Expression of transcobalamin II mRNA in human tissues and cultured fibroblasts from normal and transcobalamin II-deficient patients

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Transcobalamin II (TCII) is an important plasma transporter of cobalamin (Cbl; vitamin B_{12}). In the present study, TCII gene expression in human and rat tissues and in the fibroblasts of patients with TCII deficiency was investigated. Northern-blot analyses revealed expression of TCII mRNA in many human and rat tissues. In humans, this was 14-fold higher in the kidney than in liver, whereas in the rat the levels of expression were similar in the kidney and liver. Southern-blot analysis of genomic DNA from several species revealed sequence similarity in TCII across species. Metabolic labelling and ribonuclease protection assay revealed a 43 kDa TCII protein and a fully protected TCII mRNA band in normal fibroblasts but not in fibroblasts from three TCII-deficient patients. Southern-blot analysis of genomic DNA from all these fibroblasts revealed identical restriction patterns on *Bam*HI, *Hin*dIII, *Kpn*I, *Msp*I and *Eco*RI digestion. On the basis of these results, we suggest that TCII is expressed in multiple tissues, and its level of expression in tissues varies within the same and across species. Furthermore, the TCII deficiency characterized in this study is due to the absence of TCII protein which in turn is due to the absence or extremely low levels of its mRNA and not to detectable gross alterations in the gene structure.

INTRODUCTION

Transcobalamin II (TCII) is a 43 kDa non-glycoprotein (Quadros et al., 1986) transporter of cobalamin (Cbl; vitamin B_{12}) in plasma (Hall and Finkler, 1965). It functions in the delivery of plasma Cbl to tissues by receptor-mediated endocytosis (Youngdhal-Turner et al., 1979). After internalization, TCII is degraded in the acidic environment of the lysosomes and the free Cbl is transported out of the lysosomes to the cytoplasm (Cooper and Rosenblatt, 1987). Cbl is converted into methyl-Cbl in the cytoplasm and 5′-deoxyadenosyl-Cbl in mitochondria and these two forms are utilized as coenzyme by methionine synthase and methylmalonyl-CoA mutase respectively. The essential role of TCII in the cellular delivery of Cbl for intracellular utilization is borne out by the fact that an inherited lack of TCII leads to intracellular Cbl deficiency, resulting in megaloblastic anaemia, impaired immune defence, neurological disorders (Hakimi et al., 1971; Hitzig et al., 1974; Burman et al., 1979; Hurani et al., 1979; Hall, 1981; Frater-Schroder et al., 1981; Thomas et al., 1982) and, in extreme cases, death if not treated with Cbl.

Hereditary TCII deficiency is a rare autosomal recessive disorder (Hakimi et al., 1971) and three forms of TCII defect have been reported (Frater-Schroder, 1983). In the most common, immunoreactive TCII is absent from the plasma of TCII-deficient patients. The other two forms include the presence of immunoreactive TCII that is either unable to bind Cbl (Seligman et al., 1980; Thomas et al., 1982; Hoffbrand et al., 1984) or to promote its uptake by cells (Burman et al., 1979). This latter defect is most probably due to an inability of the TCII–Cbl complex to bind to its cell surface receptor. The three patients used in this study have been shown to have no unsaturated Cbl-binding activity in their plasma (Hakimi et al., 1971; Scott et al., 1972; Berliner and Rosenberg, 1980). In addition, cultured fibroblasts derived from these patients failed to incorporate [^{57}Co]Cbl into intracellular Cbl-dependent enzymes (Rosenblatt et al., 1987; Barshop et al., 1990). Despite these studies, it is not known whether these defects are due to the absence of TCII or an inability of TCII to bind Cbl.

Although the role of plasma TCII in the transport of Cbl to tissues is well established in all animals, including humans, no direct analysis of TCII gene expression in humans or any other species has been carried out in normal or deficient states. Using human TCII cDNA recently isolated in our laboratory (Li et al., 1993), these issues have been addressed. The results of the present study show that TCII mRNA is expressed in multiple tissues, although the levels of expression varied in different tissues, both in the same and across species. In addition, expression of TCII mRNA and protein was detected in fibroblasts from normal controls but not from the three TCII-deficient patients used in this study.

EXPERIMENTAL

Materials

Human skin fibroblasts from one control (MCH 24) and TCII-deficient cell lines from unrelated patients were obtained from the Repository for Mutant Human Cell Strains (Montreal Children's Hospital, Montreal, Quebec, Canada). Clinical and laboratory findings supported the diagnosis of TCII deficiency in WG 1221 (Hakimi et al., 1971; Scott et al., 1972; Berliner and Rosenberg, 1980; Rosenblatt et al., 1987), WG 1276 (Barshop et al., 1990) and WG 1346 (N. Li, S. Seetharam, D. S. Rosenblatt and B. Seetharam, unpublished work). The following were bought commercially as indicated: Northern blots containing poly(A)$^+$ RNA from multiple human and rat tissues, and a Southern blot containing *Eco*RI-digested genomic DNA from different species (Clonetech, Palo Alto, CA, U.S.A.); RNAase-protection assay (RPA) kit (Ambion, Austin, TX, U.S.A.); Trans[^{35}S]label (specific radioactivity > 1100 Ci/mmol, containing 70% L-[^{35}S]methionine, 15% L-cysteine and the remainder

Abbreviations used: Cbl, cobalamin; TCII, transcobalamin II; RPA, RNAase-protection assay; poly(A)$^+$, polyadenylated.
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non-amino acid derivatives; Amersham, Arlington Heights, IL, U.S.A.). Antiserum to human TCII was raised in rabbits as described previously (Ramanujam et al., 1991a).

Northern-blot analysis

A commercial blot containing human or rat tissue poly(A)$^+$ RNA was hybridized overnight with ^{32}P-labelled TCII cDNA (specific radioactivity $\sim 1 \times 10^9$ d.p.m./μg of DNA) labelled by random-priming (Feinberg and Vogelstein, 1983) in 5 × SSPE, 10% Denhardt's solution, 50% formamide, 2% SDS and 100 μg/ml salmon sperm DNA at 42 °C. The blot was washed at low stringency (2 × SSPE/0.05% SDS) at 55 °C for 1 h. In order to correct for the possible variation in the amounts of mRNA used in each lane and to make sure that mRNA used from various tissues was intact, the same blots were stripped and rehybridized with full-length (2 kb) human β-[^{32}P]actin or mouse β-actin probe (specific radioactivity $\sim 1 \times 10^9$ d.p.m./μg of DNA). Autoradiographs were visualized for TCII mRNA after exposure in the presence of an intensifying screen for 16 or 40 h (human) or 5 days (rat). For the visualization of actin mRNA, the blots were exposed in the absence of an intensifying screen for either 16 (human) or 5 h (rat). The TCII and cytoskeletal actin mRNA bands were quantified by scanning with a response spectrophotometer (Jostens Graphic Products, Chicago, IL, U.S.A.). For visualization and quantification of TCII mRNA in the blot containing mRNA from human spleen, thymus, prostate, testis, ovary, small intestine, colon and leucocytes, autoradiography was carried out for 40 h (results not shown). The ratio of absorbance of TCII mRNA to actin mRNA was calculated for each tissue and expressed as arbitrary units (testis = 1 unit).

Cell culture

Fibroblasts were cultured at 37 °C in 5% CO_2 in Eagle's minimum essential medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 2 mM glutamine, 0.25 mM non-essential amino acids and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin and 0.75 mg/ml amphotericin).

Metabolic labelling and immunoprecipitation of TCII

Confluent fibroblasts (5×10^6) were labelled with 60 μCi/ml Trans[^{35}S]label in 90% methionine-free minimal essential medium and 10% minimal essential medium for 24 h. After labelling, the medium was collected and the cells were extracted with Tris/HCl buffer, pH 7.4 (50 mM), containing 150 mM NaCl and 1% Triton X-100 at 5 °C for 18 h. The labelled medium (7 ml) or the cell lysate (2 ml) was first incubated with preimmune rabbit serum (15 μl) for 2 h at 5 °C, followed by incubation with a 10% suspension of washed formalin-fixed Staph-A cells (100 μl) for 2 h and centrifugation. The precleared medium or cell lysate was then incubated with human TCII anti-serum (15 μl) for 12 h at 5 °C. The labelled TCII bound to the antiserum was precipitated with a 1:1 suspension of Protein A–Sepharose beads. The radioactive immune pellet collected by centrifugation was repeatedly washed with 10 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl and 1% Triton X-100. The washed immune pellets were boiled in the loading buffer (1% SDS) and the radioactivity was separated on an SDS/(10%) polyacrylamide gel as described by Laemmli (1970).

RPA

Total RNA from confluent fibroblasts was isolated by the guanidine thiocyanate/caesium chloride method (Chirgwin et al., 1979). Two riboprobes (see Figure 5), one from the 3′ end and the other from the 5′ end of the TCII cDNA, were generated as follows. The 3′-end probe was generated by linearizing the recombinant plasmid V_2 (Li et al., 1993) containing the full-length coding region of TCII cDNA with *Nde*I, followed by *in vitro* transcription in the presence of [^{32}P]UTP using T7 RNA polymerase. To prepare the 5′-end probe, TCII cDNA derived from the V_2 clone was digested with *Hin*dIII and the 5′-*Hin*dIII fragment (328 bp) was subcloned into the Bluescript KS-II (Stratagene) vector. The recombinant plasmid was subjected to double-stranded sequence analysis (Sanger et al., 1977) to confirm the predicted sequence and linearized with *Eco*RV. The 5′-riboprobe was synthesized by *in vitro* transcription in the presence of [^{32}P]UTP using T7 RNA polymerase. The specific radioactivity of these riboprobes was $\sim 0.8 \times 10^8$ d.p.m./μg. After synthesis, the riboprobes were purified by urea/PAGE and subjected to solution hybridization with 20 μg of total cellular RNA for 16 h at 43 °C. The RNA was digested with RNAase A (1 unit/ml) and RNAase T1 (200 units/ml) at 37 °C for 30 min, followed by proteinase K (0.16 mg/ml) digestion for 15 min at 37 °C in the presence of SDS (0.8%). The digest was then subjected to phenol extraction and ethanol precipitation. The protected fragment was analysed by electrophoresis on an 8 M urea/6% acrylamide gel and visualized by autoradiography after exposure in the presence of an intensifying screen for 3 days at −80 °C. In order to test the integrity and relative amounts of total RNA used in RPA, total RNA (5 μg) from the same batch of RNA was subjected to RPA using mouse β-[^{32}P]actin riboprobe (250 bp; specific radioactivity 0.8×10^8 d.p.m./μg). The bands were visualized after exposure without an intensifying screen for 12 h at room temperature.

Southern-blot analysis

Genomic DNA from control and TCII-deficient fibroblasts was isolated as described by Gross-Bellard et al. (1973). DNA (7.5 μg) was digested with *Eco*RI, *Hin*dIII, *Kpn*I, *Msp*I or *Bam*HI, electrophoresed on a 0.9% agarose gel and transferred to a Nytron nylon membrane. Full-length ^{32}P-labelled TCII cDNA (specific radioactivity $\sim 1 \times 10^9$ d.p.m./μg) was generated by random-priming (Feinberg and Vogelstein, 1983) and used for hybridization. Hybridization was carried out at 42 °C for 16 h in hybridization buffer containing 6 × SSPE, 1% SDS and 100 μg/ml denatured salmon DNA. After hybridization, the filter was washed in 0.1% SSPE/1% SDS for 30 min at 65 °C and subjected to autoradiography.

A Southern blot containing *Eco*RI-digested genomic DNA from various species was hybridized with full-length human ^{32}P-labelled TCII cDNA (specific radioactivity $\sim 1 \times 10^9$ d.p.m./μg). The hybridization and washing conditions were the same as that used in probing the Northern blot except that the washing temperature was 65 °C.

RESULTS AND DISCUSSION

Distribution of TCII mRNA in human and rat tissues and sequence identity of TCII across species

The tissue distribution of steady-state levels of TCII mRNA was studied in human and rat tissues by Northern-blot analysis using poly(A)$^+$ RNA obtained from these tissues. All the human tissues tested (Figure 1a) revealed a single band corresponding to 1.9 kb

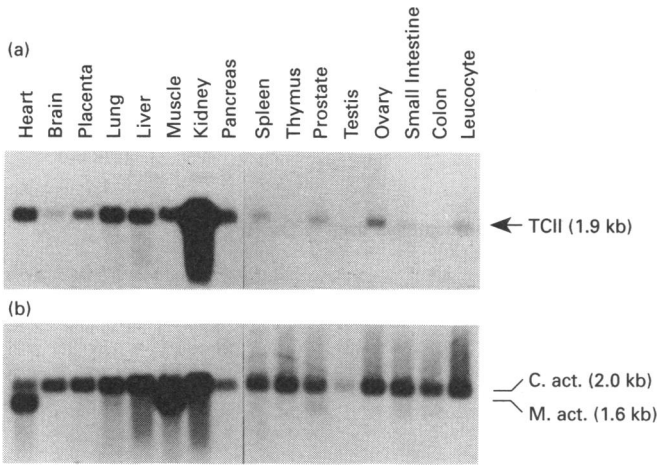

Figure 1 Expression of TCII mRNA in human tissues

Northern-blot analysis of poly(A)$^+$ RNA (2.5 μg) from the tissues indicated was probed with full-length ^{32}P-labelled human TCII (**a**) or β-actin (**b**) cDNA. See the Experimental section for details of washing and autoradiographic analysis. C. act., cytoskeletal actin; M. act., muscle actin.

Table 1 Relative expression of TCII mRNA in human and rat tissues

The relative distribution of TCII mRNA in each tissue is expressed as a ratio of the intensity of TCII mRNA to that of cytoskeletal actin mRNA. The unit is an arbitrary number and the ratio in the testis in both human and rat is taken as 1 unit.

	Human		Rat	
	units	%	units	%
Heart	6.8	17.0	2.1	37.5
Brain	2.0	5.0	1.1	19.4
Spleen	2.0	5.0	2.1	37.5
Lung	3.7	9.3	2.0	35.2
Liver	2.9	7.3	5.6	100.0
Skeletal muscle	3.2	8.0	2.0	35.2
Kidney	40.0	100.0	4.4	77.4
Testis	1.0	2.5	1.0	17.6
Placenta	2.5	6.3		
Pancreas	5.7	14.3		
Thymus	0.7	1.8		
Prostate	2.9	7.3		
Ovary	2.7	6.8		
Small intestine	1.7	4.3		
Colon	0.6	1.5		
Leucocyte	1.9	4.8		

when probed with full-length ^{32}P-labelled TCII cDNA; the band of highest intensity was from kidney. To normalize the amount of mRNA loaded in each lane, the same blot was stripped and hybridized with ^{32}P-labelled β-actin cDNA (Figure 1b). The relative distribution of TCII mRNA in human and rat tissues normalized to cytoskeletal actin mRNA levels are shown in Table 1. In humans, taking the level of TCII mRNA expression in the kidney as 100%, the relative levels of TCII mRNA were about 15% in heart and pancreas, about 7–9% in placenta, lung, liver, muscle, prostate and ovary, about 5% in leucocytes, small intestine, spleen and brain and about 2% in the colon, thymus

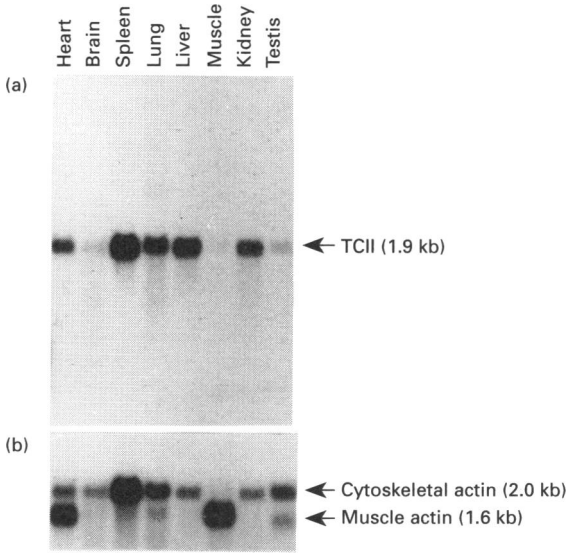

Figure 2 Expression of TCII mRNA in rat tissues

Northern-blot analysis of poly(A)$^+$ RNA (2.5 μg) from rat tissues was probed with full-length ^{32}P-labelled human TCII (**a**) or mouse β-actin (**b**) cDNA. Details of hybridization and washing conditions are provided in the Experimental section.

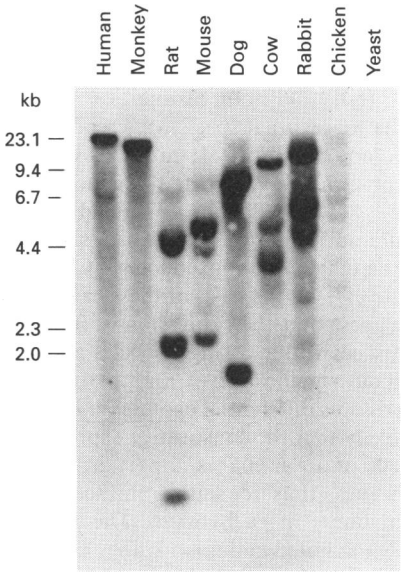

Figure 3 Southern-blot analysis of genomic DNA from different species

A blot containing *Eco*RI-digested genomic DNA from the indicated species was probed with radiolabelled full-length human TCII cDNA. Details of hybridization and washing are provided in the Experimental section.

and testis. In order to quantify TCII mRNA in the human spleen, thymus, prostate, testis, ovary, small intestine, colon and leucocyte the autoradiograph was exposed for a longer period of time (40 h) (results not shown).

The multiple tissue expression of TCII mRNA noted in humans was also observed in rat tissues (Figure 2a). However, unlike in humans, the TCII mRNA level was the highest in the liver followed by kidney (Figure 2a). As a control of sample loading

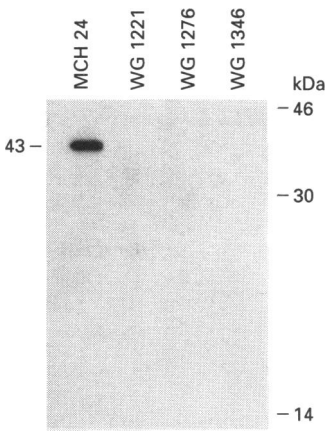

Figure 4 SDS/PAGE analysis of immunoprecipitated radiolabelled TCII from fibroblasts of normal control and TCII-deficient patients

TCII was immunoprecipitated using human TCII antiserum from the medium of metabolically labelled fibroblasts of control (MCH 24) and TCII-deficient patients (WG 1221, WG 1276 and WG 1346). The washed immune pellets were analysed on an SDS/10%-polyacrylamide gel followed by fluorography. The bands were visualized after 5 days of exposure. Other details of labelling and immunoprecipitation are provided in the Experimental section.

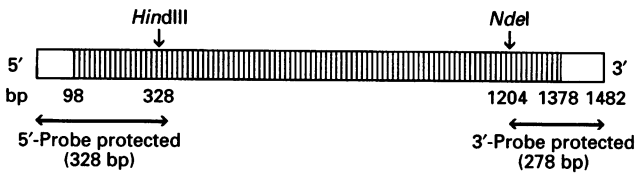

Figure 5 Antisense riboprobes used in the RPA

Full-length TCII cDNA (1.482 kb) containing the coding region (striped box) and the 5′ and 3′ non-coding regions (open boxes) are shown. The 5′-riboprobe protects 328 nt of TCII mRNA fragment consisting of 98 nt of 5′ non-coding and 230 nt of coding region. The 3′-probe protects 278 nt of TCII mRNA spanning 104 nt of the 3′ non-coding and 178 nt of coding region. The details of methods involved in the generation of these probes are provided in the Experimental section.

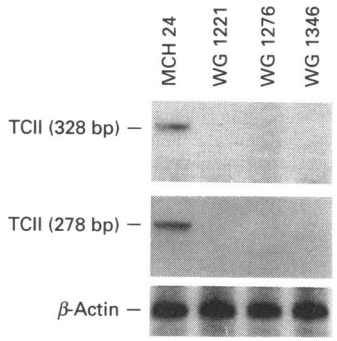

Figure 6 TCII mRNA expression in skin fibroblasts of normal and TCII-deficient patients

RPA was carried out using total RNA (40 μg) from skin fibroblasts of normal (MCH 24) and TCII-deficient patients (WG 1221, WG 1276, WG 1346). The fully protected bands of 328 bp and 278 bp indicated were obtained using the 5′- and 3′-riboprobes (see Figure 5) respectively. Details of RPA and the preparation of the riboprobes used are provided in the Experimental section.

and integrity of mRNA, the same blot was stripped and probed with ^{32}P-labelled β-actin cDNA. On normalization with β-actin mRNA and taking the level of TCII mRNA expressed in the rat liver as 100%, the relative expression was about 77% in the kidney, about 36% in the heart, spleen, lung and muscle and about 18% in testis and brain (Table 1).

These results suggest that species differences exist with respect to the relative amounts of TCII mRNA expressed in different tissues. This observation supports the conclusions from earlier studies with mice (England et al., 1973) and dogs (Sonneborn et al., 1972; Hall and Rappazo, 1975) which showed that, in these two species, the major organ producing TCII was the liver and kidney respectively. On the basis of these earlier and our present studies, we suggest that the expression of TCII is differentially regulated in tissues within a single species and across species.

The functional significance of multiple tissue expression of TCII mRNA and its differential expression in these tissues is not known. It is likely that the constitutive expression of TCII in all the tissues/cells of an animal is important to mediate Cbl flux across membranes, thus regulating intracellular levels of Cbl within certain limits in each tissue. The high levels of TCII mRNA expression noted in human kidney could arise from both the endothelial and the epithelial cells. Although direct evidence (Ramanujam et al., 1991b) has been provided for the synthesis of TCII in renal epithelial cells, evidence for its synthesis by the renal endothelium is only indirect. Perfusion studies by Hall and Rappozo (1975) suggested that TCII production in the kidney may occur in endothelial cells which are directly exposed to the circulation and the TCII secreted from these cells could therefore contribute to plasma TCII levels. This proposal may have some significance as endothelial cells derived from human umbilical vein in culture have been shown to synthesize and secrete TCII (Carmel et al., 1990; Quadros et al., 1990). In addition, Quadros et al. (1990) have proposed that, because of the large surface area that vascular endothelium provides, TCII synthesized by endothelial cells in the vasculature may contribute significantly to plasma TCII levels. It is interesting to note that another plasma protein in humans, Von Willebrand factor, is also derived from the vascular endothelium (Jaffe et al., 1974). However, further *in situ* hybridization studies are needed to identify the specific cell type in the kidney that produces abundant TCII mRNA transcripts.

The expression of TCII mRNA in the human intestine (Figure 1) is an interesting finding in view of its proposed role in the transport of Cbl across the mucosal barrier (Chanarin et al., 1978). Consistent with this proposal are the results of Cbl-absorption studies in TCII-deficient patients (Cooper and Rosenblatt, 1987), including two of the three patients (Rosenblatt et al., 1987; Barshop et al., 1990) reported in the present study, who failed to absorb orally administered Cbl. These studies suggest that TCII expression in the small intestine absorptive enterocytes is important in transcytosis of Cbl from the lumen to the circulation. Consistent with this suggestion are the results of *in vitro* studies (Ramanujam et al., 1991a) demonstrating *de novo* synthesis and unidirectional secretion of TCII by human colon adenocarcinoma (Caco-2) cells and expression of TCII mRNA (Li et al., 1992) in these cells. Taken together, these *in vitro* and *in vivo* studies strongly suggest that TCII synthesized by intestinal epithelial cells may play a role in the final stages of Cbl transcytosis. Although it is very difficult to measure TCII mRNA levels in the biopsy specimens from these patients (because of lack of availability of the tissue), it is likely that TCII is not expressed in the intestine, leading to Cbl malabsorption.

As human TCII cDNA recognized rat TCII mRNA on Northern blots (Figure 2), TCII sequence identity across species

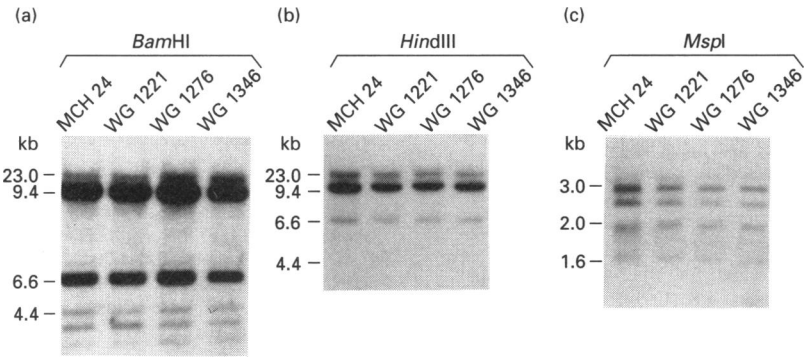

Figure 7 Southern-blot analysis of genomic DNA from normal and TCII-deficient patients

Genomic DNA (7.5 μg) from cultured fibroblasts of normal (MCH 24) and TCII-deficient (WG 1221, WG 1276, WG 1346) patients was completely digested with *Bam*HI (**a**), *Hin*dIII (**b**) and *Msp*I (**c**). The digests were subjected to Southern blotting and probed with a full-length labelled human TCII cDNA. Other details are provided in the Experimental section.

was further examined at the genomic DNA level. A Southern blot containing *Eco*RI-digested DNA from several species was probed with ^{32}P-labelled full-length human TCII cDNA (Figure 3). Hybridization bands were detected in all the species examined except yeast. DNA derived from human and monkey revealed similar patterns, indicating the absence of an *Eco*RI site in the TCII gene in these two species. DNA from chicken showed very faint cross-hybridization bands, suggesting low sequence identity of TCII between chicken and human. Cross-species sequence identity of the TCII gene noted in this study is similar to that observed for the intrinsic-factor gene (Hewitt et al., 1991) but not the haptocorrin gene (Hewitt et al., 1990). These cross-species identity studies at the genomic level of the three Cbl binders support the earlier observations of Nexo and Nexo (1982), who, using a peptide-mapping technique, demonstrated that a greater degree of homology exists across species for intrinsic factor and TCII than for haptocorrin.

Defective TCII gene expression in patients with TCII deficiency

In order to examine whether fibroblasts from TCII-deficient patients were able to synthesize and secrete TCII, cultured fibroblasts from three TCII-deficient patients and a normal control patient were metabolically labelled. Immunoprecipitation of the labelled medium and SDS/PAGE analysis revealed a 43 kDa band in the medium of normal fibroblasts only (Figure 4). Immunoprecipitation of the detergent extract of fibroblast lysate revealed a faint TCII band in the normal control but not in the TCII-deficient patients (results not shown). This observation demonstrates that the lack of TCII Cbl-binding activity in the three patients was not due to the production of a mutant TCII that was not secreted or had lost its Cbl-binding function, but was due to the absence of TCII protein. The lack of TCII protein in these patients could be due to rapid degradation of mutant TCII or to the absence of its mRNA. Owing to low levels of TCII protein and mRNA expression in fibroblasts, we chose to measure the steady-state levels of TCII mRNA by RPA, a technique that is at least ten times more sensitive than Northern-blot analysis. Two riboprobes (Figure 5) and total RNA from the fibroblasts of normal control and TCII-deficient patients were used. Total RNA from normal fibroblasts revealed a fully protected band of 328 bp (5′-probe) and 278 bp (3′-probe) (Figure 6) in this assay, whereas no protected bands were observed using RNA from the three TCII-deficient patients. As a control of the integrity and the amount of RNA used, RPA was also carried out to measure β-actin mRNA in the same RNA samples. Mouse β-actin riboprobe revealed a single protected β-actin-protected mRNA band of similar intensity in all the lanes (Figure 6). These results suggest that TCII protein deficiency in these patients is due to the absence of its mRNA transcript.

Southern-blot analysis was then performed to examine whether the lack of TCII mRNA noted in these patients, who were homozygous for TCII deficiency, was a consequence of gross alterations in the TCII gene. Hybridization of a full-length TCII cDNA probe to genomic DNA digested with *Bam*HI, *Hin*dIII, *Msp*I or *Eco*RI and *Kpn*I (results not shown) revealed restriction banding patterns that were indistinguishable in the normal control and the three TCII-deficient patients (Figure 7). *Eco*RI and *Kpn*I digests of the genomic DNA revealed only a single band corresponding to about 23 kb (results not shown). These results suggest that the TCII gene was preserved in the three patients with TCII deficiency and that no gross insertions, deletions, rearrangements or point mutations at the restriction-endonuclease-recognition sequences tested had occurred. At present, the cause of the TCII mRNA deficiency in these patients is not known. It may be a transcriptional or processing defect or unstable TCII transcripts. Further studies are needed to explore these possibilities.

In summary, the results of the present study have provided important insights into TCII gene expression in normal human and rat tissues and for the first time have provided evidence that some forms of TCII deficiency are due to the absence of TCII mRNA.

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