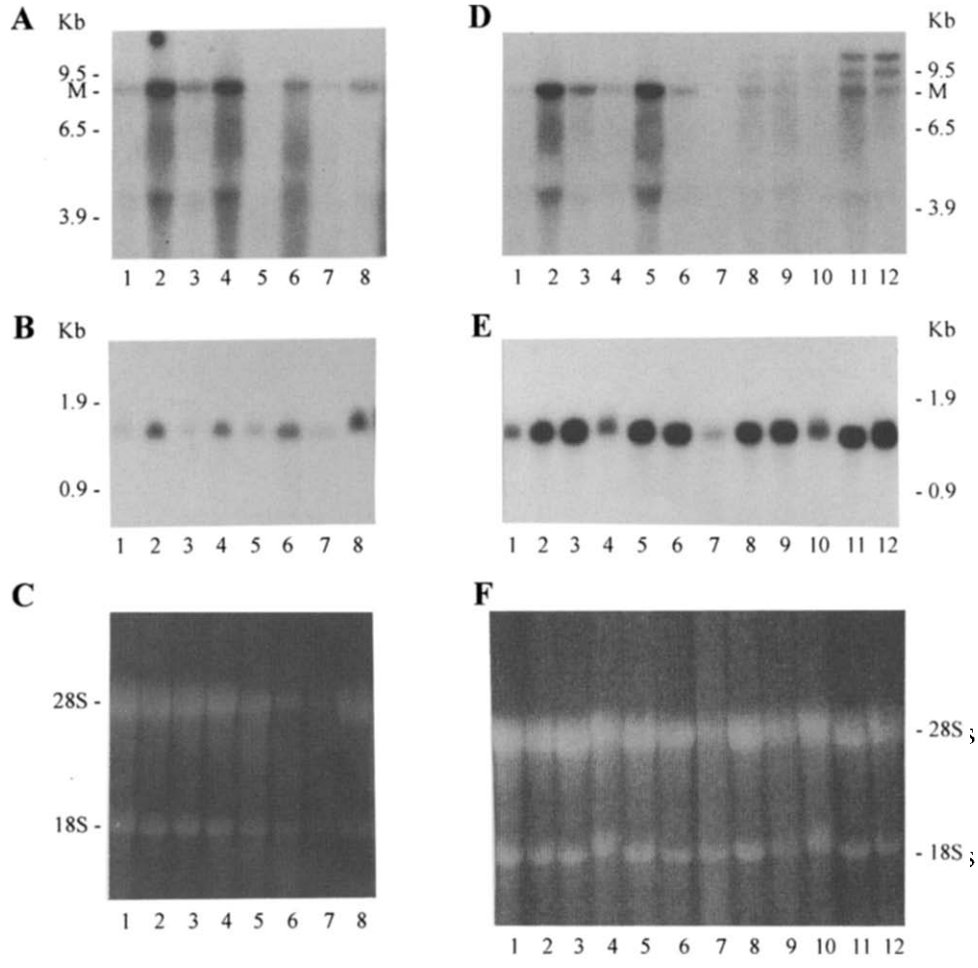

Fig. 3. Expression of Mnk in various mouse mutants. *Br* = brindled mouse, *blo* = blotchy, *tx* = toxic milk mouse. (A) Mnk probing: lane 1, 15 day *+/y* liver; lane 2, 15 day *+/y* kidney; lane 3, 10 day *br/y* liver; lane 4, 10 day *br/y* kidney; lane 5, adult liver DL strain; lane 6, adult kidney DL; lane 7, adult brain DL; lane 8, liver adult *tx* mouse. (B) same filter probed with GAPD. (C) Ethidium bromide stained gel used for (A) and (B). (D) Lanes 1,4, *+/y* liver 15 day; lanes 2,5, *+/y* kidney 15 day; lanes 3,6, *+/y* brain 15 day; lanes 7,10, *blo/y* liver 10 day; lanes 8,11, *blo/y* kidney 10 day; lanes 9,12, *blo/y* brain 10 day. (E). Same filter as (A) probed with GAPD. (F) Ethidium stained gel used for (D) and (E).

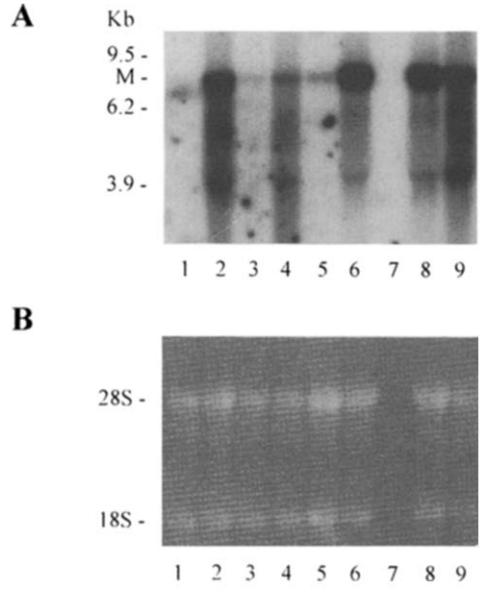

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Fig. 4. Effect of copper loading on Mnk expression. RNA was isolated from adult mice which had been loaded with copper as described in text. Lanes 1 and 3, liver Cu-loaded mouse; lanes 2 and 4, kidney Cu-loaded mouse; lane 5, liver untreated mouse; lane 6 and 8, kidney untreated mouse; lane 7, no RNA; lane 9 testis adult untreated.

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