Decreased carbonic anhydrase III levels in the liver of the mouse mutant 'toxic milk' (*tx*) due to copper accumulation

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The mouse mutant 'toxic milk' (*tx*) is characterized by marked hepatic accumulation of copper, similar to that found in patients with the genetic disorder of copper transport, Wilson disease. In addition, lactating *tx* females produce copper-deficient milk. To characterize further the biochemical basis of this defect, Western blots of tissue extracts from normal and *tx* mice were probed with various heavy-metal radioisotopes (^{63}Ni, ^{65}Zn and ^{64}Cu). A 30 kDa Ni/Zn-binding polypeptide was found to be markedly decreased in the livers of the *tx* mice. This protein was isolated from normal adult mice using a procedure based on Ni-chelation chromatography. The amino acid sequences of two CNBr peptides were identical with portions of the mouse skeletal muscle carbonic anhydrase III (CAIII) sequence. Two other peptides sequenced had closely related sequences to that of CAIII, but with two differences in 45 amino acids. These two peptides may be derived from a novel CAIII isoform, which we term CAIIIB to distinguish it from the published form, CAIIIA. We isolated a cDNA clone corresponding to CAIIIA and used this to show that CAIIIA mRNA was also decreased in the mutant liver, but not in muscle. Copper loading of normal mice also decreased hepatic CAIIIA mRNA, suggesting that the decrease in CAIII mRNA in the *tx* mouse liver is a secondary consequence of the high copper levels in the liver.

INTRODUCTION

'Toxic milk' (*tx*) is an autosomal recessive mutation in mice which causes hepatic copper to accumulate to about 800 μg/g dry weight in mutant adults, compared with 12 μg/g dry weight in normal animals [1,2]. Transport of copper across the placenta and into milk is reduced in mutants, and pups from such dams are born severely copper-deficient and usually die in the second postnatal week if allowed to consume the mutant dam's milk [1]. Mutant pups fostered to a normal female usually survive and commence accumulation of hepatic copper in their second postnatal week. The high concentration of copper in the liver of the adult *tx* mouse is similar to that found in patients with Wilson disease [1] and another rodent model of Wilson disease, the LEC rat [3,4]. We have recently found a mutation in the Wilson disease gene homologue in the *tx* mouse, demonstrating that this mouse is a true model of Wilson disease [5], despite some differences in the pattern of hepatic copper accumulation compared with Wilson disease patients [2,6].

Most of the excess copper in the mutant *tx* liver is bound to metallothioneins (MTs) [7,8]. Hepatic MT mRNA was found to be normal in younger *tx* mice, but increased markedly in adults as copper accumulated, suggesting that regulation of MT synthesis is normal in *tx* mice and that MT mRNA is induced by the accumulation of copper [9]. Koropatnick and Cherian reported that MT mRNA levels are normal in the liver of the adult *tx* mouse, and they proposed that the *tx* mutation may affect MT stability [8]. We have re-examined MT mRNA levels in *tx* liver and found at least a 10-fold elevation, so we consider that the *tx* defect is unlikely to affect MT stability directly [10]. The lower copper concentrations found in the milk of *tx* dams may be a secondary consequence of abnormal hepatic transport, or could conceivably be due the mutation directly affecting a copper transporter in the mammary gland.

Further analysis of the biochemical changes in the *tx* mouse liver will assist understanding of copper transport and add to the knowledge of this process, which is increasing rapidly following the cloning of the genes affected in Menkes disease and Wilson disease [11–15]. In this paper we use radioactive copper, zinc and nickel to detect possible alterations in metal-binding polypeptides in the *tx* mutant using Western blots. A 30 kDa Ni/Zn-binding protein was found to be decreased in the liver of the mutant, and was purified by Ni-chelation chromatography. Amino acid sequencing suggested that the protein was a mixture of two carbonic anhydrase III (CAIII) isoforms. Only one isoform of this enzyme has been previously reported in mouse liver. CAIII mRNA was also decreased in the mutant liver. Since copper loading of normal mice decreased hepatic CAIII mRNA, the loss of CAIII in the *tx* mouse liver is likely to be a consequence of copper accumulation rather than a direct affect of the mutation.

EXPERIMENTAL

Animals and copper treatment

The animals used all came from the inbred strain DL in which the *tx* mutation originally occurred [1]. Animals are genetically homogeneous, segregating only at the *tx* locus. Livers were collected and stored at −70 °C until analysis. Copper-loaded animals were 60-day-old Balb/C male mice injected intraperitoneally twice at 24 h intervals with 2 mg/kg Cu as copper acetate. This treatment elevated the mean hepatic copper from 16 μg/g dry wt. to over 200 μg/g, as determined by atomic absorption spectrometry as described previously [9].

Gel electrophoresis and metal Western blots

Approx. 300 mg of mouse tissue was homogenized in 1.2 ml of 100 mM Tris/HCl, 50 mM NaCl, adjusted to the indicated pH

Abbreviations used: CA, carbonic anhydrase; MT, metallothionein.

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with HCl (buffer A), and centrifuged at 45000 ***g*** for 15 min at 4 °C in am SS34 rotor using a Sorval RC5B centrifuge. The supernatant (100 μg of protein) was fractionated in an SDS/Tricine/polyacrylamide gel (10 % total, 3 % cross-linker) [16]. Proteins were detected by staining with Coomassie Blue. For transfer, the gel was not stained and the proteins were transferred to reinforced nitrocellulose (0.45 μm; Schleicher and Schuell) using a Bio-Rad Transblot Electrophoretic Cell [17] with 25 mM Tris, 192 mM glycine, 20 % methanol, pH 8.3, at 30 V for 18 h at 4 °C. The membrane was then treated as follows: (1) buffer A + 20 mM EDTA (100 ml) for 5 min; (2) buffer A + 10 mM $MgCl_2$ (2 × 200 ml) for 5 min; (3) as (2), but 20 ml for 30 min and with one of the following radioisotopes included: 5 μl of ^{63}Ni (3–12 Ci/g; Dupont–NEN; typically 50 μCi/gel), 10 μl of ^{64}Cu (50–150 Ci/g; ANSTO; typically 1.6 μCi/gel) or 20 μl of ^{65}Zn (10–100 Ci/g; Dupont–NEN; typically 20 μCi/gel); (4) repeat of step (2).

Purification of the 30 kDa protein

A column of chelating Sepharose Fast Flow (1.5 cm × 10 cm) (Pharmacia) was washed with 50 mM EDTA, pH 8.0, to remove bivalent cations; 50 ml of 1 M $NiCl_2$, pH 6, was then passed through the column followed by buffer A (20 ml). Excess blood was removed from livers of adult (60-day-old) mice by perfusion with 15 ml of 5.4 mM KCl, 116 mM NaCl, 20 mM Hepes, 250 mM $NaHCO_3$ and 5.5 mM glucose at 37 °C through the portal vein. Livers were homogenized in buffer A, pH 6, at 4 °C (4 ml/g) using a Dounce homogenizer. Chelex 100 resin, pH 6 (1 g/3 g), was added, stirred for 5 min and removed by centrifugation at 45000 ***g*** at 4 °C (SS34 rotor). The supernatant was filtered through Miracloth (Calbiochem) and adjusted to pH 7.2 (using buffer A, pH 9), and then applied to the Ni-Superose column and washed with buffer A, pH 7.2, until the A_{280} of the effluent was zero. Subsequent washes were: buffer A, pH 6.5 (50 ml); buffer A, pH 6.0 (50 ml); buffer A, pH 5.5 (50 ml). The remaining proteins were eluted with buffer A, pH 5.5, containing 20 mM EDTA. The eluate was dialysed against 10 mM Tris/HCl, pH 7.5, and applied to a DEAE Fast Flow column (Pharmacia) (1.5 cm × 20 cm) in the same buffer. The 30 kDa protein was in the non-bound fraction, which was subsequently dialysed against 20 mM ethanolamine, pH 9.0, and applied to a Mono Q HR 5/5 column (Pharmacia), and was eluted using a linear salt gradient (0–1 M NaCl).

CNBr cleavage

The purified protein was dialysed against 50 mM ammonium carbonate, pH 8, at 4 °C, lyophilized and treated with CNBr using the method of Scott et al. [18]. Briefly, samples were digested for 18 h at 1 mg/ml in a solution of 50 mg of CNBr/ml of 70 % formic acid. Residual CNBr/formic acid was removed with a stream of nitrogen, and the peptides were redissolved in 0.5 M acetic acid, centrifuged at 8000 ***g*** for 2 min to remove insoluble material and then lyophilized.

Peptide isolation

Peptides were separated in a Tricine/SDS/polyacrylamide gel (16.5 % total, 6 % cross-linker) [16]. Peptides were stacked at 30 V and electrophoresed at 130 V. After electrophoresis the gel was either stained with Coomassie Blue or equilibrated in 10 mM Caps, 20 % methanol, pH 11, for 10 min and transferred in the same buffer to an Immobilon membrane (Millipore) at 0.2 mA/cm for 2 h [19]. The membrane was stained with 0.1 % (w/v) Coomassie Blue and destained using 50 % aqueous methanol to identify the location of the peptides, and the excised bands were sequenced directly.

Amino acid sequencing

Immobilon strips containing peptides of interest were subjected to automated Edman degradation using an Applied Biosystems Inc. Model 471A Protein Sequencer equipped with a Brownlie Laboratories Microgradient Delivery System for the chromatographic identification of phenylthiohydantoin amino acids. All sequencing reagents used were purchased from Applied Biosystems, and phenylthiohydantoin amino acids were separated using a 2.1 mm (internal diam.) C18 column by the application of a linear gradient of acetonitrile buffered with sodium acetate as described by the column manufacturers. Detection was by absorbance at 269 nm.

Isolation of cDNA clones

A mouse liver cDNA library from Clontech (cat. no. ML 1017a; Balb/C) in λgt10 was screened with two oligonucleotide primers based on the published CAIII cDNA sequence [20]. Primer 1 (antisense) was: 5′ GAGAGGACCACCCCTCAGCAT 3′, corresponding to the amino acid sequence from Met-78 to Leu-84. Primer 2 (antisense) was: 5′ AGAGGCACCGGGGGCTCATT 3′, corresponding to the amino acid sequence from Asn-234 to Val-241 and including the Pro-239 residue specific to the A form of CAIII. Clones which were positive with both oligonucleotides were isolated, and the largest (30.2) contained an insert of 1 kb which was sequenced and found to be almost identical with the published sequence of mouse CAIII [20]. The insert from this clone was labelled by random priming and used to probe the Northern blots.

RNA isolation and Northern blots

Total RNA was isolated from fresh tissues using a guanidinium chloride procedure [21]. For Northern blots, 10 μg of RNA was denatured by heating for 10 min at 65 °C in 50 % formamide, 2.0 M formaldehyde and 40 mM Mops, and fractionated on a 1.5 % agarose gel containing 0.66 M formaldehyde, 40 mM Mops, 10 mM sodium acetate and 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 rRNAs, which provided an indication of the consistency 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 sodium citrate). CAIII mRNA was detected by hybridization with the CAIIIA cDNA probe, which had been labelled with [^{32}P]dCTP to a specific radioactivity of greater than 1.0×10^8 d.p.m./μg by random priming, using a kit supplied by Boehringer 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 BSA per ml), 100 ng/ml sheared denatured salmon sperm DNA, 5 mM EDTA, 10 mM Tris/HCl, 5 % SDS and 0.5 M sodium phosphate, pH 7.0 (20 °C). Filters were washed in 0.2 × SSC/0.1 % SDS at 60 °C. The filters were exposed overnight using X-Omat XAR film (Kodak) at −70 °C. Filters were also probed with a mouse MT-I probe [22] and a glyceraldehyde-3-phosphate dehydrogenase cDNA clone [23].

RESULTS

Western blot analysis using radioactive nickel, copper and zinc

Tissue extracts from *tx* and normal mice were prepared, and the

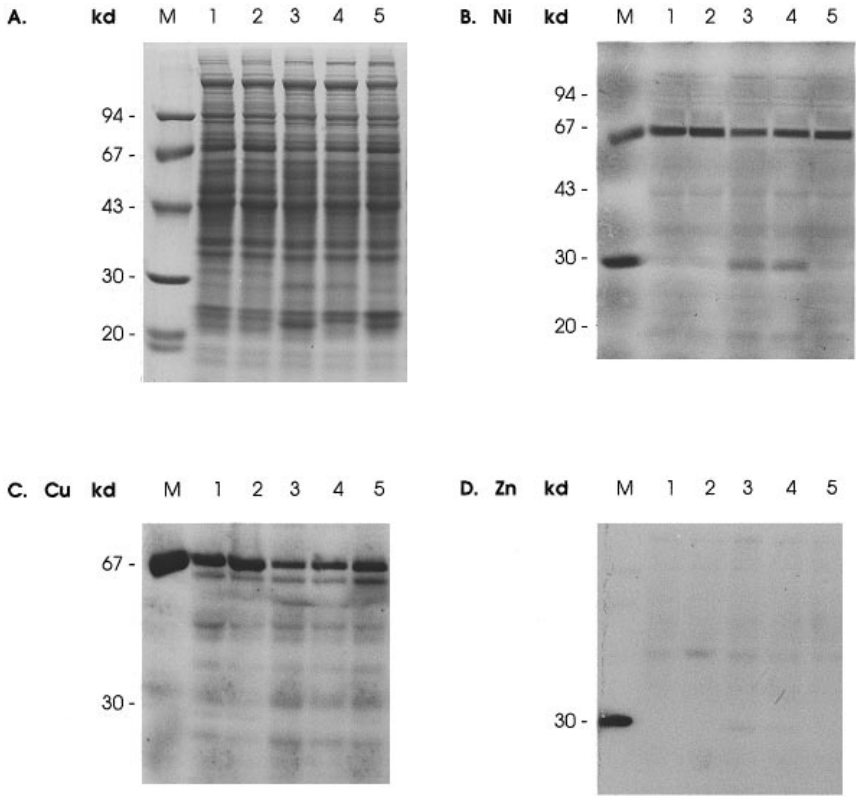

Figure 1 Liver extracts from *tx* and normal mice fractionated by SDS/PAGE and probed with Ni, Cu or Zn

Liver homogenates were prepared and electrophoresed as described in the Experimental section. The gel was either stained with Coomassie Blue or transferred to nitrocellulose and probed with the indicated radioactive metal. Molecular masses of the markers used are shown on the left in kDa, and the marker lanes are labelled M. (**A**) Coomassie Blue-stained gel; (**B**) membrane probed with ^{63}Ni; (**C**) membrane probed with ^{64}Cu; (**D**) membrane probed with ^{65}Zn. Lane 1, 20-day-old normal female; lane 2, 20-day-old *tx* female; lane 3, 150-day-old normal male, Cu-loaded; lane 4, 150-day-old normal female; lane 5, 150-day-old *tx* female.

proteins were separated using SDS/PAGE and transferred to nitrocellulose as described in the Experimental section. To detect the metal-binding components, the same blot was probed successively with ^{64}Cu, ^{65}Zn and ^{63}Ni, using EDTA to remove the previous isotope. As can be seen in Figure l, some bands were detected with only one isotope but others were detected with all three isotopes. For example, a band at 67 kDa was detected with nickel (Figure 1B) and copper (Figure 1C), but not significantly with zinc (Figure 1D); this band was thought to be serum albumin from residual blood in the tissues. Most significantly, as seen clearly in lanes 4 and 5 of Figure 1(B), a 30 kDa band in the liver, detected by nickel and weakly by zinc in normal mice, was absent from the liver extract from the *tx* mouse. In this case normal and mutant female mice were compared, but the same result was seen with male mice (not shown). There appeared to be a corresponding band difference detected by Coomassie Blue staining (lanes 4 and 5, Figure 1A). The 30 kDa band was not substantially decreased in livers of normal mice that had been treated with copper acetate as described in the Experimental section (lane 3). The 30 kDa component was barely detectable in livers from 20-day-old animals (lanes 1 and 2). Although the 30 kDa band co-electrophoresed with CA and had similar metal-binding properties (binding to nickel and zinc but not copper), there was no reason to connect CA with the defect in the *tx* mouse. This component was purified for amino acid sequencing.

Purification of the 30 kDa band using Ni-affinity chromatography

Since the protein bound radioactive Ni, an Ni-affinity column chromatography procedure was used as the first step in purification [24]. Liver homogenate prepared from normal female mice as described in the Experimental section was applied to the Ni-affinity column and, after extensive washes of the column to remove non-bound material, the 30 kDa component was eluted with EDTA. Substantial enrichment of the 30 kDa component was obtained with this step (compare lane 1, Figure 1A, with lane 1, Figure 2A), and the Ni blotting demonstrated that the 30 kDa component was indeed present (Figure 2A, lane 2). Purification to about 80–90 % was achieved by passage of the EDTA eluate through Mono Q (Pharmacia; see the Experimental section). A final product of approx. 95 % purity was obtained by chromatography on a Mono Q column with elution using an NaCl gradient (Figure 2B, lane 1). We consider that further improvements in yield and procedure are possible, but the present method gave a product of sufficient purity for peptide sequencing. Only small amounts of the 30 kDa protein were obtained when the same procedure was carried out with extracts from *tx* mouse livers (results not shown).

Peptide sequencing of the 30 kDa protein

Attempted N-terminal amino acid sequencing was not successful,

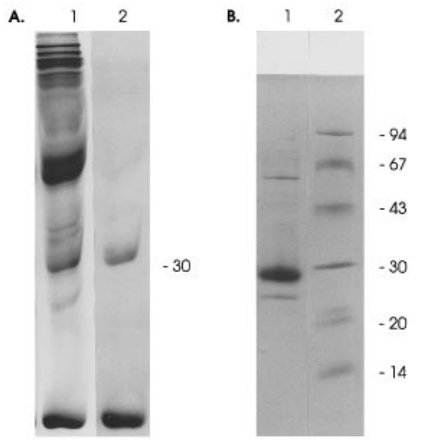

Figure 2 Purification of the 30 kDa polypeptide

Fractions from various stages of the purification of the 30 kDa protein were electrophoresed using SDS/PAGE and either stained with Coomassie Blue or probed with ^{63}Ni. (**A**) Proteins eluted from the Ni-affinity column with EDTA. Lane 1, Coomassie Blue stain; lane 2, corresponding track probed with ^{63}Ni. (**B**) Fractions of the 30 kDa protein eluted from Mono Q with an NaCl gradient. Lane 1, Coomassie Blue stain of combined fractions; lane 2, protein molecular mass markers (indicated on the right in kDa).

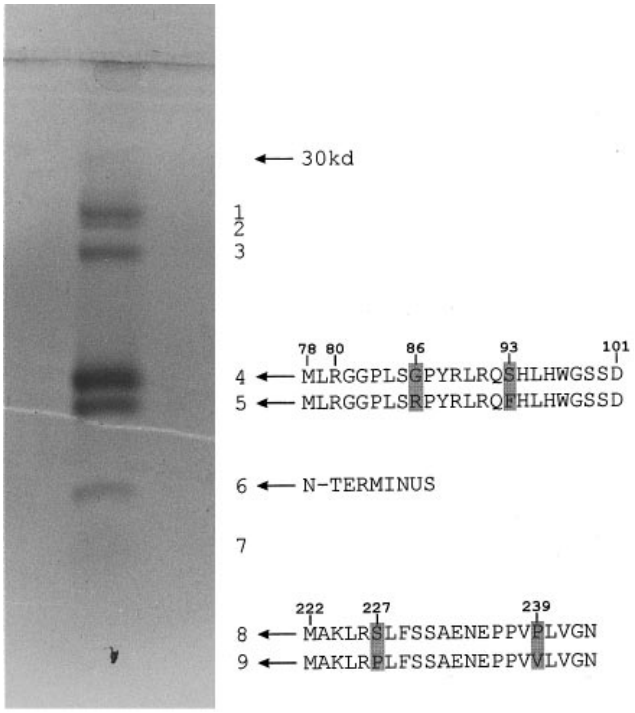

Figure 3 Amino acid sequences of CNBr peptides of the 30 kDa polypeptide

The purified 30 kDa polypeptide was cleaved with CNBr and peptides were fractionated on a 16% polyacrylamide gel as described in the Experimental section. The peptides were transferred to an Immobilon membrane and sequenced. The amino acid sequences of the various peptides are shown, with numbers based on the CAIII sequence. Peptides 8 and 9 do not show in the reproduction but were detectable on the original gel.

suggesting that the N-terminus was blocked, so CNBr cleavage was used to produce peptides for sequence analysis. Nine fragments were resolved on Tricine/16.5%-PAGE (Figure 3), and the peptides were transferred to an Immobilon membrane and subjected to amino acid sequencing. As shown in Figure 3, peptide 5 gave a sequence of 24 residues which exactly matched the published sequence of CAIII from mouse muscle from Met-78 to Asp-101 [20]. Interestingly, the sequence of peptide 4 was a closely related sequence of 24 residues but with two alterations: amino acid 86, arginine in the published sequence, is glycine in peptide 4; and amino acid 93 is altered from a phenylalanine in the published sequence to a serine in peptide 4. In addition, a sequence of 22 amino acids was obtained from peptides 8 and 9, which is not clearly visible in the stained gel shown in Figure 3. The sequence of peptide 8 corresponded exactly to the published sequence from Met-222 to Asn-243 of CAIII, but peptide 9 had two differences: amino acid 227 is proline instead of serine, and amino acid 239 is valine instead of proline. These findings may indicate that there are two forms of CAIII in mouse liver, one corresponding to the published sequence and the other a closely related isoform. Both forms, which we term CAIIIA and CAIIIB respectively, are present in only trace amounts in the livers of the *tx* mutant. The relative intensities of the two CAIII polypeptides (CAIIIA/CAIIIB) are about 1:2 (e.g. compare peptides 4 and 5 in Figure 3), so the novel form is a significant species in the liver.

Decreased CAIII mRNA levels in the *tx* liver

The identification of the 30 kDa protein as CAIII allowed further investigation of the molecular basis of its decreased levels in the livers of *tx* mice. Two oligonucleotides based on the published DNA sequence of murine CAIII [20] were used to screen a mouse liver cDNA library as described in the Experimental section. A number of clones were isolated, and DNA sequencing showed that all of these corresponded to the published form (results not shown). No clones were found that corresponded to the novel form. Attempts to isolate the CAIIIB isoform either by using the CAIIIA cDNA at low stringency, or by using mixed probes based on the CAIIIB amino acid sequence shown in Figure 3, have not been successful. It is possible that the nucleotide sequence of the CAIIIA clones is too divergent from that of CAIIIB to permit cross-hybridization.

When the CAIIIA clone was used to probe Northern blots, a 1.9 kb mRNA was detected (Figure 4A) in liver (lanes 1–4) and more strongly in muscle (lanes 5–7). The amount of CAIIIA mRNA was markedly reduced in the livers of the two *tx* animals (lanes 1 and 2) compared with the normals (lanes 3 and 4). This was not due to lower loading of RNA in these samples, as shown by the 18 S and 28 S staining intensity (Figure 4B). There was no decrease in CAIIIA mRNA in the muscle of *tx* mice (compare lanes 5 and 6 with lane 7 in Figure 4A). Thus the decrease in CAIII in the *tx* liver is most probably due to a lower amount of CAIIIA mRNA.

To determine if hepatic CAIIIA mRNA levels are reduced by copper loading, normal mice were treated with two successive intraperitoneal injections of copper acetate, which elevated the copper concentration in the liver from a mean value of 16 μg/g dry wt. to about 200 μg/g. The untreated mice showed high levels of CAIIIA mRNA in the liver (Figure 4C, lanes 1 and 2), but in the copper-treated animals the CAIIIA mRNA was barely detectable. RNA loading for the Cu-treated samples was somewhat lower (Figure 4D, lanes 3 and 4), but this was not sufficient to explain the virtual absence of the CAIII mRNA signal in these samples. The same blot was probed with a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (low specific activity probe yielding a weak signal), and this confirmed the relative uniformity of RNA loading. The blot was also probed with a MT-I cDNA, demonstrating the induction of this gene by copper

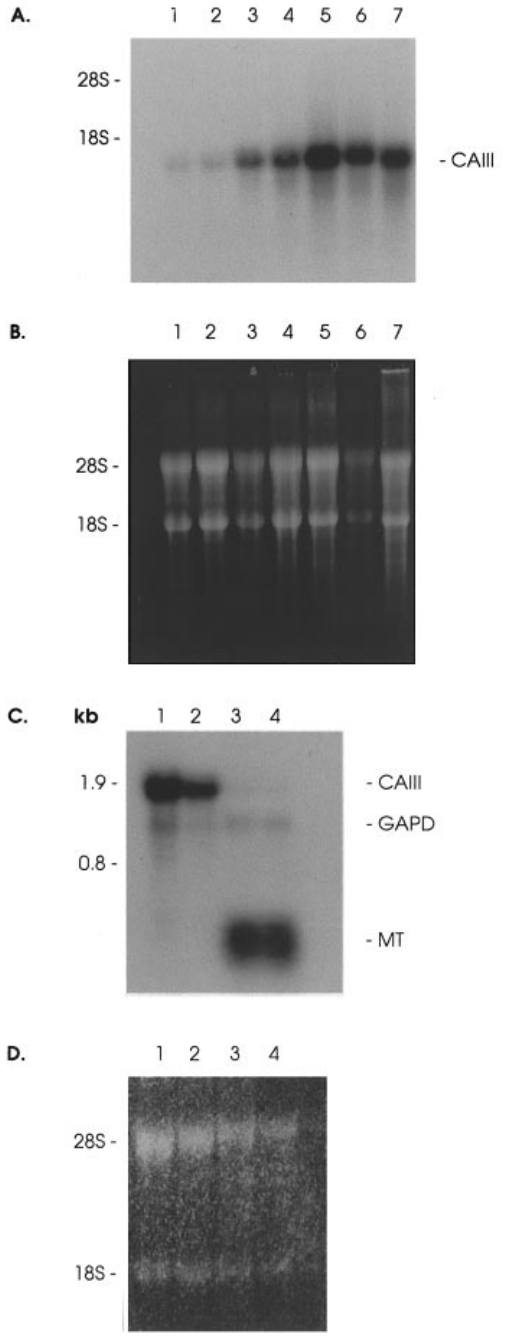

Figure 4 Northern blot analysis of RNA from normal and *tx* mice

RNA was isolated from the indicated mouse tissues, electrophoresed in agarose, blotted on to a nylon membrane and probed with CAIII, glyceraldehyde-3-phosphate dehydrogenase (GAPD) and MT cDNAs, as described in the Experimental section. (**A**) RNA from *tx* and normal mouse liver and muscle. Lane 1, *tx* liver, 145-day-old adult female; lane 2, *tx* liver, 129-day-old male; lane 3, normal liver, 143-day-old female; lane 4, normal liver, 129-day-old male; lane 5, *tx* muscle, 145-day-old female; lane 6, *tx* muscle, 129-day-old male; lane 7, normal muscle, 129-day-old male. (**B**) Ethidium bromide stain of gel used in (**A**); the positions of the 28 S and 18 S rRNAs are indicated. (**C**) RNA from livers of saline- and copper-injected 60-day-old male Balb/C mice. Lanes 1 and 2, controls; lanes 3 and 4, Cu-injected. (**D**) Ethidium bromide stain of gel used in (**C**).

treatment as has been reported previously [25,26]. These results show that the concentration of hepatic CAIIIA mRNA is markedly reduced by copper loading.

DISCUSSION

The results presented here show that levels of total CAIII and CAIIIA mRNA are both decreased in the livers of adult *tx* mutant mice. Since copper loading of normal mice produces an even greater reduction of CAIII mRNA than that seen in the *tx* mouse, it is most likely that the decrease in CAIII in the mutant is a secondary consequence of copper accumulation. The mechanism of the dramatic decrease in CAIII mRNA induced by copper has not been established, but could be mediated by either transcriptional or post-transcriptional events. It is possible, since CAIII is a zinc-containing enzyme, that zinc is required for the transcription or stability of the CAIII mRNA and that excess copper interferes with this process. We have preliminary data suggesting that zinc can partially prevent the copper-induced decrease in CAIII mRNA (A. Grimes, J. Paynter and J. Mercer unpublished work).

Another possible explanation for the effect of copper on CAIII mRNA is that CAIII is has some role in copper transport which requires its regulation by copper. The primary role of CAs is to interconvert bicarbonate and carbon dioxide. There is an extended gene family of these enzymes, but the physiological significance of each remains in doubt [27,28]. In particular, the role of CAIII in the rodent liver is unclear [28]. Since zinc and copper transport in red blood cells is reported to involve bicarbonate ions [29,30], the biliary excretion of copper may in some way depend upon bicarbonate generation. If this were the case, however, excess copper would be expected to increase rather than decrease CAIII. Moreover, if CAIII were needed for expression of hepatic copper transport, this enzyme should be found in all mammalian livers, but high levels are only found in rodents [31]. Thus it is unlikely that CAIII has a direct role in copper transport.

The sequences of the CNBr peptides provide suggestive evidence for the occurrence of closely related CAIIIs in mice, which we have termed CAIIIA and CAIIIB. There has been no previous report of two isoforms of CAIII in mouse or any other species. Since CAIII is a predominant protein in muscle, we prepared the protein from this source and the CNBr digestion showed the same pattern of bands, suggesting that the two putative isoforms are also present (results not shown). The conclusive proof of another isoform of CAIII will require full sequence characterization. None of the cDNA clones isolated using oligonucleotides based on the published sequence, or by screening with the CAIIIA cDNA probes, corresponded to the novel form, possibly because the novel isoform sequence may not cross-hybridize with CAIIIA probes. If the occurrence of CAIIIB is confirmed, it may be that the novel isolation procedure used here was instrumental in its isolation.

We conclude that copper accumulation in the *tx* mouse liver depresses the levels of CAIII mRNA and, in turn, CAIII protein. It will be of interest to examine further the molecular basis of the effect of copper on CAIII mRNA and to determine whether CAIII does have any role in copper transport.

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