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Functional rescue of the glomerulosclerosis phenotype in Mpv17 mice by transgenesis with the human Mpv17 homologue

JOHANNES SCHENKEL, RALF MICHAEL ZWACKA, CHRISTIANE RUTENBERG, ALEXANDER REUTER, RÜDIGER WALDHERR, and HANS WEIHER

Forschungszentrum Karlsruhe, Institute of Genetics, Karlsruhe, and Institute of Pathology, University of Heidelberg, Heidelberg, Germany

Functional rescue of the glomerulosclerosis phenotype in Mpv17 mice by transgenesis with the human Mpv17 homologue. The germ line insertion of a defective retrovirus into the Mpv17 gene of mice is associated with a recessive phenotype. Mice homozygous for the integration develop glomerulosclerosis at a young age. The phenotype resembles human glomerulosclerosis in its physiological parameters as well as in histology. A human homologue of the Mpv17 gene has been identified, isolated and analyzed. We here show that this gene, which has a role in the production of reactive oxygen species, can rescue the phenotype of Mpv17 deficient mice when introduced by transgenesis. This provides formal proof for the hypothesis that the phenotype is caused by the loss of function of the Mpv17 gene. It also provides evidence for the functional conservation of the Mpv17 gene in mammals and points to a potential role of this gene in human kidney disease.

The Mpv17 mouse strain was generated by insertion of a defective retrovirus into the germline of mice. Mice homozygous for the integration develop a phenotype of glomerulosclerosis at age three months or older and die later as a consequence of renal failure. A gene was identified at the integration site and its expression was found to be completely abolished by the integration [1]. This gene, the Mpv17 gene, is therefore a good candidate for a recessive disease gene causing glomerulosclerosis. A human homologue has been identified, isolated and studied [2]. It is a single copy gene with very high homology to the mouse gene. Both genes code for proteins of 176 amino acids in size, and we have recently identified the Mpv17 gene product as a player in the peroxisomal reactive oxygen metabolism [3]. We show here that the human homologue expressed from a heterologous promoter in the kidney after introduction as a transgene can rescue Mpv17 homozygous animals from developing glomerulosclerosis. This provides proof that the loss of Mpv17 gene expression is indeed causal to the phenotype. It also demonstrates functional homology between the mouse and the human gene and thereby underlines the value of the Mpv17 deficient mouse as a model of human glomerulosclerosis.

Methods

Cloning of the rescue construct

The mouse MT-1 promoter was cut out of the vector Mthgh-Dhfr (provided by E. Wagner, Vienna, Austria) [4, 5] with Eco RI and Bam HI. The 1.8 kb fragment was gel-purified and cloned into pBluescript II KS using the above-mentioned restriction sites. From a human Mpv17 cDNA, containing intron 6 due to incomplete splicing [2], the vector p6/2 was released with Sac I, the 1.2 kb fragment isolated and ligated into the according site of the pBluescript already containing the MT promoter. The correct orientation of the cDNA was verified by several asymmetric digests. The MT-Mpv17 (6/2) fragment was then released with BssHII from the pBluescript vector, purified over an agarose gel, and used for microinjection of mouse zygotes.

Generation of transgenic mice and genotyping

The 3 kb promoter/gene DNA fragment was dissolved in injection buffer (10 mM Tris pH 7.5, 0.1 mM EDTA) at a concentration of 5 ng/μl. The DNA solution was sterile filtered and injected into the pronuclei of zygotes of female Balb/c mice mated to male Balb/c or Mpv17 mice (the latter are on a CFW/Balb/c background).

Females were superovulated by intraperitoneal injections of 10 U of pregnant mare serum (PMS, Sigma) and after 48 hours with 10 U of human chorionic gonadotropin (HCG, Sigma). Eggs surviving microinjection were transferred into the oviducts of pseudopregnant foster mice according to Hogan, Constantini and Lacy [6]. Mice born were tested for the integration of the construct DNA by Southern blot analysis of tail DNA. For this purpose the DNA was digested with Sac I and hybridized with a human cDNA probe [2]. The mutation of the endogenous Mpv17 gene was monitored accordingly, whereby the tail DNA was restricted with Bam HI and hybridized with a genomic 350 bp Sau 3A restriction fragment localized close to the integration site within the Mpv17 gene [1]. Hybridizations and washes were carried out under high stringency [3].

Reverse transcriptase-PCR (RT-PCR)

Total RNA was prepared from mouse kidneys by the guanidiniumisothiocyanate method [7] and was enriched for polyadenylated RNA. For that 300 μg of total RNA were incubated overnight with oligo(dT)-cellulose type VII (Pharmacia) in 500 mM NaCl, 20 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.1% SDS, and proteinase K (300 μg/ml). After several washing steps with 400 mM NaCl, 20 mM Tris pH 7.4, 10 mM EDTA, 0.2% SDS and 100 mM NaCl, 20 mM Tris pH 7.4, 10 mM EDTA, 0.2% SDS, RNA was eluted with diethyl pyrocarbonate (DEPC) treated water and ethanol precipitated. Subsequently the RNA pellet was purified with phenol and chloroform and again precipitated. One hundred

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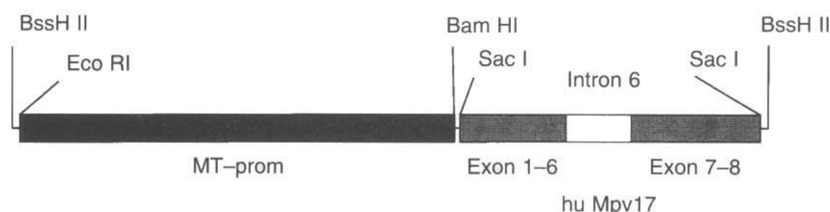

Fig. 1. *The human MT Mpv17 rescue gene construct.* The human Mpv17 cDNA gene including intron 6 is under control of the mouse metallothioneine (MT) promoter. The 3 kb BssH II restriction fragment was used for the generation of transgenic mice.

nanograms of each polyA$^+$ RNA were then used as a template to synthesize Mpv17 cDNA in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 1 mM of each dNTP (Boehringer, Mannheim, Germany), 10 units of RNase inhibitor (USB), 2.5 μM of oligo d(T) primer (Boehringer), 2.5 units of avian myeloblastosis virus reverse transcriptase (AMV-RT, Promega) and filled up to 20 μl with water. The samples were incubated for 20 minutes at 42°C and subsequently denatured at 95°C for 5 minutes. For the PCR reaction the $MgCl_2$ concentration was adjusted to 2 mM and the final reaction volume was scaled up from 20 μl to 100 μl. In addition, the samples were supplemented with Mpv17 specific primers from the human cDNA to a final concentration of 0.15 μM and 2.5 units of Taq polymerase (Amersham) were added. The sequences of the primers used are:

1s: 5′GAATTCGAGGCTCGGCGCTCAGGAAGC3′ and

4r: 5′GAATTCAAACGATGGAGTGAGGCAGGC3′

representing nucleotide positions 9–29 (1s) and 582–561 (4r), respectively [2]. After 35 cycles 20 μl of each sample were analyzed on a 1% agarose gel. The observed size for correct cDNA expression was as expected 585 bp [2]. In the RT negative controls the identical procedure was carried out omitting the reverse transcriptase enzyme.

Histology

Kidneys from homozygous and heterozygous Mpv17 mice as well as MT670 mice were fixed in 4% buffered paraformaldehyde, dehydrated and embedded in paraplast. Three micrometer sections were prepared, stained with PAS (Sigma Diagnostics) and Masson trichrome stain.

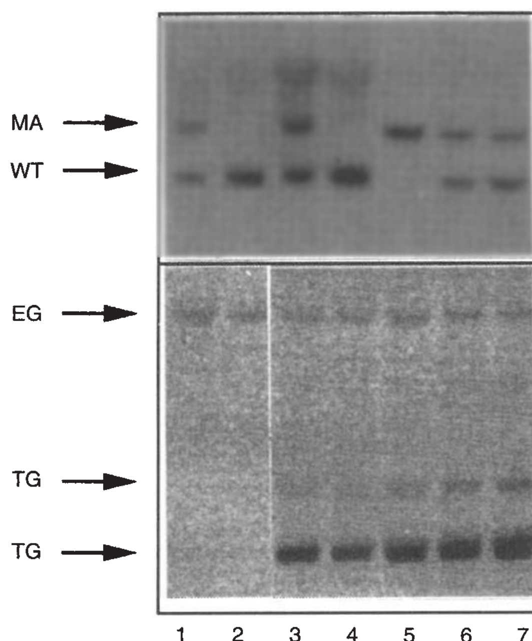

Fig. 2. *Genotyping of transgenic mice.* Southern blot analysis of tail DNA of the transgenic mice (numbers 1 to 7 indicated at the bottom). **Top panel.** Endogenous Mpv17 locus. BamHI cleaved DNA was hybridized with a 350 bp Sau 3A fragment diagnostic for the mutation [1]; WT: wt Mpv17 allele; MA: mutated Mpv17 allele. **Lower panel.** Presence of the MT-Mpv17 transgene. Sac I digested DNA was hybridized with a fragment representing the human cDNA. TG: transgene fragment. EG: cross hybridizing endogenous Mpv17 locus. The kidney histology of mouse 5 (homozygous mutant Mpv17; transgenic for MT-Mpv17) is shown in Figure 3 e and f.

Results

Construction of a vector expressing the human Mpv17 gene and generation of transgenic lines

The complete human Mpv17 cDNA was cloned previously as a fragment of 1.0 kb [2]. In the process of this procedure an incompletely spliced cDNA molecule was also isolated, in which the 263 bp intron between exon 6 and exon 7 had not been removed [2]. For several reasons we used this molecule to assemble the expression vector. Firstly, it has been reported earlier that introns enhance the expression of transgenes in general [8, 9]. Secondly, when the transgene expression is analyzed by reverse transcription PCR, artifacts due to contamination of the mRNA with genomic DNA can be readily excluded on the basis of the size of the amplification product (see below). We placed this gene, including its 3′untranslated region and its polyA addition signal, under the control of the mouse metallothioneine I promoter contained on a 1.8 kb fragment [10]. This promoter has been used before successfully to express transgenes in various tissues of mice [11]. Figure 1 depicts the resulting construct used for microinjection. Mice developed from injected eggs were tested for the integration of the construct DNA by Southern blot analysis of tail DNA. Therefore, the tail DNA was cleaved with Sac I restriction endonuclease and hybridized to the radiolabeled human cDNA fragment [2]. The transgene was detected as hybridizing fragment of 1.2 kb as shown in the bottom of Figure 2 (lower band). The second transgene specific fragment (TG) presumably represents a transition fragment at the border of the transgene integration generated by the loss of the Sac I site flanking the construct at its 3′end. Under this assumption, the number of tandem transgene copies in the particular strain displayed in Figure 2 is about 15, judging from the intensity of the transgene specific bands. Despite of the stringent hybridization conditions the endogenous Mpv17 locus cross hybridized with the human cDNA. Figure 2 shows the analysis for one of the three transgenic strains generated. None of these displayed any obvious phenotype.

Rescue of the glomerulosclerosis phenotype of Mpv17 mice by genetic introduction of the human homologue

The Mpv17 mouse strain contains a silent retroviral insert in both alleles of the Mpv17 gene. We were interested to investigate

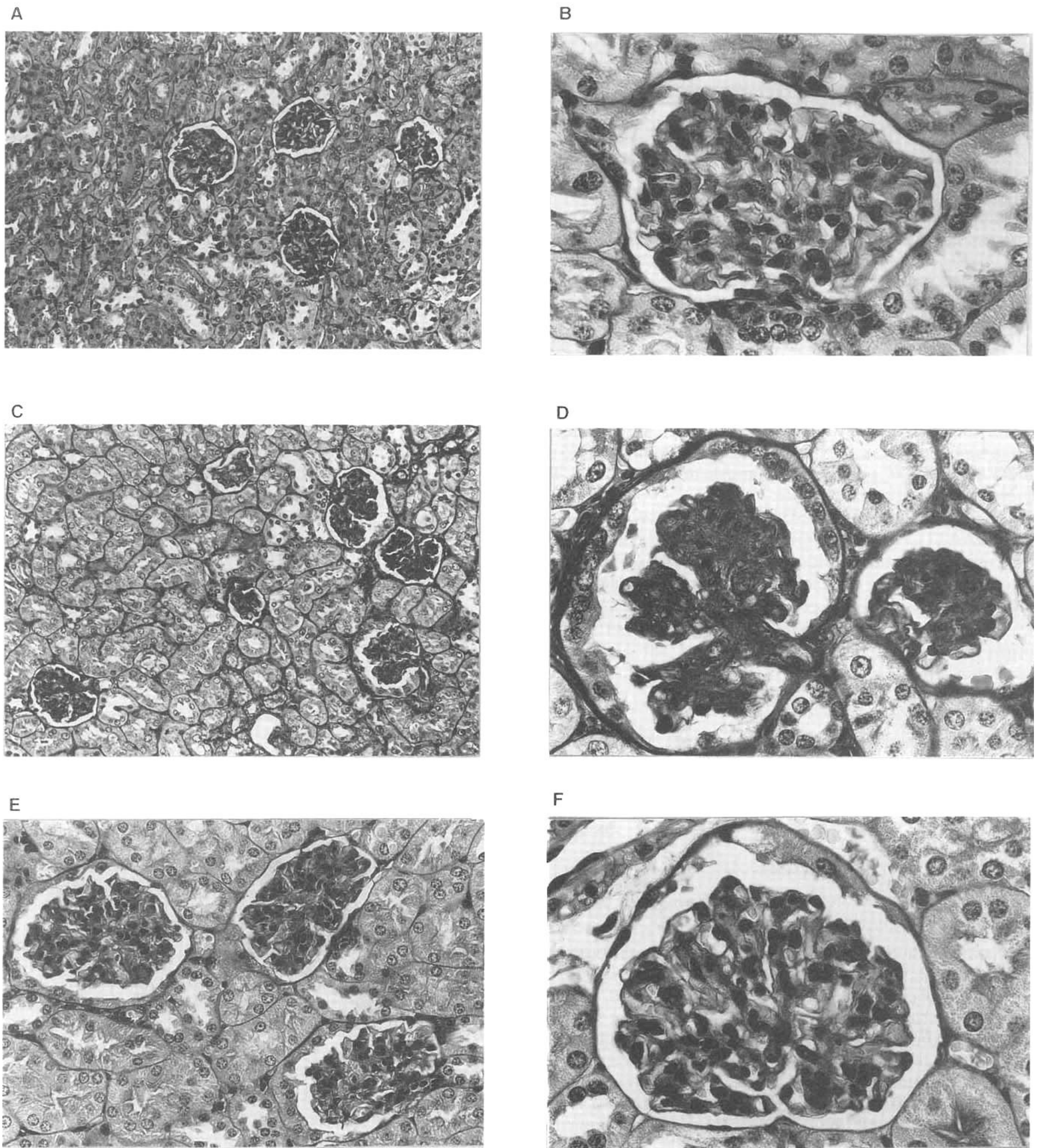

Fig. 3. *Representative histology (PAS stain) of six months-old mice with different Mpv17 genotype.* **a** (×140) and **b** (×550). Heterozygous Mpv17. **c** (×140) and **d** (×430). Homozygous mutant Mpv17 mice demonstrating an early stage of glomerular sclerosis; tubular interstitial changes have not yet developed. **e** (×270) and **f** (×550). Strain MT670 (homozygous mutant Mpv 17, transgenic for MT-Mpv17; mouse 5 in Fig. 2).

whether the human gene, expressed as a transgene, could complement the glomerulosclerosis phenotype in such mice. Therefore transgenic mice carrying the human Mpv17 gene were mated with homozygous Mpv17 mice. This is possible because Mpv17 homozygotes are fertile and live long enough to reproduce. To follow the mutant endogenous allele in these breedings, we used

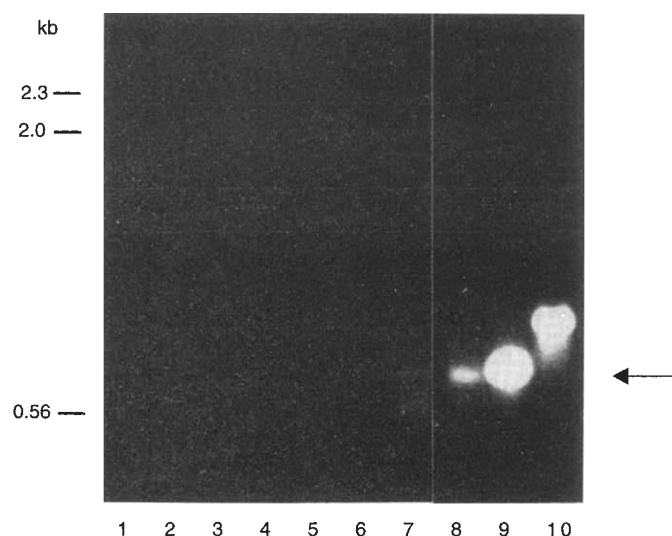

Fig. 4. *Analysis of the transgene expression.* Transgene expression was examined using the RT-PCR technique on polyA$^+$ RNA of kidneys from different mice. Lanes 1 and 5. Homozygous mutant Mpv17 mouse. Lanes 2 and 6. MT670 mouse (mouse 5 in Fig. 2). Lanes 3 and 7. Human GM637 fibroblasts. Lane 4 and 8. RSV 7 cells, murine transfected fibroblasts, constitutively expressing the human Mpv17 gene [3]. Lanes 1 to 4. Reverse transcriptase was omitted from the RT-PCR protocol as control for DNA contamination. Lanes 5 to 8. Complete RT-PCR protocol. Lane 9. Amplification on the human cDNA clone p21 [2]. Lane 10. Amplification on the MT-Mpv17 rescue construct, containing intron 6. For correctly spliced transgene derived mRNA a product of 585 bp is expected (lane 9). Unspliced transgene mRNA or contaminating transgene DNA results in a fragment of 848 bp (lane 10).

a polymorphism between the wt and mutated alleles [1]. In particular, a genomic probe (Sau 3A 350) from the integration site detects a 3 kb fragment in the wt allele (WT in Fig. 2) as opposed to a 6 kb fragment (MA in Fig. 2) in the mutant allele in Bam H1 digested DNA. Thus, heterozygous mice show both the WT and the MA band, whereas hybridization only to the MA band indicates homozygosity for the mutation. The initial breeding produced transgene positive Mpv17 heterozygous offspring which did not show any phenotype. These were interbred and the genotype of the resulting F2 generation was analyzed. Homozygous Mpv17 mice carrying the MT-Mpv17 transgene (this genotype is shown in lane 5 of Fig. 2) were interbred further and designated MT670. So far over 50 animals of this genotype were produced, none of which died of or developed symptoms of nephrotic syndrome. By contrast, all Mpv17 homozygous mice without the human transgene eventually developed the disease and died from it, although the onset of disease was variable due to the genetic heterogeneity of the background strain. Thus, the human transgene obviously affects the survival of Mpv17 mice. To determine whether the presence of the transgene prevented these mice from developing the glomerulosclerosis phenotype, several animals were sacrificed at the age of six months and analyzed histologically. A standard PAS staining is shown in Figure 3 demonstrating the sclerosis in Mpv17 mutant mice (c,d), while the heterozygous control mice showed no pathology in the glomeruli (a,b). Likewise, mice of the MT670 strain showed normal glomerular histology (e,f). Thus, on this level, transgene-dependent successful rescue was seen.

Expression of the human transgene in the kidney of MT670 mice

To investigate transgene expression mRNA was analyzed prepared from the kidneys of MT670 mice and Mpv17 mice, respectively, and reverse transcription PCR analysis was performed (Fig. 4). The primers used in this analysis specifically detected transgene derived transcripts, because they were chosen from the 5′ and 3′ untranslated regions of the human cDNA, where the murine and human sequences significantly differ [2]. A product of the expected size of 585 bp for authentic, fully spliced mRNA was detected in the sample from the MT670 mouse (lane 6). Likewise, a band of the same size was detected in human GM637 fibroblasts (lane 7), as well as in murine fibroblasts, transfected with a construct constitutively overexpressing the human Mpv17 gene (lane 8) [3]. By contrast, no expression was detected in nontransgenic Mpv17 homozygotes (lane 5). The amplification products do not originate from contaminating genomic DNA in the RNA samples because the transgene represents a cDNA in which exon 6 of 263 bp has not been removed [2]. Amplification of genomic transgene DNA therefore results in a larger fragment, as shown in a control reaction (lane 10). These results demonstrate transgene expression in the kidney of MT670 mice, and we conclude that the expression of the human homologue in these mice complements for the loss of Mpv17 function in homozygous Mpv17 mutant mice. This functional rescue not only proves that the phenotype in the mutant is caused by the loss of function of the Mpv17 gene, but is also evidence for the functional homology of the two genes in mouse and humans.

Discussion

In contrast to transgenic mouse or rat models where glomerulosclerosis has been dominantly induced by expression of a foreign transgene [12–16], the Mpv17 mouse strain is the only recessive mouse model of glomerulosclerosis so far. Therefore, it defines an endogenous gene whose loss of function correlates with this disease and constitutes a valuable genetic animal model of the human disorder. The gene inactivated by the retroviral insertion therefore is a candidate for a recessive disease gene in mice. The experiments described here show that the failure to express the gene is causal to the phenotype, since complementation can be achieved by transgenesis.

The Mpv17 gene product is a peroxisomal protein of 176 amino acids in humans and mice alike. We have shown recently that this protein is involved in the generation of reactive oxygen species (ros), most likely superoxide [3]. Remarkably, the Mpv17 defect results in decreased rather than increased ros levels, which is in contrast to chemically-induced glomerulosclerosis models in rodents. There, a direct action of ros on the glomerular basement membrane has been established [17]. Thus, we assume that the mechanisms inducing the disease are triggered by low intracellular superoxide levels, and preliminary evidence suggests that the presence of the Mpv17 gene product regulates the expression of genes involved in glomerular basement membrane turnover (Reuter, Zwacka and Weiher, manuscript in preparation). In this regard, it is intriguing that Mpv17 protein expression is normally found in podocytes (Waldherr, Zwacka and Weiher, unpublished results), the cell type mostly responsible for the biosynthesis of the glomerular basement membrane [18]. Although the combined

evidence may suggest that the kidney disease constitutes the primary defect in the mutant mice, thus far it cannot be ruled out that it may be a manifestation of an earlier defect elsewhere in the organism. The latter is in agreement with the widespread expression of the Mpv 17 gene in normal mice [1]. To distinguish between these two possibilities, the transgenic approach will be used to study whether rescue can be achieved by Mpv17 expression targeted to different tissues.

In the MT670 mice, the level of transgene expression is very low and cannot be detected by Northern blot analysis (data not shown). However, these low amounts, which we have demonstrated in the kidney of these mice, are sufficient to rescue the phenotype.

The human Mpv17 protein can functionally complement the missing Mpv17 protein in the mutant mice. This points to a conservation of its function between mammals and renders the Mpv17 gene a candidate for a human disease gene. So far, we have analyzed several cases of familial glomerulosclerosis and not found alterations in the respective Mpv17 genes. Among them we have excluded a contribution of the Mpv17 gene product to the most frequent hereditary glomerulosclerosis, the so-called Finnish nephrosis [19]. However, the Mpv17 gene still remains an interesting candidate gene for these types of diseases. Understanding the mechanisms by which the Mpv17 mice develop disease may also help to understand the mechanisms of development of the more frequent acquired glomeruloscleroses, for instance, as a consequence of diabetes.

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Reprint requests to Hans Weiher, Ph.D., Forschungszentrum Karlstruhe, Institute of Genetics, P.O. Box 3640, D-76021 Karlsruhe, Germany.

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