Forschungszentrum Karlsruhe
Technik und Umwelt

Wissenschaftliche Berichte
FZKA 5621B

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August 1995

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THE HUMAN HOMOLOGUE OF THE MURINE GLOMERULOSCLEROSIS-GENE MPV17

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A thesis submitted in partial fulfilment of the requirements of the Open University for the degree of Doctor of Philosophy

Forschungszentrum Karlsruhe GmbH, Karlsruhe
1995

Als Manuskript gedruckt

Forschungszentrum Karlsruhe GmbH
Postfach 3640, 76021 Karlsruhe

ISSN 0947-8620

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Zusammenfassung

Mäuse, homozygot für eine retrovirale Insertion am Mpv17 Lokus, entwickeln Glomerulosklerose und ein Nephrotisches Syndrom in frühem Alter. Das unterbrochene Gen, das Mpv17-Gen, ist daher ein rezessives Glomerulosklerose-Gen in Mäusen. Um herauszufinden, ob ein mögliches menschliches Mpv17 Gen in humanen Nierenkrankheiten eine Rolle spielt, haben wir das menschliche Homolog des Mpv17 Gens isoliert und analysiert. Die Sequenzanalyse ergab, daß das murine und das humane Mpv17 Gen hochgradig homolog zueinander sind (92%).
Unterschiedliche chromosomale Lokalisationen des menschlichen Mpv17 Gens und des Lokus für das Congenitale Nephrotische Syndrome des finnischen Typus, die bekannteste genetische Glomerulosklerose, schlossen eine Beteiligung des Mpv17 Gens an dieser Krankheit aus. Dies bedeutet, daß das Mpv17 und das putative CNF Gen verschiedene Funktionen haben.
Sowohl das murine als auch das menschliche Mpv17 Gen kodieren für ein 176 Aminosäuren-Protein mit hydrophoben Regionen. Eine Computeranalyse ermittelte eine signifikante Homologie zwischen dem Mpv17 Protein und dem peroxisomalen Membranprotein (pmp) 22. Die anschließende Immunlokalisierung des Mpv17 Genprodukts mit Antikörpern gegen ein rekombinantes Mpv17 Fusionsprotein ergab eine peroxisomale Lokalisation des Mpv17 Proteins. Daher ist das Mpv17-System eine mögliche Verbindung eines Verlustes einer peroxisomalen Funktion und Glomerulosklerose in der Maus. Es gibt jedoch keine signifikanten phänotypischen Ähnlichkeiten zwischen den etablierten humanen Peroxisomenkrankheiten, wie z.B Zellweger Syndrome (ZS), und der Mpv17-mutanten Maus. Deshalb denke ich, daß das Mpv17-Gen an einer bisher unbekannten peroxisomalen Funktion beteiligt ist.
Da Peroxisomen verschiedene enzymatische Aktivitäten besitzen, die den intrazellulären Redox-Status kontrollieren, und eine zu hohe Produktion von reaktiven Sauerstoff Specien (ROS) mit glomerulären Krankheiten in Verbindung gebracht worden ist, haben wir diese Parameter im Mpv17-System untersucht. Im Gegensatz zu Wildtypzellen waren

Fibroblasten, die von homozygoten Mpv17 Mäusen stammen, eingeschränkt in ihrer Fähigkeit ROS zu produzieren. Weiterhin haben NIH3T3 Zellen, die das humane Mpv17 Gen konstitutiv überexprimieren, (RSV1 und RSV7 Zellen) eine höhere Anzahl von ROS als Kontrollzellen, was darauf hindeutet, daß Mpv17 eine ROS-produzierende Aktivität besitzt.
Matrixmetalloproteinase-2 (MMP-2) ist ein Enzym, welches möglicherweise am Umsatz der glomerulären Basalmembran beteiligt ist. Während es in RSV1 und RSV7 Zellen nicht detektiert werden konnte, konnte es problemlos in Mpv17-negativen Zellen nachgewiesen werden. Eine Deregulation von MMP-2 in den Glomeruli von Mpv17 defizienten Mäusen könnte eine Erklärung für die Glomerulosklerose sein. Damit übereinstimmend haben immunhistologische Experimente gezeigt, daß die Proteinexpression von MMP-2 in MPV17-negativen Glomeruli erhöht war.
Schließlich habe ich demonstriert, daß das menschliche Mpv17 Gen den Nierenphänotyp komplementieren kann, wenn es in die Keimbahn von homozygoten Mpv17 Mäusen eingeführt wird. Daher kann die Beteiligung von anderen Genen in der Nähe der proviralen Integrationsstelle an der Krankheitsentstehung ausgeschlossen werden. Das Experiment zeigt überdies die Funktionalität des humanen Proteins.
Diese Studien weisen auf eine wichtige Rolle des Mpv17 Proteins in Peroxisomen hin und identifizieren ein wichtiges und neues Gen, das mit der Pathogenese von humaner Glomerulosklerose verbunden ist.

Abstract.

Mice homozygous for a retroviral insertion at the Mpv 17 locus develop glomerulosclerosis and nephrotic syndrome at an early age. The disrupted gene, the Mpv17 gene, is therefore a recessive glomerulosclerosis gene in mice. In order to determine whether a putative human Mpv17 gene could be involved in human kidney disease, we isolated and analysed the human homologue of the Mpv17 gene. Sequence analysis revealed that both the murine and the human gene are have a high dgree of homology (92%). Different chromosomal localisations of the human Mpv17 gene and the locus of congenital nephrotic syndrome of the Finnish type (CNF), the most prominent inherited form of glomerulosclerosis, excluded an involvment of the human Mpv17 gene in this condition, suggesting that the Mpv17 gene is involved in a function distinct from the CNF locus.

Both the human and murine Mpv17 genes can code for a protein of 176 aa containing hydrophobic regions. A computer analysis showed a striking homology to the peroxisomal membrane protein pmp 22. Subsequent immunolocalisation of the Mpv 17 protein product using antibodies against recombinant Mpv17 fusion proteins revealed peroxisomal localisation of the Mpv 17 gene product. Thus, the Mpv 17 system a likely link between the loss of a peroxisomal function and glomerulosclerosis in mice. There are, however, no significant phenotypic similarities between well-characterised human peroxisomal disorders, such as Zellweger syndrome (ZS) and the Mpv17 mutant mice. Therefore I believe that the Mpv17 gene is involved in a so far unknown peroxisomal function.

Since peroxisomes possess several enzymes that can contribute to the intracellular redox status, and since excess production of reactive oxygen species (ROS) has been implicated in glomerular diseases, we investigated these parameters in the Mpv17 system. In contrast to wild type cells, fibroblasts derived from homozygous Mpv17 animals were found impaired in their ability to produce ROS. In addition, NIH3T3 cells constitutively overexpressing the human Mpv17 gene (RSV1 and RSV7 cells) show a higher level of ROS than control cells, indicating that the Mpv 17 protein has a ROS-producing activity.

Matrix metalloproteinase-2 (MMP-2), an enzyme potentially involved in the turnover of the glomerular basement membrane, could not be detected in the RSV1 and RSV7 cells, while in cells negative for Mpv17 expression MMP-2 transcripts were readily detectable. Deregulation of MMP-2 in glomeruli

deficient for Mpv17 protein could therefore be a possible explanation for the glomerulosclerosis. Consistent with this notion, immunohistological experiments revealed that the MMP-2 protein level was indeed increased in glomeruli from Mpv17 mutant mice.

Finally, I showed that the human Mpv17 gene can complement the kidney phenotype, when transferred to the germline of homozygous Mpv17 animals. This excludes the involvement of genes other than Mpv17 in the vicinity of the proviral integration site and further shows the functionality of the human protein. These studies indicate that Mpv17 is an important protein in the peroxisome, and identify a potentially important and new gene linked to a human kidney pathogenesis.

Contents

Chapter 1. Introduction.

The basic function of the kidneys is the detoxification of the blood by removing the final waste products of the body`s metabolism, such as urea, creatine, phosphates, and sulfates. The functional unit of a kidney is the nephron. In the nephron the blood is first filtrated and then the wanted substances are selectively reaborbed, leaving the waste products to be excreted. Furthermore active removal of waste products takes place. There are numerous sites in the kidney where this equilibrium between filtration and reabsorption can be impaired, resulting in an array of different kidney disorders that often lead to renal failure if left untreated. Treatment often consists of dialysis, or transplantation of a donor kidney, which are both associated with numerous problems. Kidney disease thus poses a major health problem and though today the clinician is able to treat many but not all renal defects very little is known about their etiology.

One site of potential pathological changes which leads to serious renal defects is the glomerulus. In many cases of glomerular disease the integrity of the basement membrane, part of the capillary wall, is obstructed giving rise to increased permeability and eventually to proteinuria. When this condition is accompanied by an increase in extracellular matrix deposition in the glomeruli it is referred to as glomerulosclerosis. However, the initial molecular events that cause the glomerular lesion as well as how the different histopathological changes in the glomerulus are connected are so far unknown.

In order to understand and study renal disorders, animal models are required. Animal models for human disease produced either by external, often chemical, stimuli, by spontaneous mutations or created by transgenesis are valuable tools to study these defects at the molecular level within the complexity of a multicellular organism.

The transgenic mouse strain Mpv17 (Weiher et al., 1990) studied in this work is a model for human glomerulosclerosis and nephrotic syndrome. While the gene responsible for the Mpv17 phenotype has been isolated (Weiher et al., 1990), the molecular mechanism by which it causes renal disease is unknown. The aim of my project was therefore to define the function of the Mpv17 gene product at the cellular and molecular level. This should then permit possible diagnostic and therapeutic approaches

to the treatment of human renal disease to be developed. In this introduction I will describe the physiology and function of the kidney, and survey the pathology of kidney diseases, with particular reference to the Mpv17 model. Finally, I will summarise the aims of my project.

General function of the kidney

Removing waste products from the body and controlling the concentrations of several ionic substances such as sodium, potassium as well as the pH value in the extracellular fluid is the principle function of the kidneys. The amount of extracellular fluid in a human body is around 15 l and is the sum of blood plasma (3 l) and the so called interstitial fluid (12 l) which is the fluid in the spaces between the cells (Guyton, 1984). There is a ready exchange among these two systems through pores in the capillary membranes. In this way nutrients are transported from the blood system to the cells and excreted waste products are transported in the other direction. As these often toxic excreta are collected in the blood the body needs ways to get rid of these substances without having to exchange the complete volume of the extracellular fluid.

Kidney anatomy

In most mammals the kidney is normally supplied by a single renal artery that enters the hilar region (hilus), which is a slit located on the concave side of each kidney. This main stem artery divides to form a anterior and a posterior branch from which smaller arteries arise. These supply the lower, middle, and upper parts of the anterior segment of the kidney, and also the posterior half, which is divided into apical, posterior, and lower segments, respectively. The renal pelvis, vein, the lymphatics and a nerve plexus also pass into the kidney via the hilar region. A bisected kidney reveals two distinct regions: a pale outer region called the cortex and a darker inner section, the medulla. In humans, the medulla is divided into between 8 to 18 so called renal pyramids with each apex extending towards the renal pelvis forming a papilla. In rats and mice only a single pyramide can be found, which is called a unipapillate kidney. The base of each pyramid is located at the boundary of the cortex and the medulla. Despite this slight difference,

the rodent kidney resembles the human organ in its appearance. A tough fibrous capsule surrounds the kidney on the outside.

Development of the kidney

During embryogenesis three distinct stages of the developing kidneys are defined: the pronephros, the mesonephros, and the metanephros. All three forms originate from the nephrogenic cord, which is of mesodermal derivation. The pronephros develops in the human embryo at the end of the third week after conception. It is composed of seven tubules that form nephrostomes at their proximal ends and open into the coelomic duct. The distal ends create the pronephric duct, that finally empties in the cloaca. It is however thought that the pronephros in mammals is not yet a functional unit, but is just a precursor of the later stages of kidney development. During the middle of the fourth week the mesonephros starts to develop, which contains more nephrons than its predecessor. At this stage, glomerular structures begin to appear as part of the mesonephric tubule, that ends in the the mesonephric duct. In the male the mesonephric tubules and the mesonephric duct form several components of the reproductive system later on, while in the female the mesonephros mainly regresses. The final developmental stage of the mammalian kidney is the metanephros. While the excretory portion including the collecting ducts, calyces, pelvis, and ureter develops from an outgrowth of the mesonephric duct called the ureteric bud, the renal corpsule is formed from the metanephric blastema in the nephrogenic cord. The kidney of a new-born baby contains nephrons of different developmental stages that mature with time, but new nephrons are not created. At birth the most mature nephrons are located near the medulla, while the the less developed nephrons are seen in the outer cortex. It is thought that nephrons in the growing metanephros start functioning around the 11th week after conception. The post-natal maturation process is indicated by a dramatic increase of the glomerular filtration rate (GFR) from 0.045 ml/min/g kidney weight in 1-3-day-old rats to 1.0 ml/min/g kidney weight in 40-day-old rats, which is due to the enlargment of the endothelial pores and the epithelial slit area.

Kidney Physiology

The principle functional units of the kidney are the two million nephrons in the two kidneys of humans. Rodents only possess around 70,000 nephrons. It is essential to understand the structure and function of a nephron in order to conceive the events leading to renal defects. A nephron consists of the renal corpuscle that itself is composed of the glomerulus, a network of capillaries, and the Bowman´ s capsule surrounding the glomerulus, and tubules being subdivided into the proximal part, the loop of Henle, the distal tubule, the collecting duct and the pelvis (Figure 1.1a). Since the blood pressure in the glomerulus is as high as 60 mm Hg compared to 30 mm Hg at the arterial end of an average capillary it is possible to passage large quantities of fluid through the glomerular capillary wall. This fluid is called the glomerular filtrate and contains most of the waste products but also essential ions, sugars and other small molecules. The glomerular filtrate is passed into the Bowman´ s capsule (space) from where it is transported to the tubular system. As the filtrate passes through these tubules, substances elementary to the body such as glucose, amino acids, certain ions, and a large volume of water are reabsorbed by the peritubular capillaries, that entwine the tubular system. This reabsorbtion occurs in a selective manner leaving most of the waste substances behind, which are passed on through the collective duct and pelvis into the ureter. By this way more than 180 litre of glomerular filtrate is produced each day, but due to the reabsorbtion process only 1.5 litre leave the body in form of urine and need to be replaced. Hence, the waste products being composed of mainly urea, uric acid, creatinine, phosphates, sulfates and certain acids are excreted in a rather concentrated form in the urine that is finally passed on from the ureter to the bladder. The basic function of the nephron can therefore be summarised as a two step process: (1) A large amount of plasma is filtered through the glomerular membranes, thereby clearing the blood from unwanted products and (2) in the tubular system the substances which have been passed through the filter together with the obsolete waste products, but which are still required, are selectively reabsorbed into the blood system. During this second process a few substances that have failed to be cleared from the blood are actively removed by secretion through the tubular walls. Hence, the urine is formed by a

combination of filtration and secretion processes. In glomerular diseases the filtration process is impaired for numerous different reasons often leading to renal failure.

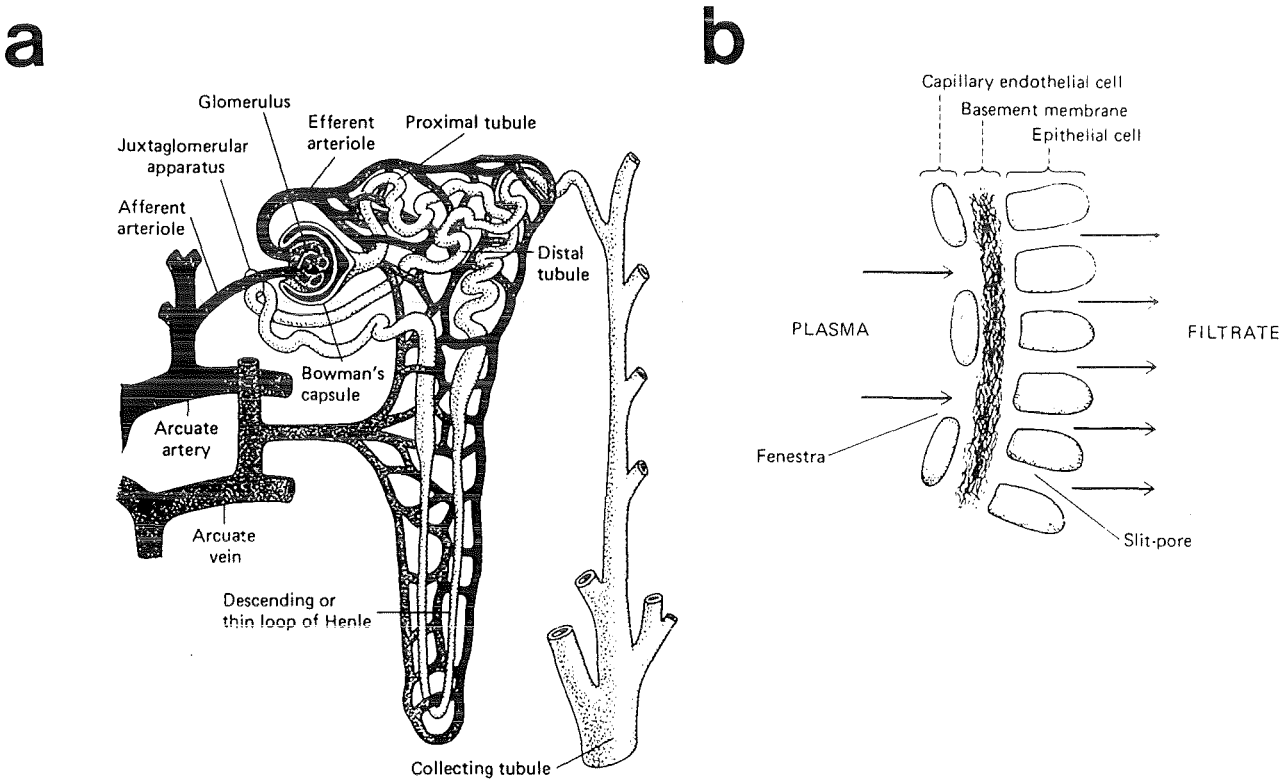

Figure 1.1 a: The functional nephron. **b**: Functional structure of the glomerular membrane (both taken from Guyton: Physiology of the Human Body. Saunders College Publishing, Philadelphia, 1981)

Cellular and molecular composition of the glomerulus

The filtration step is accomplished by the glomerular membrane that is comprised of a layer of endothelial cells of the capillary itself, a basement membrane composed of collagenous and proteoglycan fibers, and an epithelial cell layer on the other side (Figure 1.1b).

However, these cell layers do not form an impermeable layer, but are interrupted by openings called fenestrae in case of the endothelial cell layer and by slit pores between the adjacent foot processes of the

epithelial cells. The basement membrane between the two layers is also highly porous because it is a meshwork of fibers. Therefore, it is thought that the fenestrae, the slit pores, and the spaces between the fibers together constitute the filtration barrier between capillary blood and the urine space, also called Bowman´ s space. The collagenous fibers of the glomerular basement membrane, a specialized extracellular matrix, consist mainly of members of the type IV collagen family. This family so far includes six different α chains: α1 (IV), α2 (IV), α3 (IV), α4 (IV), α5 (IV), and α6 (IV) (Hudson et al., 1994). It is known that mutations in the α5 (IV) chain are involved in the etiology of Alport syndrome a familial kidney disease associated with sensory neural deafness (Barker et al., 1990). Recently mutations in the α3 (IV) and α4 (IV) chains have been shown to play a causative role in non X-linked Alport syndrome (Mochizuki et al., 1994) indicating that genes coding for the proteins comprising the GBM are all candidate genes for inherited glomerular disease. Goodpasture syndrome, an autoimmune disease also characterized by glomerulonephritis, is caused by generation of antibodies directed against the collagen α3 (IV) gene product (Saus et al., 1988). The structural homology among the six type IV collagen chains is charaterized by a 230 amino acid long non-collagenous domain at the carboxyl terminus, and a 1400 residues long stretch of Gly-X-Y repeats being designated the collagenous domain, that is interrupted at different sites by variable sequences. Three α chains are joined together to form a triple-helical structure, a so called protomer, that can differ with respect to the type and stoichiometry of chains thereby confering a high level of diversity on the protomeric structure. Protomers themselves can interact with each other in either a head to head or tail to tail fashion resulting in the collagenous meshwork of the GBM. The reason for the existence of at least six different type collagen chains in the GBM is still unknown, but raises questions about their possible involvement in kidney disorders other than those already described. An abnormal synthesis of collagen type IV is often found in polycystic kidney disease or glomerulosclerosis. In addition to collagen IV, the major structural component of the GBM, laminin, nidogen, and proteoglycans are bound into the collagen meshwork in an unknown manner. The heparan sulfate proteoglycan is believed to repel large proteins with its high anionic charge and interestingly its synthesis is

upregulated in glomerulosclerosis, as are collagen IV and laminin. On the electron microscopic level three layers can be distinguished in the GBM. In the centre is a dense layer, the lamina densa, that is enclosed by the lamina rara externa and the lamina rara interna. This layered appearance is due to the fusion of the endothelial and epithelial basement membrane during development (Abrahamson et al., 1985). Negatively charged glycosaminoglycans have been found in all three layers of the GBM, constituting a charge selective barrier in addition to the size-selectivness established by the collagenous meshwork. Removal of these sugar residues by enzymatic digestions resulted in an increased permeability of the GBM to serum albumin for example, that is normally not able to pass through glomerular capillary wall, suggesting a possible role of the glycosaminoglycans in establishing the permeability characteristics of the GBM (Glassock et al., 1991). Usually proteins of the size of albumin or bigger are almost completely restricted from being passed on into the urinary space. It is commonly believed that the GBM is the principle structure responsible for the permeabilty properties of the glomerulus, although the other constituents certainly have their role in the filtration process either actively or indirectly by maintaining the integrity of the GBM.

The visceral epithelial cells or podocytes are the largest cells in the glomerulus. They form long multiple processes called foot processes outward from the main cell body that wrap around individual capillary loops. These pedicels come into direct contact with the lamina rara externa of the GBM. The part of the membrane of the podocytes that is attached to the GBM is referred to as the basal cell membrane while the domain facing the urinary space, which is by far the majority of the podocyte´ s surface, is called the luminal membrane. The attachment to the GBM is thought to be facilitated by integrin molecules. Immuno-chemical studies have revealed the presence of β_1-integrin and corresponding α_3 chains on the basal membrane. However, the adhesion mechnism through this particular integrin cannot be the sole one, as experiments using peptides competing for binding to the RGD site (Ruoslathi et al., 1986) on the GBM do not impair the attachment of the cells to the matrix.

Since the podocytes on the one side face the urinary space and on the other side adhere to the basement membrane, the two sides require

different biochemical and biophysical properties. This polarisation, a common feature of epithelial cells, is usually created by tight junctions (Farquhar et al., 1963), but glomerular epithelial cells employ a different method to achieve the separation of the luminal from the basal side. The gap between two adjacent foot processes forms the filtration slit or slit pore that is spanned by a thin membrane, the slit diaphragm (Rodewald et al., 1974). It is thought to be involved in the permea-selective filtration process. A prominent feature of the podocytes is the presence of a negatively charged surface coat of the membrane due to numerous sialic acid residues on the plasma membrane of the pedicels. The major protein carrying these negtively charged sugar moieties has been identified as a 140 kd sialoglycoprotein in rats (humans form a doublet of 160 and 170 kD) named podocalyxin (Kerjaschki et al., 1984). It could be shown that in an experimental rat model for human glomerular minimal change nephropathy, generated by intoxification with puromycin aminonucleoside, the amount of sialic acid residues was reduced by about 75 %, while the number of podocalyxin molecules was unchanged, suggesting a loss of sialic acid rather than down regulation of protein synthesis is causative for the drop in negative charge on the surface of the podocytes of the treated rats (Kerjaschki et al., 1985). The dramatic decrease in the number of sialic acid residues was associated with a flattening of the foot processes, typical for several glomerular lesions (Mostofi et al., 1971). Interestingly, the same phenotype could be observed when rat kidneys were perfused with medium containing polycationic compounds (e.g. poly-L-lysine) being capable of neutralizing the anionic character of the visceral epithelial cells, suggesting a causative role of the loss of negative charge for the morphological changes of the podocytes (Seiler et al., 1975). The question of whether this flattening of the podocytes is the primary defect leading to the dysfunction of the epithelial cells and thereby eventually to the glomerular disease is still open. However, it is commonly accepted that the collapse of the normal shape of the podocytes is not due to a passive loss of the negative surface charge, but is an active process involving complex cellular activities as a response to the treatment with poly-L-lysine, for example. Other antigens that have been identified on the surface of visceral epithelial cells are the

Heymann nephritis antigen (gp 330) and the human glomerular C3b receptor (Kerjaschki et al., 1992).
There is evidence that pododcytes are at least one site of the synthesis and maintenance of the GBM. Cultured human visceral epithelial cells have been found to produce type IV collagen and glycosaminoglycan synthesis has been detected in cultures of rat podocytes. In addition, certain prostaglandins can be synthesised by cultured epithelial cells. A subtype of these glomerular cells are the parietal epithelial cells which line the outer wall of the Bowman´s capsule and form a continuous epithelium with the podocytes.
The glomerular capillaries are confined by a thin endothelium, thereby forming the initial barrier that substances from the capillary lumen have to pass before they encounter the fibrous network of the basement membrane and finally the layer of epithelial cells on their passage to the Bowman´ s space. The endothelium is perforated by pores called fenestrae, which have diameter of around 100 nm in the human kidney. Thin diaphragms have been observed spanning these fenestrae. As for the podocytes, the negative charged glycoprotein podocalyxin has been identified on the surface of the glomerular endothelial cells, giving the membrane an overall anionic character. This also contributes to the charge selective properties of the filtration apparatus.
A third cellular component of the glomerulus are the mesangial cells which together with the surrounding matrix material make up the mesangium. The matrix is similar but not identical to the glomerular basement membrane. It contains sulfated glycosaminoglycans, fibronectin, and laminin. The mesangial cell is irregular in shape, possesses a dense nucleus and elongated cytoplasmic processes. It is thought that mesangial cells are specialized pericytes having the functional properties of smooth muscle cells. In addition to the structural support they give to the capillary loops in the glomerulus, they might be involved in the control glomerular filtration by regulating the blood flow in the glomerular capillaries with their contractile properties. Stimulation of contraction by various substances such as angiotensin II, vasopressin, and platelet-activating factor (PAF) of mesangial cells in culture have been observed, as well as a relaxant reaction in response to agents like prostaglandin E_2 (PGE_2). The latter can be produced by mesangial cells themselves thereby counteracting

and regulating the vasoactive substances, atrial peptides (ANP), and dopamine. In addition, mesangial cells are thought to possess phagocytotic properties. The prostaglandin production can influence the local cell proliferation and the production of cytokines, which are known to be involved in glomerular diseases. Therefore, the study of the interactions between cytokines, glomerular cells, and prostaglandins is important in order to understand the mechanisms of such disorders. The different entities of glomerular disease as well as their etiology will be introduced in the next section. (The general anatomy and phyiology of the kidney is discussed in depth in Tisher and Madsen, 1991 and Guyton, 1984.)

Glomerular diseases

Kidney diseases can be subdivided into seven classes including vascular diseases, glomerular diseases, tubular disorders, interstitial diseases, cystic kidney diseases, renal disease as part of multi-system disease, and nephrothialises. A simplified classification of glomerular diseases is to distinguish between glomerulosclerosis and glomerulonephritis.

Glomerulosclerosis in children usually has a bad prognosis and due to the lack of adequate therapeutic treatment often requires a kidney graft in the last instance (Müller-Wiefel and Waldherr, 1992). A study collecting data of four years from 73 pediatric transplantation centers in the US revealed that glomerulosclerosis is the second most common reason leading to kidney transplantation (McEnery et al., 1992). Overall 200 transplantation cases in this study were ascribed to glomerulosclerosis, indicating that it is a relatively rare disease. Besides these congenital and infantile diseases, the importance of glomerulosclerosis arises from its appearance in connection with various kidney and multi-organic diseases.

Glomerular defects frequently give rise to the nephrotic syndrome which can have multiple causes and are characterized firstly by massive proteinuria often associated by lipidurea, hypoalbuminemia and hyperlipidemia.

Glomerulosclerosis and nephrotic syndrome are not single disease entities having a defined etiology or a clear cut pathological picture. The nomenclature and classification of glomerular diseases is complex and often deals with several synonyms for the same clinical condition.

Differences between distinct pathological entities are sometimes very subtle and hard to define.

First of all I will discuss the different causes for the nephrotic syndrome. The literature subsumes different scleroses and nephritises leading to the nephrotic syndrome in the absence of any underlying inherited, multisystem disease (e.g. diabetes or AIDS), drug, or microbial exposure. The definitions of primary gomerular diseases that evoke the nephrotic syndrome or ideopathic nephrotic syndrome (INS) have been introduced for this group in the literature (Glassock et al., 1991). The second group sums up specific etiologic events and disorders in which glomerular lesions arise secondary to other diseases leading to the nephrotic syndrome. The kidney phenotype in this group is a consequence of medication or intoxification (e.g. contrast media, interferon, and heroine abuse), allergens, immunisations, infections (e.g. hepatitis B, HIV-1), neoplastic changes (e.g., leukemia, lymphomas, and solid tumors), multisystem diseases (e.g. rheumatoid arthritis, Goodpasture`s syndrome), and heredofamilial and metabolic diseases (e.g. diabetes mellitus, Alport´s syndrome, congenital nephrotic syndrome). This group also include glomerular lesions resulting from chronic renal allograft rejections or pregnancy. (For a complete list see Glassock et al., 1991).

Very little is known about the etiology of INS. However, much knowledge has been accumulated regarding its morphology and pathogenesis, making it possible to classify idiopathic nephrotic syndrome into several groups of clinicopathological entities. These are minimal-change disease, mesangial proliferative glomerulonephritis, focal (and segmental) glomerulosclerosis, diffuse glomerular sclerosis, membranous glomerulonephritis, mesangiocapillary glomerulonephritis, endocapillary proliferative glomerulonephritis, other chronic, sclerosing lesions, and other unclassified lesions. This classification is based on light microscopic examinations of renal biopsis complemented by immunofluorescence and electron microscopic studies. It is also possible that one or more members of this group now being viewed as discrete entities are in fact heterogeneous disorders and the classification must be regarded as being in a constant state of development. Focal and segmental glomerulosclerosis is, for example, frequently found to be superimposed on minimal change disease, mesangial proliferative

glomerulonephritis and sometimes on other primary glomerular lesions (e.g. IgA nephropathy), and the pathological differences are often so subtle that even in the clinical practice it is sometimes difficult to distinguish between these entities.

Minimal change disease is the dominant primary glomerular condition in children (83%) thereby often resembling the characteristics of a congenital (birth to 3 months of age in humans) or infantile (3 months to one year of age) nephrotic syndrome. The minimal change disease, also called minimal change lesion, minimal change nephropathy, or by its old name lipoid nephrosis due to lipid droplets in the cells of the proximal tubules, was first described by Munk (1913). The lesions in the tubules are accompanied by heavy proteinuria, but no abnormalities of the glomeruli, i.e. hyaline material deposits can be seen by light microscopy. However, when these glomeruli are examined by electron microscopy it is found that the visceral epithelial cells are affected. The foot processes are fused, leading to closing of the slit-pores. However there is normally no detachment of the podocytes from the basement membrane, which is usually of normal thickness. This parameter discriminates between minimal change disease and focal and segmental glomerulosclerosis. Another marked difference between these two conditions is the presence of IgM and complement depositions in focal and segmental glomerulosclerosis, which are thought to be due to unspecific trapping of immunglobulins leading to subsequent binding of complement substrates in the glomeruli. No distinct mesangial hypercellularity has been observed for both conditions. This again distinguishes them from the third clinicopathological condition of this group which can also be sometimes superimposed by a focal and segmental glomerulosclerosis which is termed mesangial proliferative glomerulonephritis. The latter is a relatively uncommon glomerular lesion being found in less than 10% of patients with idiopathic nephrotic syndrome. In uncomplicated cases of mesangial proliferative glomerulonephritis no segmental sclerosis can be detected, but IgM deposits have been observed. The epithelial foot processes are also diffusely swollen (fusion) and the basement membrane is sometimes found to be slightly enlarged (Glassock et al., 1991). Two other differences between minimal change disease and glomeruloscleroses are that patients suffering from the latter often fail to respond to treatment

with corticosteroids and that spontaneous remissons are frequently seen in minimal change patients, but rarely found in glomerulosclerosis cases. Glomerulonephrites include an inflammatory element and will not be further discussed even though they also exibit glomerulosclerotic characteristics. Thus glomerulosclerosis is the major common feature of renal defects associated with nephrotic syndrome.

So far quite, a few general etiologic factors leading to glomerulosclerosis have been suggested. Gonzalez-Rubio (1992) report on the role of oxidative stress in the development of glomerulosclerosis, while Diamond and Karnovsky (1992) see accumulating evidence for the involvement of hypercholesterolaemia as one of the major features of glomerulosclerosis in the initial glomerular injury. In a study by Kasiske et al. (1990), with rats fed a high-cholesterol diet and developing glomerular lesions, a relative increase in cholesteryl esters was the principal alteration in the major lipid classes in the kidneys of these animals. How cholesteryl ester accumulation might lead to renal injury is unclear. In another experiment feeding rats a cholesterol-rich diet over a short term led to an increase in the glomerular capillary pressure and a decrease in the glomerular filtration rate (Kaplan et al., 1990). These effects were prevented by adding an antioxidant to the diet. The authors therefore concluded that native low density lipoprotein (LDL) oxidised in vivo initiates events leading to vasoconstriction and, postulated that these abnormalities contribute to progressive glomerulosclerosis. Another potential effect of oxidised LDL is direct mesangial cell injury (Keane, et al., 1990). Furthermore, it is hypothesised that oxidised LDL might be involved in the increased recruitment of circulating monocytes into the glomerulus (Diamond and Karnovsky, 1990) leading to elaboration of peptide mitogens or to the perturbation of the glomerular eicosanoid balance, which could thereby lead to glomerulosclerosis. It is known that certain cytokines and growth factors are upregulated in glomerular disease (Sedor, 1992 and Floege et al., 1993). Recently Imai et al., (1994) showed that kidney specific overexpression of TGF-β and PDGF-β in rats led to glomerulosclerosis. Other factors that have been reported to lead to glomerulosclerosis are physical force, such as lasting hypertension (Neuringer et al., 1992; Kaplan, et al., 1990) or increasing tension in the capillary wall and or stretching of the mesangial cells (Riser et al., 1992). Kritz et al. (1994)

report that the GBM expands in response to an increase in the hydrostatic pressure gradient across the capillary wall, and that the contractile system of the podocytes formed by microfilament bundles counteracts this pressure-dependent dilation of the glomerular capillary wall. Podocytes possess receptors for various vasoactive substances including endothelin (Rebibou et al., 1992; Hori et al., 1992), angiotensin II (Yamada et al., 1990), and ANP (Sharma et al., 1992). Any alterations in the cytoskeletal apparatus could impair the response to these substances, potentially leading to glomerular injury. Additional elements that are associated with the development of glomerulosclerosis are vascular injury (Klahr et al., 1986) and continual immunological injury of glomeruli (Wilson, 1991). In the case of diabetic nephropathy, which is the leading cause of end stage renal failure in the United States (Kashgarian et al., 1994), it is suspected that nonenzymatic glycosylation of proteins due to the hyperglycemia (Crowley et al., 1991; Flier and Underhill, 1988; Skolnik et al., 1991) leads to an increase in the normal GBM components. This effect is mediated by receptors for the so called advanced glycosylation end products on macrophages and mesangial cells which elicit a series of intacellular signalling events.

Glomerulosclerosis is considered to be the final common pathway leading to end-stage kidney disease caused by various initial events. The understanding of the mechanisms involved in this development is regarded to be of primary importance in order to be able to interfere with the progression of the renal disease. At which stage of this final common pathway the different factors intervene and whether there is one factor to which they can all be reduced remains an open question.

The genetic kidney diseases giving rise to the nephrotic syndrome in young children are among the best defined pathological entities. The congenitial nephrotic syndrome of the Finnish type (CNF) is a disease very frequent in Finland. A familial history has been descibed for most cases and an autosomal recessive transmission has been established for them. It is characterised by dilations of the proximal tubules and focal and segmental glomerulosclerosis (Norio and Rapola, 1989). Habib and Bois (1973) first introduced the condition called diffuse mesangial sclerosis (DMS) and have so far reported 30 cases, of which 30% were familial (Habib et al., 1993). While the phenotype of DMS is similar to

CNF in many respects, it differs in its rapid progression to end-stage renal failure and the hypertrophy of mesangial cells. Interestingly, DMS resembles the nephropathy seen in a subset of Drash syndrome and Wilm´ s tumor (WT) patients (Habib et al., 1989). Norio and Rapola (1989) speculate about numerous other familial cases of congenitial and infantile nephrotic syndromes that are not classified in any of the main conditions, because of their isolated occurence. In the already mentioned Alport syndrome, mutations in the genes for collagen type IV cause the nephrotic syndrome and hematuria.

Animal models for kidney disease

Recently a gene for dominant autosomal polycystic kidney disease (The European Polycystic Kidney Disease Consortium, 1994) and one responsible for tubular sclerosis (European Chromosome 16 Tubular Sclerosis Consortium, 1993) have been cloned. The functions of the respective normal proteins as well as the mutated forms are at present unknown. Modelling the diseases in mice is a way to elucidate the mechanisms leading to the renal failure. Other kidney diseases that await the generation of animal models are the Alport syndrome and, as soon as the particular gene for it has been identified, Finnish Nephropathy (CNF). The biochemical events that have been identified as being involved in kidney disease can also be imitated by transgenesis, i.e. over-/ecotopic expression or elimination of genes coding or controlling these factors. Other transgenic mouse lines have been found incidentally to develop disorders of the kidney. The following list shows transgenic mouse strain that develop glomerulosclerosis or exhibit abnormalities that are found in connection with it.

Transgene	species	renal lesion
SV40 large T antigen	mouse	glomerulosclerosis,cysts
c-myc	mouse	glomerulosclerosis,cysts
Il-6	mouse	mesangial proliferation
Renin	rat	glomerulosclerosis
Pax-2	mouse	glomerulosclerosis
HIV-I genome	mouse	glomerulosclerosis
TGF-β, PDGF-β (gene transfer)	rat	glomerulosclerosis

Figure 1.2 Glomerular lesions in transgenic animals (ref.: MacKay et al., 1987; Trudel et al., 1991; Suematsu et al., 1989; Bachmann et al., 1992; Doi et al., 1988; Dressler et al., 1993; Kopp et al., 1992).

Mice that carried the viral oncogene SV40 large T antigen under the control of its own enhancer elements died at an early age from choroid plexus ependymoma (brain tumor). The transgene was expressed in the tumors, and at lower levels in kidney tubules and thymus. In accordance with this finding the tubular epithelial cells of the animals underwent proliferation leading to cystic dilatations. Though not expressed in glomerular cells, the transgene caused glomerulosclerosis with increasing proteinuria and nonspecific IgG deposits.

The transgenic mouse line ecotopically expressing the c-myc gene in renal tubular cells, using the same control elements as in the first example, emphasises the point that defective regulation of epithelial cell growth is involved in the development of cystic kidney disease and possibly in the development of glomerulosclerosis, although the latter could be a secondary effect due to the the tubular changes.

Interleukin-6 (Il-6) is a pleiotropic cytokine that is also a growth factor for mesangial cells. Overexpression of human Il-6 in B-cells resulted in plasmacytosis and hyperproliferation of mesangial cells in transgenic mice. When the same gene was hooked up to the mouse metallothionein-1 gene promoter, the kidney phenotype was more severe. The initial membranous glomerulonephritis was followed by focal glomerulosclerosis and finally extensive tubular damage.

A transgenic rat model overexpressing the mouse renin gene showed severe hypertension and glomerulosclerosis. This demonstrated the importance of arterial blood pressure control for normal glomerular function. Thus, renin that is secreted by the kidneys acts on the plasma protein angiotensinogen, eventually resulting in an activation of angiotensin that increases the blood pressure.

Pax-2 is a transcription factor that is thought to be implicated in normal kidney development and in the development of Wilms tumor. Dressler et al. (1993) deregulated the expression of the mouse Pax-2 gene and produced a dominant gain-of-function mutation in transgenic mice. The transgene was expressed in heart, liver, lung, lung, pancreas, and gut, while the endogenous gene was only expressed in kidney and hindbrain. The kidneys of the mutant mice exhibited microcystic tubular dilatation, loss of foot processes and poorly developed endothelial fenestrae. These histological findings and the proteinuria resemble the Finnish Nephropathy, suggesting that Pax-2 is involved in CNF.

The transgenic mouse strain bearing a 7.4 kb long transgene containing the HIV-I genes env, tat, nef, rev, vif, vpr, and vpu, but lacking the gag and pol genes, developed focal and segmental glomerulosclerosis, that closely resembled the renal syndrome seen in AIDS patients.

TGF-β and PDGF are known to participate in the process of accumulation of ECM that is a central biological feature of glomerulosclerosis. Both growth hormones were found to be overexpressed in experimental models for glomerulosclerosis and the

excessive matrix production could be stopped by injecting anti-TGF-β serum into the animals (Okuda et al., 1990). Imai et al. (1994) transfected PDGF and TGF-β into rat kidneys in vivo by transfer of expression constructs containing an actin promoter and the two respective human cDNAs. The pathological changes of the TGF-β transfected rats consisted of a moderate increase of mesangial cell proliferation and an extensive ECM expansion. The PDGF transfected rats exhibited a slightly different phenotype. However, in both animals the onset of glomerulosclerosis occured within a short period after the transfection.

All these models for glomerulosclerosis include the accumulation of extracellular matrix that eventually leads to proteinuria and end-stage renal failure, regardless whether the initial stimulus to injury was immunological, hemodynamic or metabolic. They help to elucidate the final common pathway leading to glomerulosclerosis by defining the different factors involved.

The Mpv17 Mouse: a model for human kidney disease

The Mpv17 mouse strain is a recessive transgenic mouse mutant that develops glomerulosclerosis and nephrotic syndrome at young age (Weiher et al., 1990). This transgenic line was generated by infection of CFW mouse embryos with the recombinant retrovirus MPSVneo (Seliger et al. 1986; Weiher et al. 1987). The virus, a derivative of the myeloproliferative sarcoma virus, which is an ecotropic murine RNA tumor virus related to Moloney murine leukemia virus, was not able to replicate, and carried a neo gene as a reporter. As a retrovirus it integrates into the genome of its host and could thereby lead to activation or repression of certain genes, possibly giving rise to dominant (e.g. gain of function) or recessive (gene inactivation) phenotypes. This process is called insertional mutagenesis. After having been exposed to virus producing cells, 4-16 cell CFW mouse embryos were transferred to pseudopregnant foster mothers. The resulting mosaic founder animals were bred until germline transmission of the retroviral insert was observed. The created transgenic lines were screened for single copy integration. None of these animals expressed the transgene or exhibited a dominant phenotype. The mice possessing only a single proviral integration were bred to homozygosity for the

insert. Two of these mutants resulted in recessive lethal phenotypes, of which one was the Mpv17 mouse. The Mpv17 animals exhibited no apparent phenotype up to the age of two or three months of age. At this time they began to develop pallor, weight loss and showed a generally reduced activity. Subsequent serum analysis of the diseased animals demonstrated that pathological changes already began at the age of six to eight weeks. These included elevated cholesterol levels as one of the first signs, and increased blood urea nitrogen and creatinine levels. Albumin levels were reduced due to the proteinuria and finally a decreased red blood cell count, low hematocrit, and reduced hemoglobin levels could be measured. The massive proteinuria and other abnormalities were indicative of renal failure and were characteristic of the nephrotic syndrome in man (for an account on the nephrotic sydrome see the section on kidney diseases). The survival times of individual mice varied between six weeks and one year. Figure 1.3 (taken from Weiher et al., 1990) depicts a comparison of wild type, Mpv17 heterozygous animals and Mpv17 homozygous animals and shows that only animals homozygous for the retroviral insert at the Mpv17 locus eventually died from renal failure (Figure 1.3), while wild type and heterozygous animals are indistinguishable, which was also true for the physiological parameters listed in Figure 1.3.

wt/wt	wt/Mpv17	Mpv17/Mpv17
0/44	0/103	48/48

b. Physiological Parameters in End-Stage Disease

	wt/wt or wt/Mpv17	Mpv17/Mpv17
Serum		
BUN (mg/dl)	22 ± 3.9	30 ± 12.5
Creatinine (mg/dl)	0.32 ± 0.04	0.89 ± 0.19
Cholesterol (mg/dl)	110 ± 15	424 ± 130
Albumin (g/dl)	3.5 ± 0.21	2.33 ± 0.34
Blood		
RBC ($\times 10^6$)	9.8 ± 0.6	5.9 ± 0.8
HCT (%)	48.8 ± 3.3	26.3 ± 5
HGB (g/dl)	16.6 ± 1.4	8.8 ± 1.9
Urine		
Protein (mg/dl)	30	500

Serum, blood, and urine samples were analyzed at Tufts University School of Veterinary Medicine. Samples (100–200 μl) were taken from homozygous animals at end stage when mice showed signs of pallor, which was between 54 days and 6 months of age. Control samples were taken from age-matched heterozygous or wt animals. In this group, no changes were seen over time. Serum data are means of 20 homozygous animals and 12 animals in the control group. Blood values are means of 6 animals, and urine was analyzed in 2 animals of each group. Protein values of urine from more than 20 homozygotes were also tested quantitatively using test strips and all showed positive signal (data not shown). BUN: blood urea nitrogen. RBC: red blood cell count. HCT: hematocrit. HGB: hemoglobin.

Figure1.3 Genetics and phenotype of the Mpv17 locus (taken from Weiher et al., 1990)

In order to elucidate the exact nature of the renal disease, kidneys from diseased and control mice were examined by light and electron microscopy. Early stages of the disease showed glomerular lesions, which were first confined to a small number of glomeruli and involved only a portion of each affected glomerulus, but later involved more glomeruli and bigger segments of them. These phenotypes are known as focal and segmental glomerulosclerosis. The glomeruli became progressively obliterated by hyaline material, which consisted of basement membrane components such as collagen, laminin, heparan sulfate proteoglycan, and entrapped plasma proteins. The basement membrane itself, however, showed no apparent changes except for a slight thickening. In end-stage disease renal tubules showed microcystic dilatation and accumulation of hyaline material. Electron microscopic studies of the affected kidneys revealed degenerative changes in the visceral epithelial cells (podocytes). The so called foot processes were, disturbed manifested by simplification (fusion) of these processes.

At later disease stages vacuolisation of the epithelial cells was observed and some cells were detached from the basement membrane. The subendothelial space of capillaries contained homogeneous electron-dense material, reflecting the deposition of plasma derived macromolecules in the subendothelial space and hyaline formation, which finally resulted in the complete obliteration of the glomeruli reminiscent of global glomeruloscerosis. Throughout the course of the disease there were no signs of an increased cellularity or cellular infiltration, which would be indicative of an inflammatory process as in glomerular nephritic disorders. All the parameters were characteristic of glomeruloscerosis and nephrotic syndrome leading to renal failure, and the lack of involvement of other organs, suggested that the phenotype was primarily caused by an impaired renal function. Thus, the Mpv17 mouse should be a valuable model to approach the open questions regarding the molecular connections between the different pathological findings in glomerulosclerosis.

The recessive nature of the defect suggested that loss of an essential function by insertional mutagenesis caused the phenotype. In order to analyse the mutation at the Mpv17 locus the flanking sequences of the proviral insert were cloned. Therefore a genomic library using λEMBL 3 phage (Frischauf et al., 1983) vectors was generated from Mpv17 mouse liver DNA. Screening with a probe comprising the MPSV long terminal repeat gave rise to clones representing the 3´ end of the provirus and about 10-15 kb of flanking mouse DNA sequences. Subsequently, a cloned 1.5 kb sub-fragment (H1.5) from the integration region was used to isolate a 16kb insert around the pre-integration site form a wild type mouse genomic λ-library. Using a 340 bp non-repetitive Sau3A subfragment it was shown that this clone represented the viral pre-integration site by hybridizing it to DNA from wild type, heterozygous, and homozygous Mpv17 mice on a Southern blot. The Sau3A probe detected a 3 kb band in wild type mice and a 6 kb band representing the Mpv17 insertional allele in DNA digested with BamHI. This finding showed that the Sau3A fragment detected a restriction fragment length polymorphism (RFLP) and therefore this probe could serve as a useful tool for genotyping Mpv17 mice. Additional restriction digests made it clear that the integration of the provirus was not accompanied by any detectable rearrangements. The observed phenotype

and its recessive nature made it very likely that a gene being necessary for normal kidney function was affected by the retroviral insertion.
Genes that have an important function are often found to be evolutinary conserved among different species due to seletion pressure. The cloned region representing the pre-integration site was therefore dissected into 12 subfragments, which differed between 500 bp and 1.5 kb in size, and were used as probes on genomic Southern blots of DNA from different species. In this so called zoo blot a probe from one species representing a conserved sequence should give rise to one or more distinct bands on DNA from other species. Indeed, one of the twelve subclones, a 800 bp fragment, was found to be conserved and non-repetitive as it hybridised to a single fragment of human, cow, rabbit, and rat DNA under stringent hybridisation and washing conditions. It detected a 1.7 kb transcript in Northern analyses on RNA from different organs of wild type and heterozygous mice, but not on RNA from homozygous animals. This probe thus fulfilled the criterion for the gene whose expression was inactivated by the retroviral insert. The cellular transcript was called the Mpv17 transcript and the gene coding for it was termed the Mpv17 gene.
Organs found positive for Mpv17 gene expression were: heart, brain, kidney, testis and to a lesser extent liver and spleen. This expression pattern could also be seen in newly born mice and during development, as demonstrated in 8 day old mice and in day 16 embryos, respectively. The gene is transcribed in EC (F9) and ES (D3) cells, in lines derived from kidney epithelial cells (TMCK-1, RAG) as well as in NIH3T3 fibroblasts. The Mpv17 gene was found to be ubiquitously expressed throughout embryonic development and in adult animals, but no transcription could be found regardless of age or disease status in homozygous animals, indicating that the provirus inhibits the stable expression of Mpv17 RNA. This raised the question of how the lack of Mpv17 expression during embryonal development and in all the organs in which it is normally expressed leads to the very specific kidney defect described above.
Subsequently the entire Mpv17 cDNA was cloned and sequenced. A cDNA library created from wild type kidney RNA was screened using the 800 bp fragment as a probe. The resulting complete cDNA clone was sequenced, revealing a total sequence of 1426 nucleotides with an

open reading frame of 176 amino acids. The first ATG codon was preceeded by a nearly perfect Kozak consensus sequence (Kozak, 1987) suggesting that this ATG codon is indeed the start point of translation. The 3´ region of the cDNA consisted of two repetitive elements with several stop codons in all reading frames. They had a high homology to the so called B2 and B1 mouse repeats, respectively (Krayev, 1980, 1982). No upstream consensus poly (A) addition signal were found, which is either due to the fact that the Mpv17 transcript is a gene without such a signal or that the clones were primed in a poly (A) stretch, that often follows repetitive sequences (Birnstiel et al, 1985).
When analysed with the ALOM program (Klein et al., 1985), the sequence of the derived 176 amino acid protein revealed two major hydrophobic regions, which indicated a possible membrane association of the supposed protein. Furthermore, there are four cysteine residues that could give rise to disulphide bridges and several serine and threonine residues, sites of potential phosphorylation, present in the amino acid sequence. Besides this, the sequence gave no hints of a possible function. A homology search revealed no significant similarity with any known protein sequence.

Aim of the presented work

The Mpv17 mouse strain develops glomerulosclerosis and nephrotic syndrome at young age. Due to recessive transmission and the specific kidney phenotype that closely resembles glomerulosclerosis and nephrotic syndrome in man, a putative human Mpv17 gene would be a prime candidate gene for inherited glomerular disease such as the congenital nephrotic syndrome of the Finnish type (CNF).

The Mpv17 mouse line was created by insertional mutagenesis which gave rise to the loss of expression of the Mpv17 gene. The zoo-blot analysis (Weiher et al., 1990) indicated that the Mpv17 gene is conserved between different species including man. The first objective was therefore to isolate and analyse the putative human Mpv17 (hMpv17) gene. In order to try to demonstrate an involvement of the human Mpv17 gene in inherited glomeruloscleroses, biopsy material from patients suffering from such diseases could be screened for mutated expression of Mpv17. In addition, regardless of its involvement in familial human kidney disease, understanding the normal function of the Mpv17 gene product should give insights into the general etiology of glomerulosclerosis. The unravelling of the intracellular localisation and the cellular distribution of the Mpv17 protein would be the first step towards the elucidation of this function. For this purpose I set out to raise antibodies against the Mpv17 gene product. The acquired information on the localisation and distribution of the Mpv17 protein should give indications towards further studies of its function. The elucidation of the cellular distribution should also unravel whether the Mpv17 protein is normally present at sites in the glomerulus that are affected in the mutant mouse. Such a finding would make it possible that the lack of Mpv17 expression in the glomerulus directly gives rise to the defect.

The data obtained from these experiments combined with results from the analysis of other animal models for kidney disease would help in the set-up of appropiate assays to define the function of the Mpv17 protein. Trying to explain how the loss of Mpv17 function leads to glomerulosclerosis was the subsequent step of the project. Therefore, I wanted to examine parameters that could be potentially controlled by the action of the Mpv17 gene product and could result in glomerular injury.

These result should contribute to the search for the final common biochemical pathway that leads to glomerulosclerosis.
A final objective of this project was to show that the loss of Mpv17 expression indeed leads to the renal disease in the mutant mice. This question had to be considered, because the Mpv17 mouse strain was generated by random insertional mutagenesis, so it could not be ruled out that another gene was affected by the proviral integration. The transfer of a functional copy of the Mpv17 gene into the genome of Mpv17 mutant animals to rescue the phenotype was an approach that would definitively demonstrate that the lack of Mpv17 activity gives rise to glomerulosclerosis in mice. Therefore I set out to make a construct containing the Mpv17 cDNA under the control of an appropriate promoter ensuring expression in the kidney to generate transgenic mice to rescue the phenotype.

Chapter 2. Materials and Methods.

Materials

Chemicals. Chemicals were purchased from Roth (Karlsruhe), Merck or Sigma, unless otherwise stated. Radiochemicals were obtained from Amersham. Restriction enzymes were bought from from Promega, Boehringer, Mannheim or United States Biochemicals (USB). Unlabelled nucleotides were purchased from Boehringer, Mannheim.

Antibodies and DNA constructs obtained from others. All mouse Mpv17 constructs were obtained from Dr. Hans Weiher. The human cDNA λ-library were provided by Dr. Peter Seeburg. The mouse MMP-2 DNA probe was provided by Dr. Peter Angel and the anti-MMP-2 antibody by Dr. Matti Höyhthä. The RSV cells were generated by Alexander Reuter in our laboratory.

Solutions

100xDenhardts-solution: 2% bovine serum albumin (BSA), 2% ficoll, 2% polyvinylpyrrolidone.

Formaldehyde-denaturing buffer for RNA: 35µl formaldehyd (37%), 1µl ethidiumbromide (10mg/ml), 10µl 20xMOPS, 100µl formamide (deionised), 54 µl water.

Hybridisation solution: 6xSSC, 5xDenhardts-solution, 0.5% SDS, 250µg/ml salmon sperm DNA.

Laemmli running buffer: 25mM Tris-acetate, 192mM glycine, 0.1% SDS.

5xLaemmli sample buffer: 10% SDS, 50% glycerol, 31.25mM Tris-HCl pH 6.8, 0.005% bromophenol blue, 25% 2-mercaptoethanol.

20xMOPS-buffer: 400mM morpholinopropanesulfonic acid (MOPS), 100mM Na-acetate, 10mM EDTA, pH 7.0.

PBS: 123mM NaCl, 17mM Na_2HPO_4, 2.5mM KH_2PO_4, pH 7.3.

DNA loading buffer: 8% sucrose, 50mM Tris-HCl pH 8.0, 50mM EDTA, 5% Triton X100.

SM buffer: 5.8g NaCl, 2g $MgSO_4$, 50ml 1M Tris-HCl pH 7.5, 5ml 2% gelatine.

20xSSC: 3M NaCl, 0.3M Na_3-citrate.

TAE-buffer: 40mM Tris-acetate, 1mM EDTA

TE: 10mM Tris-HCl pH 8.0, 1mM EDTA

Western transfer buffer: 29g Tris-acetate, 145g glycine, 5g SDS, 1 litre methanol ad 5 litre water.

Bacterial Culture and Manipulation

Media for bacterial growth. Liquid and solid bacterial media were made according to the recipes in Maniatis (1982), except for 2xTY media, which contain 16g/l Tryptone, 10g/l yeast extract and 5g/l NaCl. Ingredients were made by Difco.

Preparation of transformation-competent bacteria. For producing competent E. coli bacteria the method of Hanahan (1986) was used. A 100ml culture of 2xTY medium containing 1/100 of an overnight culture of E. coli K12/DH5α was grown to an OD_{550} of about 0.3, after which the bacteria were centrifuged at 1500xg at 4^0 C for 5 minutes. The supernatant was removed and the bacterial pellet carefully resuspended in 1ml ice-cold STB (100mM KCl, 45mM Mn_2Cl, 10mM $CaCl_2$, 3mM $[Co(NH_3)_6]Cl_3$, 10mM K-MES pH 6.5, and 10% glycerol). After adding another 1ml of STB the bacteria were left on ice for 30 minutes, before they were again centrifuged at 1500xg at 4^0 C for 5 minutes. The supernatant was discarded and the bacteria resuspended in 4ml of STB. Competent bacteria were either used immediately or were stored in 200µl aliquots at -80^0.

Transformation of competent bacteria. 50µl of competent bacteria were mixed with the DNA and stored on ice for 20 minutes which was followed by 3 minutes at 37^0 C and again 3 minutes at 0^0 C. Then the bacteria were pelleted at 3000 rpm in a microfuge, the supernatant removed, resuspended in 100µl 2xTY with 100µg/ml ampicillin, and subsequently plated on 1.5% Bacto-Agar, 100 µg/ml ampicillin in 2xTY.

Production of plating bacteria. A colony of E. coli LE392 was picked from an TY-agar plate and inoculated into 100ml TY which was supplemented with 1ml 20% maltose and 1ml 1M $MgSO_4$. After culturing the bacteria overnight by 37^0 C they were centrifuged at 1500xg for 10 minutes and resuspended in 50ml 0.01M $MgSO_4$.

Cell Culture And Manipulation

Media for tissue culture. Tissue culture media were made from powder obtained from Gibco, and were constituted according to the manufacture´ s instructions. Penicillin and streptomycin were added to 100 units (U) and 375U, respectively. The media were sterilised by filtration. Trypsin was obtained from Gibco and diluted to a 0.25% working solution in versene (8g/l NaCl, 0.2g/l KCl, 1.15g/l Na_2HPO_4, 0.2g/l KH_2PO_4, 0.2 g/l EDTA, 1.5ml/l 1% phenol red).

Tissue culture. NIH 3T3, mouse lung fibroblasts, GM 637 cells, and murine primary skin fibroblasts were grown in Dulbecco´ s modified Eagle´ s medium supplemented with 10% fetal calf serum (FCS). Ag8 cells were cultured in RPMI 1640 with 10% FCS and 2.5mM glutamine supplementation. All cells were grown in a humid atmoshpere containing 6% CO_2 at 37^0 C.
Adherant cells were passaged before reaching total confluency by first removing the medium and washing the cells briefly with 1 ml of 0.25% trypsine before another 1ml of trypsin was applied and the cells briefly incubated at 37^0 C. After being released from the surface of the culture dish the cells were suspended by pipetting and diluted by a factor of 3 to 5 into new culture dishes with fresh medium. For the freezing of cells they were trypsinised, transferred into a 15ml plastic tube containg

10ml medium and centrifuged for 3 minutes at 150xg. The medium was aspirated and the cells resuspended in 1 ml freezing medium which contained the respective medium complemented with 10% dimethylsulfoxide (DMSO). The cell suspensions were transferred to special freezing tubes and stored for 2-3 days at -80^0 C before they were moved to liquid nitrogen (-196^0 C), where cells can be safely stored for years. Thawing of cells was performed by quickly warming them to 37^0 C, and pipetting them into a 15ml tube containing 10ml medium. They were then pelleted at 150xg, resuspended, and transferred to a culture dish with medium.

Preparation of primary mouse skin fibroblasts. A piece of skin was taken from newborn mice, 24h after birth, and placed in Dulbecco´ s modified Eagle´ s medium containing 0.05% Collagenase A (Boehringer Mannheim). After overnight incubation at 4^{0}C epidermal cell layer was carefully removed. The remaining dermis was suspended by pipetting in normal medium containing 10% serum and cultured until cells reached confluency.

Measurement of intracellular ROS by fluorescence. Cells ($5x10^5$) were washed twice with PBS, trypsinized, pelleted and stained with Hydroethidine (Polysciences) according to the manufacturer´ s recommendations. Cells (10000) were analyzed using a Becton Dickinson FACStar with excitation and emission settings described elsewhere (Rothe and Valet, 1990).

Treatment of cells with H_2O_2. Cells derived from homozygous animals were treated with 100mM hydrogen peroxide in growth medium for 30 minutes, washed, and incubated in normal growth medium again for 2, 6, 10 hours before Poly A^+ RNA was prepared (Keyse and Tyrell, 1989).

Nucleic Acid Methods

Purification of DNA. DNA purification was achieved using phenol, phenol/chloroform, and chloroform extraction. Phenol extraction was performed using an equal volume of phenol containing 0.1%

hydroxyquinoline, and equilibrated in 10mM Tris-HCl pH 8.0, 1mM EDTA. An emulsion was formed by vortexing vigorously, and the phases were then separated by centrifugation. The aqueous layer was removed for further processing, avoiding any interface. Subsequently extractions with phenol:chloroform (1:1) and chloroform (24 volumes chloroform and one volume iso-amylalcohol) were performed.
DNA was concentrated by ethanol precipitation adding 0.1 volumes of 3M sodium acetate pH 4.8 and 2.5 volumes of absolute ethanol. Glycogen was added as a carrier where appropriate. The mixture was frozen at -80^0 C for 20-30 minutes, then centrifuged at high speed for more than 10 minutes at 4^0 C. The supernatant was removed, and the pellet washed in 70% ethanol. After drying under vacuum, the pellet was resuspended in TE.

Tail DNA isolation. Around 2cm of a mouse tail biopsy was added to 750µl of 50mM Tris-HCl, pH 8.0, 100mM EDTA, 100mM NaCl, 1% SDS, and 0.5mg/ml proteinase K in 2ml Eppendorf tubes. The tubes were incubated overnight at 55^0 C and then mixed for 5 minutes on an Eppendorf mixer. After this, 250µl 6M NaCl solution was added and mixed for 5 minutes, before the tubes were centrifuged at full speed in a microfuge. 800µl of the supernatant, avoiding the top phase and the pellet, were transferred to a new tube and supplemented with 500µl iso-propanol. After mixing for 2 minutes, the tubes were spun for 1 minute at full speed in a microfuge. The supernatant was then carefully removed and the pellet washed with 1.5ml 70% ethanol. The pellets were briefly dried and resuspended in 500µl TE. 50µl were used for restriction digests in a total volume of 100µl.

Plasmid Minipreparations. For the production of small scale plasmid DNA the method of Birnboim and Doly (1979) was used. 1.5ml of an overnight culture of bacteria were transferred to a 1.5ml Eppendorf tube and centrifuged for 5 minutes at 1500xg. The bacterial pellet was resuspended in 100µl lysozyme solution (50mM glucose, 10mM EDTA, 25mM Tris pH 8.0, lysozyme 2mg/ml) and left on ice for 2 minutes before 200µl 0.2M NaOH with 1% SDS were added. After vortexing 300µl of 3M Na-acetate pH 4.8 solution were added. This was followed by a 5 minutes incubation on ice and a centrifugation at 4^0 C

and 10000xg for 10 minutes. After the centrifugation the supernatant was carefully removed and transferred to a new tube. The plasmid DNA was then precipitated with 1ml ethanol by leaving the solution for 30 minutes at -20^0 C. Subsequently the DNA was pelleted by centrifugation at 4^0 C and 10000xg for 10 minutes and the DNA then resuspended in 100µl 50mM Tris, 100mM Na-acetate pH 4.8. The DNA was precipitated for a second time by adding 50µl of ethanol, an incubation at -20^0 C for 20 minutes, and a centrifugation using the same conditions as above. The DNA was then resuspended in 50µl TE.

Plasmid Maxipreparation. A 200ml overnight bacterial culture was centrifuged at 4000xg for 10 minutes, resuspended in 10ml 50mM glucose, 10mM EDTA, 25mM Tris pH 8.0, lysozyme 2mg/ml and left on ice for 5 minutes. Subsequently 20ml 0.2M NaOH 1% SDS were slowly added before the solution was supplemented with 15ml 3M Na-acetate pH 4.8 and incubated on ice for 30 minutes. This was followed by a centrifugation at 16000xg for 20 minutes and the supernatant was then mixed with 100ml ice-cold ethanol and left at -20^0 for at least 20 minutes. The solution was again centrifuged using the same conditions as above, the resulting pellet resuspended in 8ml 50mM Tris, 100mM Na-acetate pH 4.8, and transferred to a corex-tube. The DNA was precipitated by the addition of 20ml ethanol, incubation for at least 10 minutes at -20^0 C, and a centrifugation at 10000xg for 20 minutes. The DNA pellet was vaccum-dried and resuspended in 4ml 50mM Tris, 1mM EDTA pH 8.0. The subsequent density gradient centrifugation was performed by adding 4.1g CsCl and 320µl ethidium bromide (10mg/ml) to the 4ml DNA solution, which was then transferred to a sealable VTi65 (Beckmann) tube. The first centrifugation was performed overnight at 20^0 C and 55000rpm in a VTi65 rotor. The plasmid (lower) band was removed with a 1ml syringe and transferred to a new centrifugation tube wich was prepared with 4.1g CsCl dissolved in 4ml 50mM Tris pH 8.0, 1mM EDTA. The second centrifugation was performed at 55000rpm at 20^0 C for 6h. The plasmid band was harvested (1ml), diluted in 2ml water, and the remaining ethidium bromide extracted 3 times with butanol. After that the DNA was precipitated by adding 6ml ethanol and incubation at -20^0 C overnight. The DNA was concentrated by centrifugation at 10000xg for 20

minutes. The sediment was then washed with 80% ethanol, dried and resuspended in 300µl TE buffer.

Bacteriophage minipreparation. One plaque was placed overnight in 1ml SM buffer at 4^0 C. On the next day 100µl of the phage eluate was added to 50ml TY, 0.1ml of plating bacteria, and 100µl of Mg/Ca solution (10mM $MgCl_2$, 10mM $CaCl_2$). This was incubated while slowly shaking at 37^0 C for 7h. After that 2 drops of $CHCl_3$ were added and the suspension centrifuged at 4^0 C for 15 minutes at 4000xg. The concentrations of NaCl and PEG 4000 in the supernatant were adjusted to 2M and 40mM, respectively, and slowly stirred for 6h at 4^0 C. Subsequently, the suspension was centrifuged for 20 minutes at 4^0 C and 6000xg. The supernatant was dicarded and the pellet resuspended in 0.6ml SM buffer. 2.5µL DNase I (10mg/ml) were added and the solution incubated for 1h at 37^0 C. Then 2µl of DEPC as well as 1.2ml 1M Tris pH 8.5, 1% SDS, 0.1M EDTA were pipetted to the solution and incubated at 70^0 C for 5 minutes. Subsequently 0.6ml 5M potassium acetate were added and the solution put on ice for 30 minutes, after which it was centrifuged for 5 minutes at 4^0 C and 10000xg.
The phage DNA in the supernatant was precipitated with 2ml iso-propanol and centrifuged at 10000xg. The pellet was resuspended in 500µl TE buffer. Two phenol extractions and one with chloroform after which the DNA was precipitated with ethanol for a second time completed the procedure.

Bacteriophage Maxipreparation. A 50ml culture was set up containing 50 ml TY medium, 0.1ml plating bacteria, 0.1ml Mg/Ca solution (10mM $MgCl_2$, 10mM $CaCl_2$), and 0.1 ml phage eluate. The culture was incubated overnight at 37^0 C. On the next morning 1 or 2 drops chloroform were added and the lysate centrifuged at 4^0 C and 4000xg for 15 minutes. 1ml of the supernatant was pipetted into 1 litre TY medium, 10ml MgCa solution, and 10ml plating bacteria. This was cultured overnight at 37^0 C. After that 2ml of chloroform were added as well as 60g NaCl, and the solution was centrifuged for 20 minutes at 4^0 C and 6000xg. The supernatant was supplemented with 75g/l PEG 4000 and stirred for 4-6h in the cold room. Subsequently, the suspension was spun at 10000xg and 4^0 C for 20 minutes. The pellet was

resuspended in 20ml SM buffer and again centrifuged for 10 minutes at 10000xg and 4^0 C. The supernatant was transferred to a 50ml plastic tube, the pellet washed twice with 5ml SM buffer, and the supernatants were combined and supplemented with DNase I to reach an end concentration of 10µg/ml. The solution was incubated for 1h at 37^0 C. This was followed by a centrifugation for 2h at 30000rpm in a Beckmann Ti60 rotor. The pellet was resuspended in 5ml SM buffer to which 0.675g/ml CsCl were added and then centrifuged in a Beckmann Ti50 rotor for 24h at 35000rpm and 15^0 C. The resulting phage band was removed using a 1ml syringe and dialysed for 1h against 50mM Tris pH 8.0, 10mM $MgCl_2$, 10mM NaCl. Subsequently, the dialysate was transferred to a 10ml tube and supplemented with EDTA ad 20mM, proteinase K ad 50µg/ml, and SDS ad 0.5%, and incubated at 65^0 C for 1h. After a phenol and chloroform extraction the phage DNA was ethanol-precipitated and finally resuspended in TE buffer.

Preparation and analysis of Poly A+ RNA from tissue culture cells. Poly A+ RNA was isolated from five 140mm plates, which were grown to 90% confluency. Cells were lysed in 100mM NaCl, 20mM Tris-HCl, pH 7.4, 10mM EDTA, 0.1% SDS, and proteinase K (300µg/ml). The lysate was incubated overnight with oligo(dT)-cellulose type VII (Pharmacia). After several washing steps with 400mM NaCl, 20mM Tris pH 7.4, 10mM EDTA, 0.2% SDS and 100mM NaCl, 20mM Tris pH 7.4, 10mM 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. 5µg of poly A+ RNA were loaded on a 1% agarose gel supplemented with 2.2% formaldehyd, which was run in MOPS buffer. The RNA was transferred to a nylon membrane (Hybond N, Amersham), which was prehybridized in 6xSSC, 0.5% SDS, 1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA, and 250µg/ml denatured salmon sperm DNA for 2h at 65^0 C. The radioactively labelled probe (Feinberg and Vogelstein, 1983) was added and incubated overnight at 65°C. The filter was washed 15 minutes with 2x SSC, 1% SDS and twice for 15 minutes in 0.1xSSC, o.5% SDS. All washing steps were performed at 65°C in a rocking water bath.

Quantification of nucleic acid. The amount and purity of nucleic acids was estimated from the O.D. readings at 260 nm and 280 nm as described in Maniatis et al. (1982).

Restriction enzyme digestions. Restriction enzyme digests were performed with commercially available endonucleases in accordance with the supplier´ s directions.

Agarose gel electrophoresis. Horizontal agarose gels were used for the separation of DNA molecules as well as for the size-estimation of DNA fragments. The normal concentration of agarose was 1%. Agarose was dissolved in TAE buffer by heating in a microwave oven. After the solution had been cooled down to 60^0 C ethidium bromide was added to a final concentration of 0.5µg/ml. The respective DNA samples were supplemented with 1/5 of their volume of the 5x DNA sample buffer before loading. TAE was used as running buffer. The separation was performed at a field strength of about 7V/cm. The DNA fragments in the gel were detected on a screen with UV light of 320nm.

Southern blotting. Hybond N^+ filters were used to blot DNA agarose gels as described in Maniatis et al. (1982). The transfer was usually performed overnight in 0.4M NaOH, after which the filter was briefly washed in 2xSSC. Then the filter was either used directly in a hybridisation experiment or stored air-sealed at 4^0 C. Hybridisation of the blotted DNA to radioactively labelled DNA probes (Feinberg and Vogelstein, 1983) was usually performed at 65^0 C over night, after which the filters were stringently washed for 15 minutes in 2xSSC, 1% SDS, and then for 2h in 0.1xSSC, 0.5%SDS changing the washing solution once.

Radiolabelled probes. DNA probes were made by the random priming method (Feinberg and Vogelstein, 1983) using templates purified by electroelution from an agarose gel fragment, and alpha-^{32}P-dCTP at 3000 Ci/mmol (Amersham).

Subcloning and ligations. Vectors cut with a single restriction enzyme were treated with calf intestine alkaline phosphatase purchased

from Boehringer according to the manufacture´ s recommendations. All vectors were isolated from agarose gels by electroelution before use. Ligation was performed as described (Berger and Kimmel, 1987). Ligated DNA was used to transform competent bacteria.

DNA Sequencing. For DNA sequencing the Sequenase-2 kit (USB) was used. 1.5-2µg of the respective plasmid were denatured in 0.4M NaOH for 10 minutes at room temperature in a 1.5ml Eppendorf tube. The volume was then increased to 20µl and the denatured DNA ethanol precipitated by adding 10% of the reaction volume of 3M Na-acetate pH 4.8 and 2.5 volumes of ethanol. After mixing, the reaction tubes were left for at least 30 minutes at -80^0 C. The DNA was centrifuged at 4^0 C and the pellet washed with 70% ethanol and resuspended in 1µl water. The DNA was supplemented with 5pmoles of the particular sequencing primer in 1µl, and 2µl 5x reaction buffer (200mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 250mM NaCl). The reaction was left for 2 minutes at 65^0 C and then allowed to cool down to room temperature. In the meantime four reaction tubes labelled G, A, T, and C were prepared containing 2.5µl of the respective termination solution (80µM dGTP, 80µM dATP, 80µM dTTP, 80µM dCTP, 50mM NaCl and 8µM of the according ddNTPs). These tubes were pre-incubated at 37^0 C while the labelling reaction was performed. Therefore 10µl of the annealing reaction (plasmid DNA+primer) were added to 1µl of 0.1M dithiothreitol (DTT), 2µl labelling mix (1.5µM dGTP, 1.5µM dCTP, 1.5µM dTTP), 0.5µl gamma-^{35}S-dATP (Amersham) and finally 2U in 2µl Sequenase Polymerase (USB). The reaction was incubated for 5 minutes at room temperature and subsequently 3.5µl of the labelling reaction was transferred to each tube containing the four different pre-warmed termination solution. Subsequently, these reaction tubes were incubated for another 5 minutes at 37^0 C. The reaction was stopped by adding 4µl stop-solution (95% formamide, 20mM EDTA, 0.05% bromo-phenol-blue and 0.05% xylenecyanol), and after a 3 minutes incubation at 75^0 C loaded on a 6% polyacrylamid gel containing 7M urea.

Sequencing gel electrophoreses. The separation of DNA molecules under denaturing conditions is usually accomplished on a 6%

polyacrylamid with 7M urea. The glass plates were thoroughly washed with detergents and ethanol before one of the plates was treated with a repel solution for easier separation of the plates after the run. About 30ml of the sequence stock solution were supplemented with 180µl 10% APS and 35 µl TEMED and poured between the glassplates that had been assembled using 0.4mm spacers and were hold together by several clamps. After polymerisation the gel was pre-run for 30 minutes before the samples were loaded. The electrophoretic separation of the DNA fragments was performed at 1400V and about 35mA. After the run the gel was transferred onto a 3MM paper, covered with Saran wrap and dried on a gel-dryer for 2h at 80^0 C. The gel was then exposed to a Kodak X-ray film overnight at room temperature.

Reverse Transcriptase-PCR (RT-PCR). Total RNA was prepared from mouse kidneys by the guanidiniumisothiocyanate method (Chirgwin et al., 1979) and was enriched for polyadenylated RNA. 100 ng of each polyA+ RNA were then used as a template to synthesise Mpv17 cDNA in 50mM KCl, 10mM Tris-HCl pH 8.3, 5mM $MgCl_2$, 1mM of each dNTP (Boehringer, Mannheim), 10 units of RNase inhibitor (USB), 2.5 µM of oligo d(T) primer (Boehringer, Mannheim), 2.5U of avian myeloblastosis virus reverse transcriptase (AMV-RT, Promega) and filled up to 20µl with water. The samples were incubated for 20 minutes at 42^0 C and subsequently denatured at 95^0 C for 5 minutes. For the PCR reaction the $MgCl_2$ concentration was adjusted to 2mM, and the final reaction volume was scaled up from 20µl to 100µl. In addition, the samples were supplemented with Mpv17 specific primers from the human cDNA to a final concentration of 0.15µM and 2.5U of Taq polymerase (Amersham) were added. The sequences of the primers used are:
1s: 5´GAATTCGAGGCTCGGCGCTCAGGAAGC3´ and
4r: 5´GAATTCAAACGATGGAGTGAGGCAGGC3´
representing nucleotide pos. 9-29 (1s) and 582-561 (4r), respectively. After 35 cycles 20µl of each sample were analysed on a 1% agarose gel. The observed size for correct cDNA expression was as expected 585bp. In the RT negative controls the identical procedure was carried out omitting the reverse transcriptase enzyme.

Library screening. The human cDNA λgt10 library was obtained from Peter Seeburg, and the genomic library from human placenta cloned in λ FixII was purchased from Stratagene. The libraries were titred and propagated on the E. coli strain LE 397 on LB plates. Screening and plaque purification was performed according to Berger and Kimmel (1987), using hybridisation buffer containing 50% formamide, 1M NaCl, 10% dextran sulphate and 100μg/ml sheared, denatured salmon sperm DNA, at 42^0 C for 16h. Low stringency washes were performed at 45^0 C in 2xSSPE, 0.5% SDS, with four changes over two hours (20xSSPE contains 174g/l NaCl, 27.6g/l NaH_2PO_4 and 7.4g/l EDTA at pH 7.4). High stringency washes were performed at 65^0 C, with one half hour wash in 2xSSPE, 0.5% SDS, then three further half hour washes in 0.2xSSPE, 0.1% SDS.

Protein Methods

SDS-PAGE. Proteins were separated on the basis of size using the method of Laemmli (1970). Protein samples were boiled for five minutes in SDS sample buffer containing 5% β-mercaptoethanol for reducing sample buffer. The resolving gel contained 12.5% acrylamide, unless otherwise stated. Samples were run at 20mA into the stacking gel and then at 40mA during the day. Gels which were Coomassie-stained were incubated in 0.25% Coomassie Brilliant Blue in 10% acetic acid, 10% methanol for 1-24h. Gels were destained in 10% acetic acid, 10% methanol for 24h or more.

Production of fusion proteins in bacteria. E. coli cells harbouring the trpE-Mpv17 plasmid were cultured in M9 medium supplemented with 1% casamino acids and 10μg/ml L-tryptophan overnight at 37^0 C. The cells were diluted 1/100 into M9 supplemented with 1% casamino acids. Pilot studies used 10ml cultures; large scale production used 1 litre cultures. Indole acetic acid was added to a final concentration of 1μg/ml when the culture reached an OD of 0.5 at 600nm. Cells were harvested and inclusion bodies isolated by a modification of the method of Klein et al. (1981). Proteins were dissolved in Laemmli-sample buffer and run on a preparative SDS-PAGE. The protein was cut out and purified using an electroelution

device (Schleicher & Schuell) in 25mM Tris, 192mM glycine, 0.025% SDS.
The human Mpv17 cDNA was cloned into the pGex 3X vector and the Glutathion-S-transferase (GST)-Mpv17 fusion protein expressed in E. coli according to Smith and Johnson (1988). The fusion protein was, however, purified as described above. The purity of both proteins was checked on an analytical SDS-PAGE and the protein solutions stored at -20^0 C.

Immunological Methods

Generation of antibodies. For the production of monoclonal antibodies two Balb/c mice were injected intraperitoneally with 100µg of the GST-Mpv17 fusion protein suspended in complete Freund´ s adjuvant. Three further injections with 50µg of the same antigen in incomplete Freund´ s adjuvant were given intraperitoneally at three-week intervals. Test bleeds were used in an ELISA assay and the mouse showing the best immune response received three intravenous injections of 50µg of purified GST-Mpv17 diluted in PBS. Three days later the spleen was removed from this animal and spleenocytes were fused with Ag8 myeloma cells using the 30% PEG/spinning method as described in Harlow and Lane (1988). Hybridomas were screened for specific antibody production by ELISA, using glutathione-S-transferase (GST) and the GST-Mpv17 fusion protein on separate plates as targets. Hybridomas recognising only the fusion protein were grown into small cultures and one part of each clone frozen in liquid nitrogen while the other part was subcloned by limiting dilution until all wells containing single colonies were positive for specific antibody production. Positive clones were grown in large scale cultures and the supernatants collected. They were used for the production of affinity-purified antibodies. The monoclonal antibodies were isotyped by ELISA using a kit obtained from Biorad.
This MAb 3D8 was purified using an anti-mouse IgM affinity column (Sigma), while the 6F5 and 5D2 were purified using a protein A column (Pharmacia).
The rabbits were immunized according to standard protocols described elsewhere (Harlow and Lane, 1988) and the pre-immune sera and

antisera were purified via a Protein A sepharose column (Pharmacia). The affinity purifications were performed according to the manufacturers´ recommendations.

ELISA assays. ELISAs using peptides or fusion proteins as immobilised targets for antibody screening were performed as described in Harlow and Lane (1988), using secondary antibodies coupled to horseradish peroxidase (Dako), and 2, 2´-azinodi-[3-ethyl-benzthiazolin-sulfonate(6)] (ABTS) as the colour reaction substrate. ELISA plates were purchased from Dynatech.

Western blot analysis. Total protein extracts were prepared from tissue culture cells grown on 100mm plates by pouring off the medium and washing twice with PBS. Cells were scraped off and were spun down in 1.5ml sample tubes. The cell pellet was resuspended in Laemmli loading buffer without dye, heated to 95^0 C for 5 minutes and subsequently sonified for 10 seconds. Protein concentrations were estimated by a mini -Bradford from Biorad. 100µg of total protein were electrophoresed on a 12.5% SDS-PAGE and transferred to an Immobilon-P membrane (Millipore) using a semi-dry electroblotting apparatus (Biorad). The filter was blocked in 5% non-fat dried milk in TBS (25mM Tris pH 8.1, 144mM NaCl) containing 0.3% Tween 20 for 1h and incubated with the polyclonal rabbit anti-Mpv17 serum and pre-immune serum, respectively. The filter was then treated with horseradish peroxidase-conjugated anti-rabbit Ig (Amersham) and signals were detected using the ECL system (Amersham). Alternatively the Western blot was probed with the monclonal antibodies under the same conditions. The 6F5 and 5D2 were detected by an anti-mouse Ig conjugated to horseradish peroxidase (Amersham), while the 3D8 was detected by a secondary anti-mouse IgM antibody conjugated to horseradish peroxidase (Sigma).

Immunofluorescence microscopy. Cells were grown on glass cover slips for two days, after which the medium was removed and the cells were washed in PBS before they were fixed with freshly prepared 4% para-formaldehyde for 15 minutes. After washing with PBS cells were permeabilized using 0.2% TritonX-100 for 10 minutes before the cover

slips were rinsed with PBS. Primary antibodies were applied in 10% FCS in PBS and were incubated overnight at room temperature.
The Mpv17 gene product and catalase were visualized using the following antibodies: MAb 3D8, rabbit anti-Mpv17-ASI and sheep anti-bovine catalase (Binding Site). The primary antibodies were decorated with TRITC-conjugated goat anti-mouse (Jackson), TRITC-conjugated goat anti-rabbit and FITC-conjugated donkey anti-sheep secondary antibodies, which were incubated for 30 minutes at 37° C in PBS containing 10% FCS. After each incubation the cells were washed extensively in PBS. The cover slips were mounted on glass slides using Dako Glycergel and the fluorescent staining pattern was viewed in a fluorescence microscope (model Zeiss Axioskop). Images were recorded on Kodak Ektachrome Panther P1600x film.

Immunohistochemistry. Frozen sections of kidneys were washed in PBS for 10 minutes, after which blocking was performed with normal serum from the species the secondary antibody was raised in. Then the diluted primary antibody was applied onto the section and incubated at 4^0 C in a wet chamber overnight or 1-2h at room temperature. Then the sections were again washed in PBS for 10 minutes, before the secondary antibody was applied and incubated for 30 minutes in the wet chamber at room temperature. After washing for 10 minutes in PBS again, the sections were incubated for 30 minutes at room temperature with the horseradish peroxidase-anti-peroxidase (PAP) complex. Subsequently the sections were again washed in PBS and then treated with the freshly prepared and filtered substrate solution containing 3-amino-9-ethylcarbacole (AEC) in dimethylformamide, 50mM acetate buffer pH 4.9, and 0.03% H_2O_2 for 10 minutes. Finally the sections were rinsed with water, dried, and then mounted in a water based mounting-solution.
Alternatively an alkaline phosphatase anti-alkaline-phosphatase (APAAP) complex was used to detect the primary antibody. The blocking step can be omitted from this protocol, as the primary and secondary antibodies are diluted in normal serum. The substrate solution for the APAAP complex contained naphtole-AS-MX-phosphate (Sigma) in dimethylformamide, 0.1M Tris buffer pH 8.2, Levamisole for blocking of endogenous alkaline phosphatase activity, and Fast Red TR

(Sigma). This was incubated for 15 minutes at room temperature and then rinsed with water. The mounting solution was water based. Counterstainings were performed with hematoxilin. The secondary antibodies, complexes, and substrate reagents were bought from Dako, unless otherwise stated.

Transgenic techniques

Cloning of the rescue construct. The mouse MT-1 promoter was cut out of the vector Mthgh-Dhfr (kindly provided by Dr Erwin Wagner, Vienna) with Eco RI and Bam H1. The 1.8 kb fragment was gel-purified and cloned into pBluescriptIIKS using the above mentioned restriction sites. From a human Mpv17 cDNA, containing intron 6 due to incomplete splicing, the cDNA 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 Bss HII from the pBluescript vector, purified over an agarose gel, and used for microinjection of mouse zygotes at a concentration of 5ng/µl.

Generation of transgenic mice and genotyping. The 3kb promoter/gene DNA fragment was dissolved in injection buffer (10mM Tris pH 7.5, 0.1mM EDTA) at a concentration of 5ng/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 intra-peritoneal injections of 10U of pregnant mare serum (PMS, Sigma) and after 48 hours with 10U of human chorionic gonadotropin (HCG, Sigma). Eggs surviving microinjection were transferred into the oviduct of pseudopregnant foster mice according to Hogan et al. (1986). 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 hybridised with a human cDNA probe. The mutation of the endogenous Mpv17 gene was monitored accordingly, whereby the tail DNA was restricted with Bam HI and hybridized with a genomic 350bp Sau 3A restriction

fragment localized close to the integration site within the Mpv17 gene. Hybridisations and washes were carried out under high stringency (see above).

Histological Techniques

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 μm sections were prepared, stained with PAS (Sigma Diagnostics).

Results

Chapter 3. Cloning of the human homologue of the murine Mpv17 gene

The mutant mouse strain Mpv17 carries a retroviral insert in its genome which inactivates the Mpv17 gene leading to the development of glomerulosclerosis and nephrotic syndrome at young age. Zoo blot analysis (Weiher et al., 1990) showed that the mouse Mpv17 cDNA recognizes distinct bands on genomic DNA of humans, cows, rabbits, and rats in Southern blots under stringent hybridisation conditions arguing that the Mpv17 gene is conserved among these species (Weiher et al., 1993). Expression studies with several murine tissues and cell lines indicated a widespread expression of the Mpv17 gene. The size of the mouse transcript is around 1.7kb and can be found in brain, liver, heart, testis, kidney, and murine cell lines such as NIH 3T3.

Given the assumed conservation of the murine and human Mpv17 sequence, as shown by the zoo blot analysis, the gene was thought to be present and to have an important function in humans, too. Therefore, the lack of Mpv17 expression or a mutated form of it could be involved in human kidney disease. So far only one human genetic defect has been molecularly characterised that is associated with a predominant glomerulosclerosis phenotype. This disease is called Alport syndrome, and is caused by mutations in the various α-chains of the collagen type IV (Hostikka et al., 1990). For other glomerular diseases, based on family clustering, a genetic cause, for example in CNF (Hallmann et al., 1956), is strongly suspected. The latter disease presumably originated as a single mutation event a few centuries ago and the carrier frequency is estimated to be 1 in 200 among the Finnish population. The human Mpv17 gene is potentially one of the prime candidate genes for CNF or one of the other familial glomeruloscleroses. These familial renal defects could be caused by the lack of expression or by mutation of the human homologue of the Mpv17 gene. Biopsy material or DNA from such patients therefore needed to be screened for expression or mutations in the proposed human Mpv17 gene in order to test for an association of it with any of these genetic kidney diseases.

All other cases of primary glomerular diseases evoking the nephrotic syndrome are idiopathic, i.e. of unknown etiology. However, even in these cases the Mpv17 gene could play a causative role in the development of the condition. This is because some of the idiopathic nephrotic syndromes might have not yet been identified as familial entities since they are too rare and show no geographical clustering. In addition, the action of Mpv17 could be indirectly affected by substances controlling its expression or interacting with the gene product. Thus, studies on the regulation of Mpv17 expression are important in the context of defining other factors leading to glomerulosclerosis.

Cloning and analysis of the human Mpv17 cDNA

In order to address these issues it was first necessary to study whether a human Mpv17 gene does indeed exist and to which extent it is conserved compared to the murine gene. Hence, a λgt10 liver cDNA library (kindly provided by P. Seeburg) was screened using the coding region of the mouse cDNA as a probe. The inserts of several phages were isolated, cloned into pBluescriptIIKS and sequenced. This revealed that the three largest inserts out of a total of eight isolates altogether had identical 5´ ends and differed only in the length of their polyA tail which indicates the independence of these clones. As shown in Figure 3.1 the sequence analysis revealed a striking homology between the mouse and the human gene. It was determined to be 92% for their coding regions, while the degree of homology rapidly decreases in the 5´ and 3´ non-coding regions, as depicted in Figure 3.2.

The comparison was performed using the `bestfit´ and `gap´ application from the GCG program (Genetics Computer Group, 1991). From the striking degree of similarity it is assumed that the human and mouse gene fulfil the same function and all further experiments were therefore performed with the human cDNA if not otherwise stated. The high homology also strongly suggests an important role of the Mpv17 protein in normal human kidney function, stressing its possible role in inherited kidney disease.

Taking the first ATG which is preceded by a nearly perfect Kozak consensus sequence (Kozak, 1987) as the start point of the open reading frame, both the human and mouse nucleotide sequence have the capacity to code for a 176 amino acid protein. Furthermore, S1 analysis (Reuter,

1993) suggested that the 5´ end of the cDNA is indeed the start point of transcription. The deduced amino acid sequence of the human and murine cDNA, however, gave no hints of a possible function of the Mpv17 protein, and no functional units, could be identified, apart from two to four possible membrane spanning regions (see chapter 4).

Cloning of the genomic locus of the human Mpv17 gene

Digests of genomic DNA with the restriction enzymes EcoRI, BamHI, BglII and PstI, which were Southern blotted and probed with the human Mpv17 cDNA showed a very simple pattern of fragments (Figure 3.3a), indicating that the coding region is encompassed in only a small stretch of about 15 kb. The isolation of the cDNA made it also possible to clone this genomic region of the human Mpv17 gene. A human placenta λFIXII genomic library (Stratagene) was screened with the isolated human Mpv17 cDNA resulting in two λ-clones named λ3 and λ5 (Figure 3.3). The two clones λ3 and λ5 contain fragments of 15kb and 10kb in length, respectively. In order to test whether these clones indeed represent the Mpv17 genomic region, λ3 and λ5 DNA as well as human genomic DNA was subjected to endonuclease digests using four different restriction enzymes. It emerged from these restriction analyses that the two λ clones represent the Mpv17 genomic locus, since the two clones and human genomic DNA give rise to identical bands after digestion with the same restriction enzyme and detection with the human Mpv17 cDNA probe (Figure 3.4a). These findings are also consistent with the notion that the Mpv17 gene is a single copy gene in humans and no pseudogenes are present in the genome. In addition, it became clear that the two λ clones were overlapping (Figure 3.3), demonstrated by DNA fragments of the same size, such as the 5.6 kb BamHI fragment, that light up in the Southern analysis (Figure 3.4b).

In summary the cloning and analysis of the human homologue of the mouse glomerulosclerosis gene Mpv17 revealed that both genes are almost identical. It suggests that the human gene product is correspondingly important for the normal function of the human kidney. The identification and analysis of two λ clones representing the genomic region demonstrated that the human Mpv17 is a single gene,

and allowed further studies to investigate the role of Mpv17 in human kidney disease.

The role of Mpv17 in inherited human glomerulosclerosis

The human Mpv17 cDNA clone as well as the genomic clones and subclones were used to test whether Mpv17 is involved in CNF. The condition resembles the Mpv17 phenotype by its autosomal recessive inheritance, early detectable proteinuria and similar histopathological findings such as tubular dilations and the fusion of the foot processes of the visceral epithelial cells (Norio and Rapola, 1989). Among the various diseases responsible for a congenital or infantile nephrotic syndrome, CNF is the best known. Besides diffuse mesangial sclerosis (DMS) which is genetically less well characterised, it is the only familial nephrotic syndrome, and a causal gene defect has not been identified yet (Habib et al., 1993). Therefore, the potential involvement of the human Mpv17 gene was examined.

Expression of Mpv17 RNA in kidneys from a CNF patient was found unchanged when compared to a healthy person. No point mutations could be found in cDNA from nephrectomy material from that patient (Hans Weiher, personal communication), and restriction fragment length polymorphism (RFLP) analyses was unable to detect a linkeage between the human Mpv17 gene and CNF (Karl Tryggvason, personal communication). In the meantime the CNF locus has been mapped to the long arm of human chromosome 19 thereby excluding eight genes coding for basement membrane components (Kestila et al., 1994b) as well as Pax-2 (Kestila et al., 1994c) that has been shown in a transgenic mouse to be associated with glomerulosclerosis (Dressler et al., 1993). However, the human Mpv17 was assigned to chromosome 2p21-23 (Karasawa et al., 1993) by fluorescent-in-situ-hybridisation (FISH)-analysis using the λ5 subclone pBS 5.6 (Figure 3.5). This is further evidence that the Mpv17 gene is not involved in the development of Finnish nephropathy. The murine Mpv17 gene was assigned to chromosome 5 using somatic cell hybrid panels (Karasawa et al., 1993)

Expression of the human Mpv17 gene.

The mouse Mpv17 gene was shown to be transcribed in a number of murine tissues and several cells of rodent origin such as NIH 3T3, F9, D3, TMCK-1, and RAG cells, the latter two being derived from kidney epithelium (Weiher et al., 1990). It was also shown that the Mpv17 gene is expressed in human kidneys (Weiher, 1993). We found Mpv17 transcription in different human cells as depicted for GM637 cells in Figure 3.6. This figure also shows that the human Mpv17 cDNA probe detects the mouse transcript in NIH 3T3 cells while fibroblasts derived from Mpv17 homozygotes are negative, as expected, for expression of Mpv17. Other human cells that were positive for Mpv17 transcription include several primary fibroblasts and 293 cells (data not shown).

For the analysis of the Mpv17 protein expression the generation of antibodies against the Mpv17 gene product was required.

```
  1  GTGGGAGGGA GGCTCGGCGC TCAGGAAGCA TGGCACTCTG GCGGGCATAC
 51  CAGCGGGCCC TGGCCGCTCA CCCGTGGAAA GTACAGGTCC TGACAGCTGG
101  GTCCCTGATG GGCCTGGGTG ACATTATCTC ACAGCAGCTG GTGGAGAGGC
151  GGGGTCTGCA GGAACACCAG AGAGGCCGGA CTCTGACCAT GGTGTCCCTG
201  GGCTGTGGCT TTGTGGGCCC TGTGGTAGGA GGCTGGTACA AGGTTTTGGA
251  TCGGTTCATC CCTGGCACCA CCAAAGTGGA TGCACTGAAG AAGATGTTGT
301  TGGATCAGGG GGGCTTTGCC CCGTGTTTTC TAGGCTGCTT TCTCCCACTG
351  GTAGGGGCAC TTAATGGACT GTCAGCCCAG GACAACTGGG CCAAACTACA
401  GCGGGATTAT CCTGATGCCC TTATCACCAA CTACTATCTA TGGCCTGCTG
451  TGCAGTTAGC CAACTTCTAC CTGGTCCCCC TTCATTACAG GTTGGCCGTT
501  GTCCAATGTG TTGCTGTTAT CTGGAACTCC TACCTGTCCT GGAAGGCACA
551  TCGGCTCTAA GCCTGCCTCA CTCCATCGTT TCCACCTTGC AGTGATGCAG
601  CTTGACCCTG GAACGGTCAG ACAACCTCCT CAAAGTGGGC ATACCAGTTT
651  CCACGGGGTT GGGTTGCCGG TCAGAGCTTA AGAGGACTAG CACCTGCAAT
701  GCCCTCTTCA CTCTAAATGT ACACTGACTG CTTTAGAGCC CTTGATAATA
751  GTCTTATTCC CACCACATAC TAGGCACTCC ATAAATATCT GTTGAACCTT
801  CATGACCTTA TCAACTTTAC ACCCATATCC AGCAAATGCC ACTCATCCCC
851  ACTCTTCATA GACACATTTG TTACTCTAAC CCTGCCTAGG CTTCTTGTAG
901  CTCCAGCTCT TTAGAGACTC CGGGAACCCT TTATATGGTG CCTCAGTAAA
951  TATGTTATTA AATATGTAAT CCGG
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Figure 3.1 Nucleotide sequence of the human Mpv17 cDNA sequence.

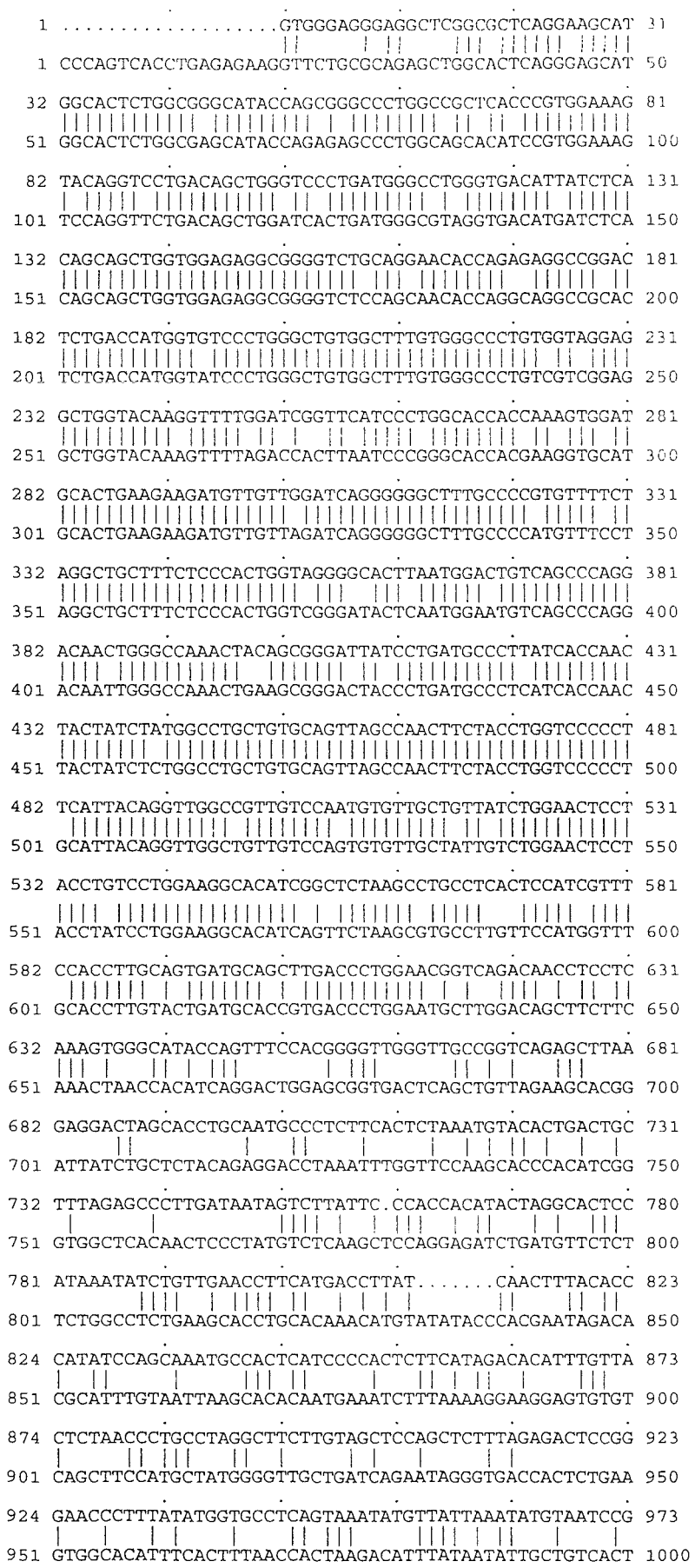


Figure 3.2 Nucleotide sequence of the human Mpv17 cDNA (top) as compared to its murine homologue (bottom). Both sequences can code for proteins of 176 amino acids.

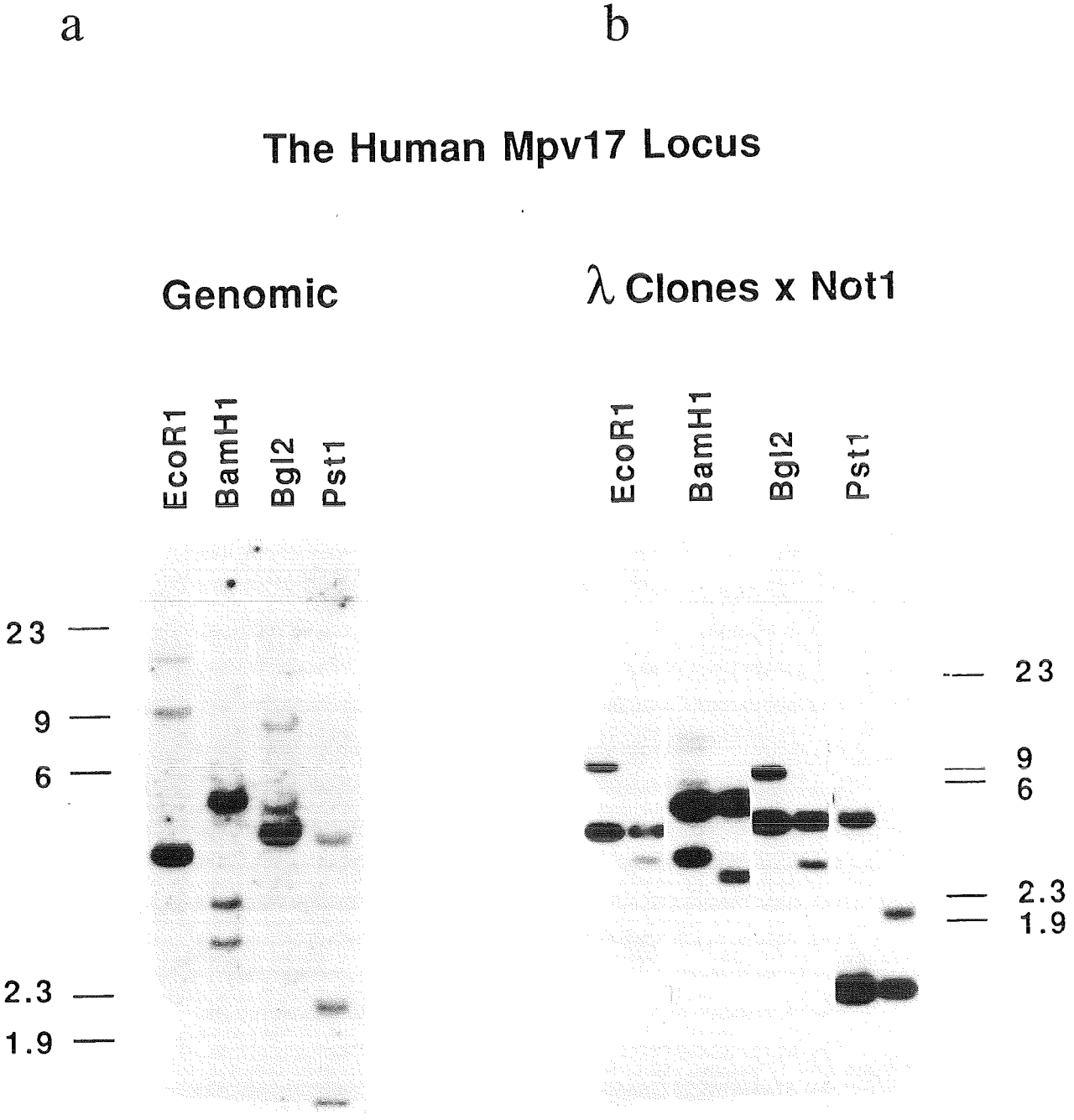


Figure 3.3 The human Mpv17 locus cloned in two λ phages. **a**: Southern blot of human genomic DNA. **b**: Southern blot of two λ phages covering the Mpv17 region. The left lane contains λ3 DNA, the right lane λ5 DNA for each enzyme, respectively. Both blots were probed with the coding region of the human cDNA (p317-10)

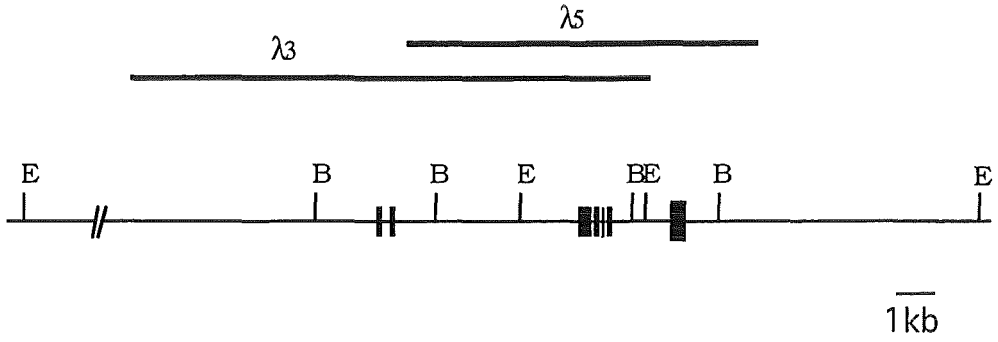


Figure 3.4 Map of human Mpv17 genomic locus and the genomic clone λ3 and λ5. BamH1 (B) and EcoR1 (E) restriction sites are indicated. The sizes of the exons are depicted as bars.

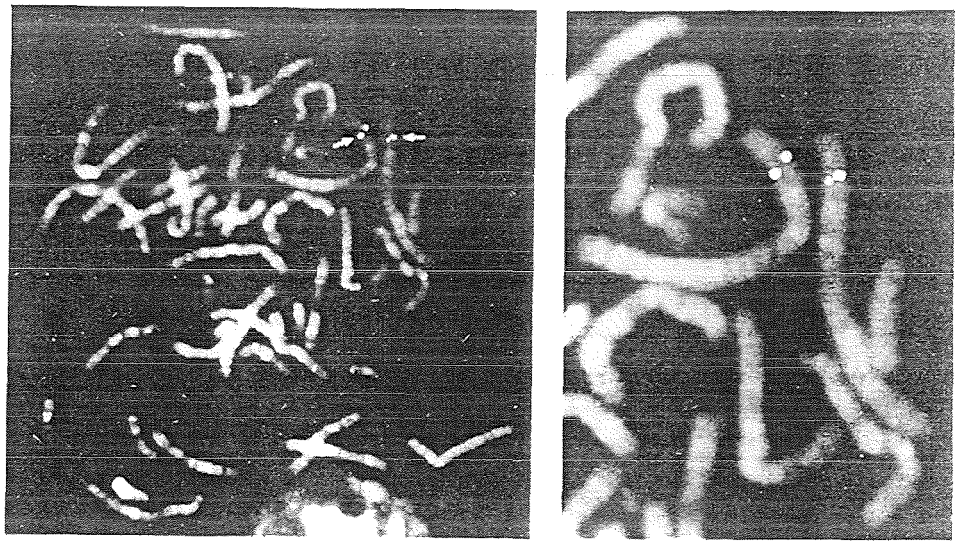


Figure 3.5 Mapping of the human Mpv17 gene to 2p23-p21. 100ng of biotin labelled λ5 subclone p5.6 DNA were combined with 10mg of human Cot1- DNA (BRL) in a 10ml hybridisation cocktail and hybridised to human metaphase chromosomes. The bound probe was visualized via FITC-conjugated to avidin (see arrow). Chromosomal counterstaining with DAPI resulted in a Q-banding like pattern. Note that the highly specific FITC signals are located within 2p23-p21. Digitized images of FITC and DAPI fluorescence were generated separately with the use of a CCD camera (Photometrics), processed and overlayed electronically. Photographs were taken from the video screen. (The FISH analysis was performed in Dr. Peter Lichters lab, Heidelberg)

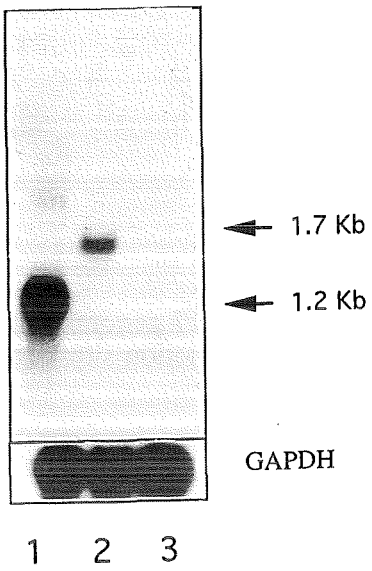


Figure 3.6 Mpv17 expression in human and murine fibroblasts. Poly(A)$^+$ RNA was prepared from GM637 (lane 1), NIH3T3 (lane 2), and fibroblasts from Mpv17 homozygous mice (lane 3). 4 µg were loaded per lane. As a probe the human cDNA clone p31710 (Karasawa et al., 1993) was used.

Chapter 4. Mpv17 protein analysis and generation of Mpv17 specific antibodies

The calculated molecular mass of the 176 aa Mpv17 protein is around 20 kD assuming that no significant post-translational modifications occur. The two hydrophobic regions predicted by the ALOM programm (Klein et al., 1985), spanning from position 94 to 114 and from 140 to 165, are conserved to the same extent between the human and mouse gene as is the rest of the sequence; only four amino acids out of together 47 are varied. In the first stretch encompassing 21 amino acids isoleucine 110 and methionine 114 in the mouse sequence are exchanged for an alanine and leucine residue, respectively. The second hydrophobic region (26 aa) long contains a switch from the murine isoleucine at position 163 to valine and from valine 164 to isoleucine in the human sequence. All four changes do not convert the hydrophobic character of the two stretches, thereby allowing for membrane association of the mouse and the human Mpv17 protein. However, both proteins contain conserved proline residues in their hydrophobic regions, which are not commonly found in typical membrane-spanning domains. Four cysteine residues at position 59, 99, 103, and 160 potentially giving rise to disulfide bridges can be found in the murine as well as in the human amino acid sequence. Threonine (80) and serine (170) residues in both sequences have been identified as potential phosphorylation targets by protein kinase C, while the serines at position 115 and the threonines at position 111 can be potentially phosphorylated by casein kinase II (Weiher, 1993).

Homology to the peroxisomal membrane protein 22

An EMBL database search revealed an interesting homology to the peroxisomal membrane protein (pmp) 22, that was recently cloned from a rat cDNA expression library and its nucleotide and deduced amino acid sequence established (Kaldi et al., 1993). This protein has been described as being a major component of rat peroxisomes (Hartl et al., 1985; Hartl et al., 1987) and a pore-forming activity has been attributed to it (Van Veldhoven et al., 1987), although its precise function in the peroxisomal membrane remains unclear. A sequence comparison according to the TFASTA algorithm (Lipman and Pearson, 1985) of

Mpv17 with the pmp 22 protein sequence revealed 28% identical amino acids in a stretch of 155 overlapping amino acids and an intriguing overall sequence similarity of 54% (Figure 4.2). When the topology of the two proteins was investigated by hydropathy analysis using the algorithm of Kyte and Doolittle (Genetics Computer Group, 1991) the profiles as shown in Figure 4.3 (a, b) turned out to be quite similar. This points to a potential relatedness between the two proteins with respect to their intracellular localisation and possibly their function. In contrast to the ALOM programme this analysis revealed four possible transmembrane stretches instead of two. It has been reported that these membrane spanning sequences are often flanked by one or more charged amino acids and that the kind of charge is important for the orientation of the protein in the membrane (Dalbey, 1990; Hartmann et al., 1989). For bacterial membrane proteins it has been noted that when viewed from the N-terminus to the C-terminus, the transmembrane stretch is preceded by a positively charged residue such as lysine and arginine and terminated by glutamic acid.

According to Singer (1990) the pmp 22 protein fits the definition of a type IIIb integral membrane protein. Type IIIb membrane proteins possess more than one transmembrane sequence and an NH_2-terminus on the exterior face of the membrane. The Mpv17 protein roughly follows these rules, but shows a slightly different picture. Trying to superimpose the topological model for the pmp 22 (Kaldi et al., 1993) onto the Mpv17 protein requires four membrane spanning regions as predicted by the Kyte and Doolittle analysis and shifting of the two hydrophobic stretches as predicted by the ALOM programme in order to better fit the hypothetical model. The existence of four membrane associated domains in the Mpv17 protein would also be in better accordance with the Chou-Fasman analysis (Genetics Computer Group, 1991) that is shown for the Mpv17 protein as well as for the pmp 22 in in Figure 4.3 (c, d). Whether the Mpv17 protein also belongs to the group of type IIIb membrane proteins and truly parallels the pmp 22 membrane topology requires further studies.

So far, no target signals have been identified for peroxisomal membrane proteins, while for peroxisomal matrix proteins two such signals have been identified (Gould et al., 1989; Swinkels et al., 1991). The Mpv17 protein and the pmp22 lack these signals, which is in accordance with

the hypothesis that the Mpv17 protein, as pmp 22, is a peroxisomal membrane protein. Due to the lack of signal sequences, the underlying mechanism of peroxisomal membrane targetting is completely unknown.

Raising antibodies against Mpv17 protein.

In order to investigate if Mpv 17 is indeed a peroxisomal protein, as suggested by the sequence and topological similarities with the peroxisomal membrane protein 22, the actual intracellular localisation of the Mpv17 protein needed to be determined. It is important to elucidate the intracellular localisation of a protein, since it gives first insights into the possible function of the protein, and in the Mpv17 case could reveal a new link between a peroxisomal function and kidney disease.

Production of recombinant Mpv17 fusion proteins as antigens for immunisation.

Antibodies were raised against two bacterially expressed recombinant hMpv17-fusion proteins and two peptides taken from the Mpv17 sequence.

One of the fusion proteins contained the 34 kD Trp E protein from Escherichia coli (Ausubel et al., 1984), while the second one was created by fusing the 27 kD glutathione-S-transferase (GST) from Schistosoma japonicum (Smith and Johnson et al., 1988) to the open reading frame of the human Mpv17 gene. The prokaryotic part was located at the N-terminus of the resulting fusion proteins, and both contained the entire coding region of the human Mpv17 gene at the C-terminus. While the Trp E fusion protein was already available in the lab (provided by H. Weiher) the construct for the GST-Mpv17 protein needed to be cloned (Figure 4.4). The expression vector system pGex offers three different plasmids for the three possible reading frames so that at least one cloning strategy will always lead to in frame fusion of the Mpv17 reading frame to the carboxyl terminus of the Schistosoma japanicum GST. Normally the GST fusion proteins are soluble in aqueous solutions and can then be purified from crude bacterial lysates under non-denaturing conditions by affinity chromatography on immobilized glutathione. We chose the strategy of cloning the hMpv17 segment in frame with the GST sequence using the Sma I site in the cloning site of

the pGex 3X vector. Therefore the pBluescript vector containing the human Mpv17 cDNA was cut with Dde I at position 20 and 557 which released the entire coding region of the human Mpv17 gene. Positive clones were identified and the correct orientation was determined using asymmetric digests with Pst I at position 160 in the cDNA and position 1340 in the pGex vector. These clones were tested for expression of the desired fusion protein in small-scale-cultures. For that purpose the respective E. coli clones were grown for two hours and then induced with IPTG for one hour before the bacteria were harvested, spun down, and directly resuspended and solublized in denaturing Laemmli sample buffer (see Material and Methods). An aliquot was loaded on a standard 12.5% SDS-Polyacrylamidgel (SDS-PAGE) and the proteins visualised by staining with Coomassie-blue. As a control, a parallel culture of each clone was grown, but was not induced with IPTG and then also loaded on the gel. In order to check for functional induction an established clone harbouring the empty GST vector only was analysed in parallel. The result of this experiment is depicted in Figure 4.5. Several independent insert-positive clones of correct insert orientation were analysed alongside with GST vector controls. A band of 27 kD can be seen in lane Ci (stimulated GST control) as opposed to the uninduced culture. Of the four clones, only clone 6 resulted in a band of the expected size of 46 kD for the desired GST-Mpv17 fusion protein. The other clones tested did not produce an inducible protein for unknown reasons. The recombinant plasmid of clone 6 was then also tested for the correct reading frame by sequencing the connection of the GST sequence to the Mpv17 cDNA segment. This had the expected sequence, ensuring the production of the genuine Mpv17 polypeptide within the fusion protein. This clone was then grown in a large scale culture and was subjected to the standard purification protocol for GST fusion proteins (see Material and Methods). In the course of this procedure it turned out that the GST-Mpv17 fusion protein was insoluble in aqueous solution, probably due to storage in inclusion-bodies (Smith and Johnson et al., 1988), and could therefore not be purified on the glutathione agarose column. Several attempts to keep the GST-Mpv17 protein in solution, such as lowering the incubation temperature of the bacteria from 37° C to 28° C, decreasing the induction time from 5h to 2h, and finally a combination of both, were not successful. Likewise, when the

fusion protein was solubilised in 6M urea and then dialysed in small steps against decreasing concentrations of urea, the protein precipitated at urea concentrations below 4M. The insolubility of the GST-Mpv17 protein could be explained by its hydrophobic regions. It should also be mentioned that insolubility of recombinant proteins in bacteria is seen occasionally in proteins not harbouring such regions (Marston et al., 1986 and R. Vallon, personal communication). For these technical reasons the GST-Mpv17 protein was purified by conventional methods from the producer-cells by preparation of inclusion bodies and denaturation in reducing Laemmli sample buffer, followed by preparative SDS-PAGE. The 46 kD band of the fusion protein was visualized by negative staining using 4 M potassium acetate, and the protein was eluted from the gel slice by electro-elution. The purity of the isolated protein was demonstrated by analytical SDS-PAGE as depicted in Figure 4.6. The purified protein was recognized by a polyclonal antibody against the GST-domain (data not shown) indicating that indeed a protein containing GST had been purified. Since no other GST protein especially of that size was expected in the preparation it was reasonably safe to assume that it was the desired GST-Mpv17 fusion protein. The concentration of SDS in the elution buffer was too high to ensure a quantitative protease digestion with factor Xa at the respective site in the fusion protein which would cleave the Mpv17 protein from the GST part. Therefore the complete GST-Mpv17 protein was used in addition to the TrpE-Mpv17 product for the immunisations (see below).

Mpv17 peptides used for immunisation

Using whole proteins as immunogens has the advantage that many different epitopes are presented to the immune system. This should ensure that the resulting antibodies are likely to recognize the respective protein even if denatured. An alternative is the use of peptides, which has the advantage that antibodies can be prepared when the corresponding protein is only available in small quantities, is difficult to purify, or when the sequence is only partially known. Additionally, the region of the protein that the antibody recognizes is known from the peptide sequence, making it possible to target antibodies to specific regions of a protein. However, the disadvantage is that one cannot be certain that the peptide sequence corresponds to an epitope on the intact

protein, perhaps generating antibodies that do not recognize the native or even the denatured protein. By following empirical guidelines according to Harlow and Lane (1988) it is nevertheless often possible to design peptides which will constitute an epitope. The peptides selected from hydrophilic regions of the human Mpv 17 sequences with the following sequences were designed according to these rules:

Peptide I:	**VERRGLQQHQAGRTLTMK**	(pos.: 38-54+**K**)
Peptide II:	**QDNWAKLKRDYPDAL**	(pos.:117-131)

Immunisation of rabbits with the peptides and fusion proteins.

The generation of polyclonal antibodies is a relatively quick and easy way to obtain an antiserum that usually functions well in commonly used protein assays such as immunoprecipitation, Western blots, and cell staining. Polyclonal antibodies suffer, however, from the disadvantage that many of them display background signals due to the presence of antibodies in the serum directed against other determinants to which the animal has been exposed.

Antibody / x	Antigen	Animal	ELISA	West. α-rec.	West. α-ext.
Pep. I (PC)	peptideI	rabbit	+	-	-
Pep. II (PC)	peptideII	rabbit	+	-	-
AS-I (PC)	GST-Mpv17	rabbit	+	+	+
AS-II (PC)	GST-Mpv17	rabbit	-	-	-
αTrpE. (PC)	TrpE-Mpv17	rabbit	+	+	n.a.
5D2 (Mab)	GST-Mpv17	mouse	+	+	n.a.
6F5 (Mab)	GST-Mpv17	mouse	+	+	+
3D8 (Mab)	GST-Mpv17	mouse	+	+	+

Table 4.1 anti- Mpv17 antibodies (PC=polyclonal antibody; Mab=monoclonal antibody, n.a.=not analysed, West. α-rec=Western on the purified recombinant protein, West. α-ext.=Western on cell (3T3; GM 637) protein extract)

The peptides I and II coupled to the hapten Keyhole limpet hemacyyamin (KLH) were each injected into a rabbits following the protocol outlined in Harlow and Lane (1988). ELISA test of the antisera anti-peptide I and anti-peptide II on the respective peptides were positive. Repeating the test with recombinant GST-Mpv17 protein revealed, however, that both anti-peptide sera were not able to recognize epitopes on the whole denatured protein. Additional experiments such as Western analysis and cyto-immunofluorescence with these sera also gave negative results (Table 4.1). Therefore these antisera were not used for further analyses.

Two rabbits were immunised with the GST-Mpv17 fusion protein. In addition, a rabbit was immunised with the TrpE-Mpv17 fusion protein according to general procedures (Harlow and Lane, 1988). From all

three animals pre-immune sera were taken before the first inoculation with antigen and designated PSI and PSII for the two rabbits that were injected with the GST-Mpv17 and PS-TrpE for the animal immunised with TrpE.
The corresponding antisera ASI, ASII, and anti-TrpE-Mpv17 were tested in ELISA and Western analysis against the isolated recombinant proteins and against whole cell protein extract (Table 4.1). Anti-TrpE-Mpv17 gave a positive response in an ELISA assay against the TrpE-Mpv17 as well as against the GST-Mpv17 protein, further indicating that the Mpv17 portion of the fusion protein was recognized. Additional analyses, however, such as Western blots and immunoprecipitations of protein extracts from cells known to express Mpv17 mRNA using the anti-TrpE-Mpv17 serum were unsuccessful. On the other hand the antisera ASI responded positively in the ELISA test and detected an additional band, in comparison to the preimmune-serum (Figure 4.7). This band had the expected size of the Mpv17 gene product of about 20 kD in whole-cell protein extracts from mouse and human fibroblasts, expressing Mpv17 RNA (Figure 3.6) in an immuno-blot experiment. This result indicated that ASI does specifically recognize the Mpv17 protein as a band of 20 kD, that is not detected by the respective preimmune serum PSI taken from the same animal before the first antigen injection. The fact that the band appears exactly at the position of the calculated molecular weight of 20 kD suggests that the Mpv17 protein does not undergo post-translational modifications changing its running behaviour on SDS-PAGE. The antiserum ASII failed to detect the GST-Mpv17 gene in ELISA and Western analysis. It was therefore not used for any other experiments.
Since the preimmune serum also reacted against several other proteins in Western analysis, it was necessary to always include the preimmune serum (PSI) in further experiments in order to distinguish between Mpv17-specific and background signals.

Generation of monoclonal antibodies

Because of the background problem with the ASI antiserum, I set out to raise monoclonal antibodies against the GST-Mpv17 fusion protein. Monoclonal antibodies have the advantage that they recognize only a single epitope, and thus should show much less background signal, and

represent a potentially unlimited source of antibody. In addition they can be generated using impure antigens, while polyclonal antibodies require relatively pure antigens to keep backgrounds adequately low.
Two 12 weeks old Balb/c mice were injected intra-peritoneally (i.p.) four times with GST-Mpv17 protein supplemented with Freund´s adjuvant at intervals of four weeks. Test bleeds were taken one week after the last i.p. injection and revealed a positive response against the fusion protein in ELISA tests for only one animal, which was then injected intra-venously (i.v.) on three following days with the antigen omitting the adjuvant. On the fourth day the spleen was removed from the animal and the suspended cells fused with mouse myeloma cells Ag8 (kindly provided by Gerd Moldenhauer, DKFZ, Heidelberg). The hybridomas were plated out into ten 96 well plates, and 32% of the wells were growth positive. The supernatants were differentially tested by ELISA, using GST only and the GST-Mpv17 fusion protein as targets. This allowed the identification of hybridomas producing antibodies which solely recognize the Mpv17 portion of GST-Mpv17. Three hybridoma supernatants were positive for the Mpv17 part only, while 15 were identified that recognized the GST. The three Mpv17-specific hybridomas were expanded and the supernatants tested in Western blot analyses, again using the GST and GST-Mpv17 proteins as targets. The monoclonal antibodies inwere detected with horseradish peroxidase conjugated anti-mouse secondary antibody and visualised with the ECL system (Amersham). The supernatants of clones 5D2 and 6F5 bound strongly to the Mpv17 protein, while the signal of 3D8 was rather weak (Figure 4.8). Subsequently the three clones were subcloned by limited dilution. The monoclonal antibodies produced by the pure clones were then isotyped. The monoclonal antibodies 5D2 and 6F5 were shown to be IgG_1 and the clone 3D8 was identified as IgM. The antibodies were purified by a Protein G column and an anti-mouse-IgM-agarose column, respectively. It was not possible to purify the 3D8 antibody via Protein A or G, because the constant part of IgM antibodies does not bind to either of them (Harlow and Lane, 1988). The 3D8 antibody was concentrated to 0.3 mg/ml, while the other monoclonals were used as 1 mg/ml stock solutions. All antibodies were then tested in Western blots of protein extracts from 3T3 cells and RSV7 cells. The latter cell line constitutively overexpresses the human Mpv17 gene

under the control of the Rous sarcoma virus (RSV) promoter (Zwacka et al., 1994). The 3D8 antibody recognizes a protein band of 20 kD, the intensity of which is significantly increased in the overexpressing cells (Figure 4.9), indicating that indeed the Mpv17 protein is recognized in these cells. However, the reactivity was not as high as expected, and the yield of antibody was very low. This is probably due to the fact that IgM antibodies are mainly generated in the primary immune response, thereby representing rather non-optimised and low-affinity antibodies. It is also known that this class of antibodies are secreted by the hybridomas at low concentrations. I therefore suspect that the high similarity between the murine Mpv17 protein and the human Mpv17 protein, that was used for the immunisation, results in the poor immune response in the mice, giving rise to weak antibodies. In order to have a better source of anti-Mpv17 antibodies we are currently in the process immunising Mpv17 homozygous mice, which lack Mpv17 protein expression, with recombinant Mpv17 protein. This was initially avoided because of concern about the life span of these mice. Since then it seems feasible to maintain these animals for more than the minimal time of 6 months required for the immunisation protocol.

The generated monoclonal and polyclonal antibodies against Mpv17 protein were then together tested in cyto-immunofluorescence experiments on cells known to express Mpv17 transcripts in order to decipher the intracellular localisation of the gene product.

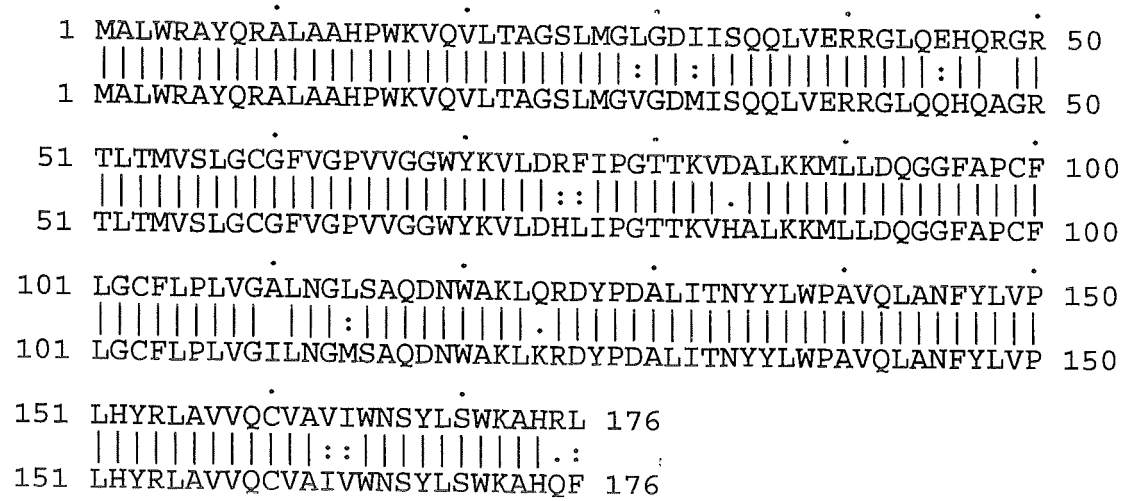


Figure 4.1 Comparison of the protein sequences of the human (top) and murine (bottom) Mpv17 genes. The percent identity between the two proteins is 92%.

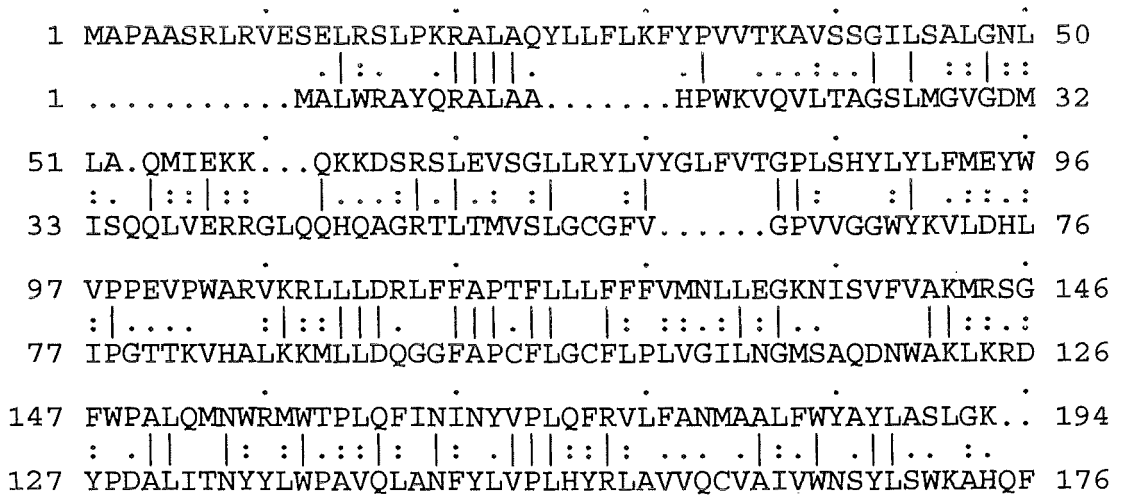


Figure 4.2 Comparison of murine Mpv17 amino acid sequence (bottom) with the rat pmp 22 (top, Kaldi et al., 1993) protein. Identities between the two proteins indicated as full lines constitute 26% of the Mpv17 sequence. Conservation on the basis of the algorithms of similarity of the GCG program (Genetics Computer Group, 1991) is more 52%.

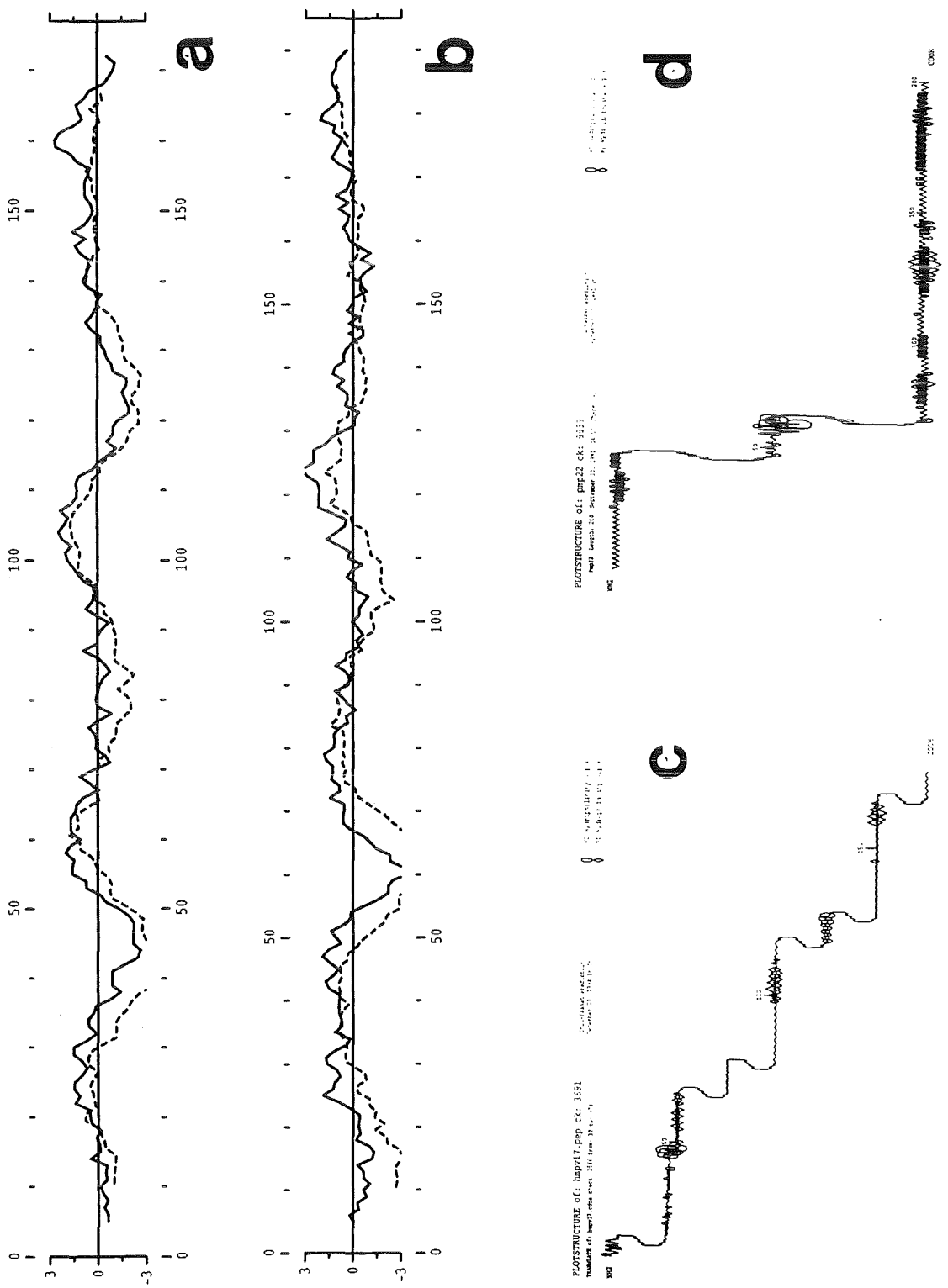


Figure 4.3 Hydropathy analysis of the human Mpv17 protein (a,c) and rat pmp 22 protein (b, d). **a, b**: Hydropathy profile using the algorithm of Kyte and Doolittle. **c, d**: Topologic prediction according to Chou and Fasman.

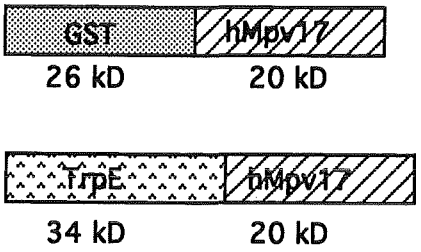


Figure 4.4 Schematic view of the TrpE-Mpv17 and the GST-Mpv17 fusion protein.

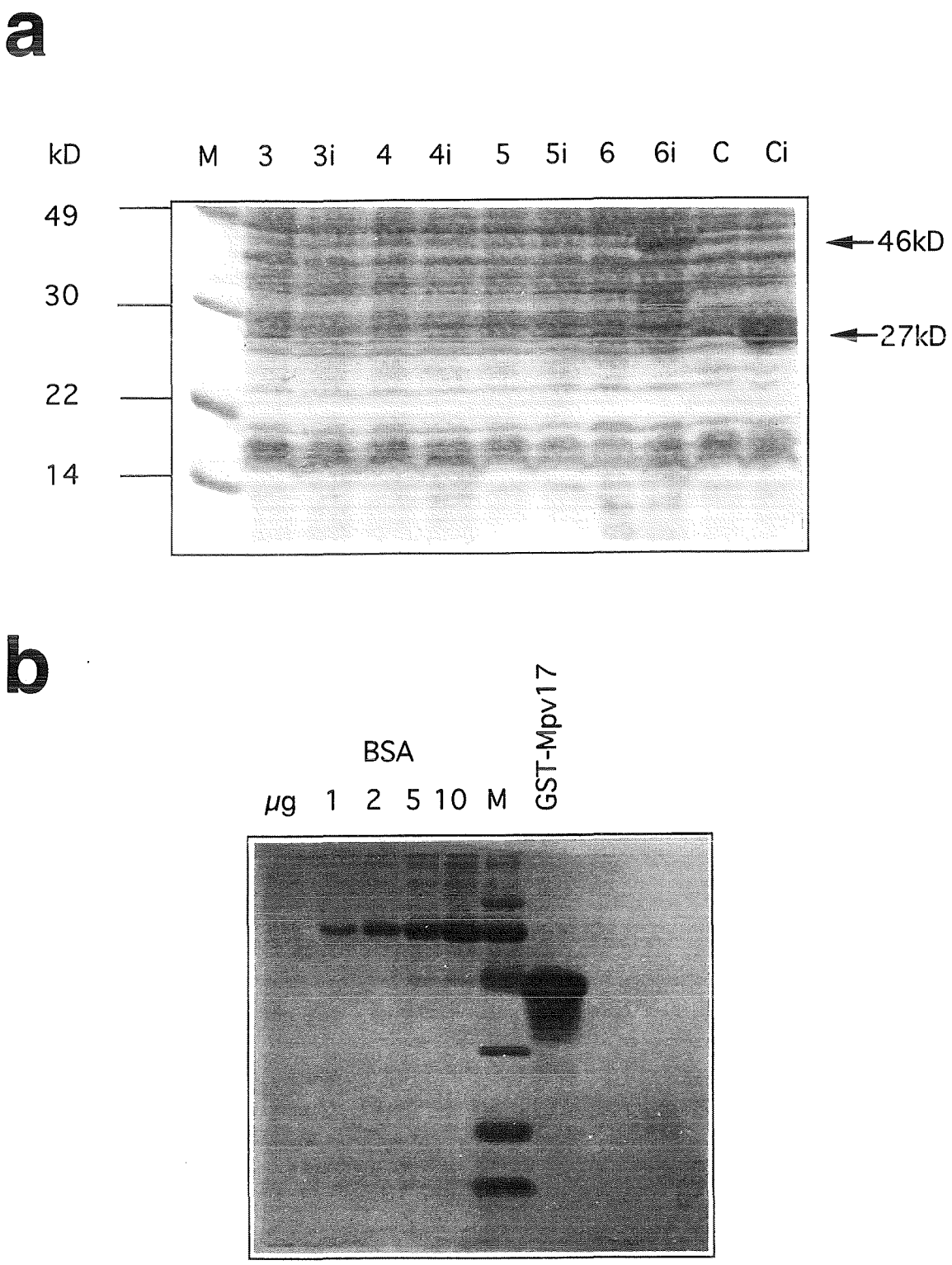


Figure 4.5 Bacterially expressed GST-Mpv17 fusion protein. **a**: Mini-scale protein preparations from pGex-Mpv17 DNA-positive clones (1-6) and a control clone (C) containing the pGex vector only. The left lane of each clone represents the non-induced culture, while the right lanes (i) show the protein extracts from cultures treated with IPTG . **b**: Purified GST-Mpv17 fusion protein from clone 6. On the right side different amounts of BSA were loaded to quantify the concentration of the purified fusion protein. (M=molecular weight marker (daltons): 106, 80, 49, 32, 27, 18).

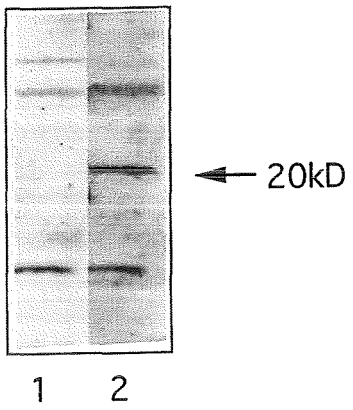


Figure 4.6 Protein extracts from NIH3T3 cells were prepared, 100µg loaded, Western blotted, and probed with preimmune serum (lane 1) and antiserum (lane 2) from rabbits immunized with GST-Mpv17 fusion protein.

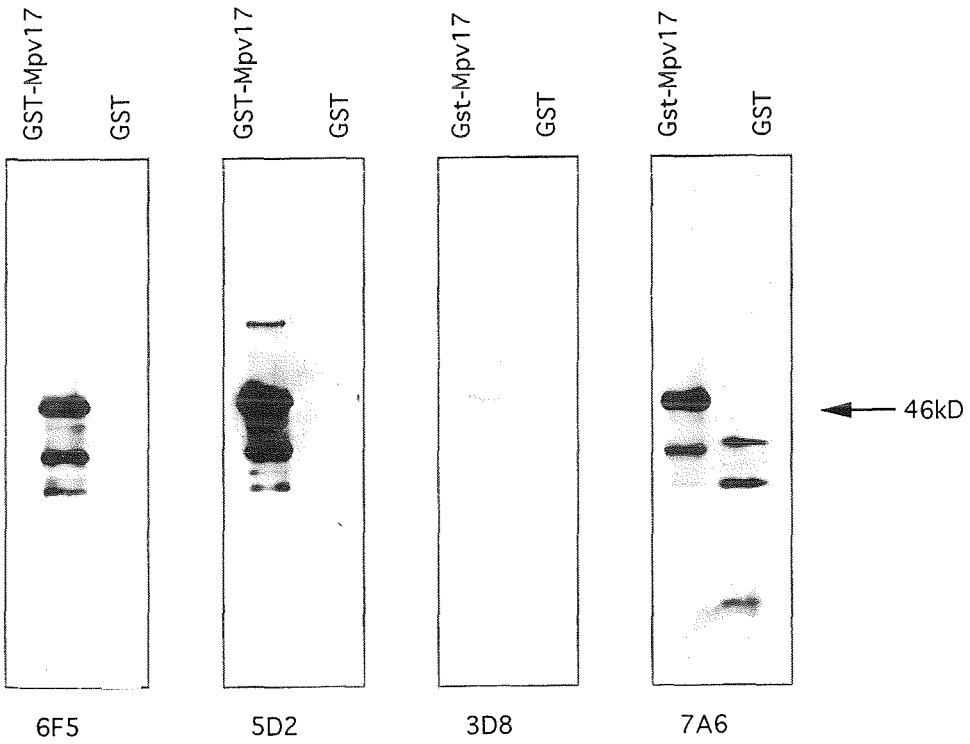


Figure 4.7 Western blots of monoclonal antibodies against 1µg of purified GST-Mpv17 and GST protein, respectively. 6F5, 5D2, and 3D8 were identified to be Mpv17 specific in ELISA-tests, while 7A6 also recognized the GST protein alone in such analysis.

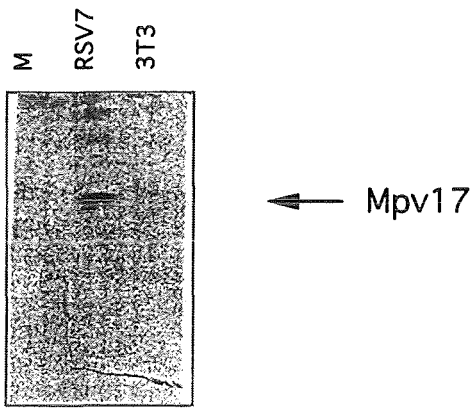


Figure 4.8 Western blot of total protein extracts from NIH3T3 cells and RSV7 cells (Zwacka et al., 1994). 3D8 recognizes the Mpv17 protein as aband of 20 kD. The increased amount of Mpv17 protein in the overexpressing cell line RSV7 is visible.

Chapter 5. Intracellular and cellular localisation of the Mpv17 gene product

The sequence homology of the rat pmp 22 and Mpv17 and the similarity between their hydrophobicity profiles gave the first hint towards a possible intracellular localisation of the Mpv17 gene product. This similarity between the two proteins led to the hypothesis that the Mpv17 gene product is a protein of the peroxisomal membrane.

Mpv17 localises to peroxisomes

NIH 3T3 cells which were shown to express Mpv17 mRNA (Figure 3.6) were subjected to cyto-immunofluorescence studies. The cells were treated with the different anti-Mpv17 antibodies ASI, anti-TrpE-Mpv17, 6F5, 5D2, 3D8. In addition, the pre-immune serum PSI as a direct control and the anti-GST antibody 7A6 were used to distinguish background from Mpv17-specific signals. In the initial experiments an intracellular punctuate pattern, consistent with a peroxisomal staining, was detected with 3D8, AS I, and with anti-Trp E-Mpv17, while the 6F5, 5D2 and as expected the PS I and 7A6 only revealed a weak overall background signal (data not shown). To corroborate the peroxisomal localisation a double label staining using a sheep anti-catalase serum, as a marker for peroxisomal localisation (Lazarow and Moser, 1989), together with the ASI and 3D8 antibodies was performed. The primary antibodies were detected by a secondary goat anti-sheep antibody conjugated to FITC-fluorescent dye and secondary anti-rabbit and anti-mouse antibodies labelled with rhodamine, respectively. The experiment revealed that the anti-catalase and the anti-Mpv17 patterns were superimposable (Figure 5.1a, b), indicating that the Mpv17 protein is indeed peroxisome-associated. Moreover, in HepG2 cells, a human hepatoma cell line which is frequently used in peroxisome studies, a similar subcellular distribution was seen (not shown). The specificity of the Mpv17 antibodies was verified using primary skin fibroblasts from homozygous Mpv17 mice and from heterozygous control animals, respectively. In an analogous double staining experiment, both anti-catalase and anti-Mpv17 antibodies showed the characteristic peroxisomal staining pattern in heterozygous cells (Figure 5.1c, d), whereas homozygous cells showed normal catalase staining (Figure 5.1f)

but lacked Mpv17 staining (Figure 5.1e). Thus, the Mpv17 antibodies specifically recognized the protein missing from homozygous Mpv17 cells. In summary, these immunofluorescence studies using various poly-and monoclonal antibodies against Mpv17 protein demonstrated colocalisation with peroxisomal markers and hence peroxisomal localisation of the Mpv17 protein. Based on hydrophobic stretches in the amino acid sequence it is suggested that the Mpv17 protein may localise to the membrane of the peroxisome, but this needs further investigation by immune electron microscopy for example.

The function of the Mpv17 gene product and any involvement of human homologue in one of the classical peroxisomal defects in man was next investigated. Interestingly, a similar degree of homology as seen for Mpv17 and pmp 22 was detected between the 70 kD peroxisomal membrane protein (Kamijo et al., 1990) and the recently cloned 69 kD adrenoleukodystrophy protein (Mosser et al., 1993), which was later shown to be also an integral membrane protein of peroxisomes (Mossser et al., 1994). They both belong to the superfamily of ATP binding cassette transporters, a fact that gave rise to the hypothesis that the two protein are possibly associated in the peroxisomal membrane. However, no evidence has been presented for this theory so far. To test for a potential association of the Mpv17 protein and the pmp 22 protein Western analysis of peroxisomal membrane fractions from homozygous Mpv17 and control mice were performed. However, no significant differences in the content of pmp 22 (data not shown) were seen. indicating that the presence of Mpv17 protein is not required for the insertion of the peroxisomal membrane protein 22 into the membrane.

Comparison of human peroxisomal disorders and the Mpv17 phenotype

In the previous section it was shown that the Mpv17 protein localises to the peroxisome. The lack of the protein leads to glomerulosclerosis and nephrotic syndrome in mice, raising questions about the nature of the link between a deficient peroxisomal function and the kidney defect. In addition, I was interested to find a human disease that is caused by a defect in the human Mpv17 gene. I therefore compared the phenotypes of several human peroxisomal disorders with the condition of the Mpv17 mouse.

Several human genetic diseases are known in which the primary defect is within the peroxisome (Lazarow and Moser, 1989). So far no animal model exists for any of them. Some of them represent defects in single biochemical pathways, while others such as Zellweger syndrome affect peroxisome biogenesis.

Peroxisomes are found in virtually all eukaryotic cells ranging from eukaryotic microorganisms to plants and animals. They were first discovered in 1954 in the proximal tubules of mouse kidneys as spherical structures (Rhodin, 1954), but were recognized as morphological and biochemical entities only 12 years later by De Duve and co-workers (Baudhuin and De Duve, 1966). At present, there are about 50 enzymes known to localise to mammalian peroxisomes covering a diverse array of functions. They are involved in anabolic functions such as the activation and β-oxidation of long chain fatty acids and their derivatives, the synthesis of ether lipids (of which the most importants ones are the so called plasmalogens), the biosynthesis of cholesterol, bile acids biosynthesis, gluconeogenesis, glyoxylate transamination. They are also involved in catabolic functions such as of purine catabolism, polyamine catabolism and finally the inactivation of reactive oxygen species such as hydrogen peroxide and superoxide anions (Lazarow and Moser, 1989).

The human peroxisomal diseases are subdivided into three classes, though often the first two groups are combined (Bioukarand and Deschatrette, 1993). The Cerebro-hepato-renal (CHR) syndrome better known as Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), infantile Refsum disease (IRD), and hyperpipecolic acidaemia (HPA) all belong to group A, described by a general loss of peroxisomal functions, due to the fact that no or only very few morphologically intact peroxisomes are present in cells from these patients. Many peroxisomal proteins are mislocalised to the cytosol while others such as the two major peroxisomal membrane proteins pmp 22 and pmp 70 are found in vesicles called peroxisomal ghosts that are suspected to be peroxisome precursors. These observations lead to the conclusion that the peroxisome biogenesis defects (PBD), a generic term for the group A disorders, are due to a structural defect and a generally impaired peroxisome assembly preventing the import of peroxisomal matrix proteins. The most prominent disorder from group A is the

Zellweger syndrome. It is a recessive genetic disease like most other peroxisomal disorders and is characterized by craniofacial dysmorphia (for example high forhead, large anterior fontanel epicanthal folds, and deformed ear lobes), neurological abnormalities (e.g. cortical dysplasia and neuronal heterotopia), impaired hearing, retinopathy, frequent cataracts, liver disease, calcific stippling of the epiphyses and, interestingly, small renal cysts. ZS is a congenital syndrome, but regressive changes can also be observed in the postnatal life mainly affecting the brain abnormalities. Patients usually die within the first few months after birth. The other three disorders of group A are milder forms, differ in their regression rate from ZS, and exhibit no glomerulocystic kidney abnormalities, reflecting the general heterogenity of peroxisomal diseases. The main biochemical characteristics of patients from group A are elevated levels of very long chain fatty acids, bile acid intermediates, pipecolic acid, phytanic acid, pristanic acid and an impairment in the biosynthesis of plasmalogens, indicating the loss of almost all peroxisomal enzymatic functions. It is assumed that the enzymes needed for the β-oxidation or cholesterol degradation, for example, are not normally compartmented, so they are subjected to rapid degradation. It is interesting to note, that the absence of peroxisomes can lead to a wide spectrum of clinical phenotypes ranging from severe ZS to the milder form of IRD.

Group B patients do possess peroxisomes, but they are deficient for a number of peroxisomal functions, thus leading to Rhizomelic chondro-dysplasia punctata (RCDP) or Zellweger-like syndrome. The most prominent disorder from this group is RCDP that is characterized by short stature, typical facial appearance, mental retardation, and biochemically by an increase in phytanic acid and deficient plasmalogen synthesis.

Group C comprises diseases which are caused by the impairment of a single peroxisomal function such as X-linked adrenoleukodystrophy (ALD), Acyl-CoA oxidase deficiency (pseudo-NALD), bifunctional protein deficiency, peroxisomal thiolase deficiency (pseudo-ZS), dihydroxyacetone phosphate acyltransferase (DHAPAT) deficiency (pseudo-RCDP), glutaryl-CoA oxidase deficiency, di- and trihydroxycholestanoic acidaemia, hyperoxaluria type I, and acatalasaemia.

X-linked adrenoleukodystrophy, which was first described by Siemerling and Creutzfeldt in 1923 and affects one out of 20 000 males with varying severity. It is the disorder that attracted most attention from the group C diseases. ALD is identified by a progressive demyelination of the central nervous system and adrenal insufficiency, while biochemically only an increase in VLCFAs can be observed. All other diseases from the last two groups have only been reported in few cases and none of them shows any kidney abnormality.

Does Mpv17 play a role in the peroxisomal biogenesis?

Since none of the described clinical pictures is similar overall to the Mpv17 phenotype, we wanted to know whether the lack of Mpv17 leads to an impaired assembly of peroxisomes in the mutant mice. In this case, the differing phenotypes in ZS patients and Mpv17 homozygotes would be due to metabolic or genetic variances between humans and mice.

So far two genes and their products have been implicated in biogenesis of peroxisomes. A 35K protein (Tsukamoto et al., 1991) was identified in complementation analyses with a rat cDNA library of the peroxisome-deficient Chinese hamster ovary (CHO) cell mutant Z65, that closely resembles fibroblasts from ZS patients. Later the highly homologous human gene was also cloned and a ZS patient (Shimozawa et al., 1992) found with mutations.in the gene. The pmp 70 gene was also identified to be mutated in two ZS patients by single strand conformational analysis (SSCP) and sequencing (Gärtner et al., 1992).

In order to elucidate the possible role of the Mpv17 gene in the peroxisome biogenesis SSCP analyses of were performed, but revealed no apparent changes in the nucleotide sequences of Mpv17 cDNA generated from the isolated RNA of ZS cells (Dr. Mika Karasawa, personal communication).

We examined the morphological integrity of peroxisomes in mutant Mpv17 mice by electron microscopy. As shown in Figure 5.2, mutant liver peroxisomes showed no structural abnormality at the level of electron microscopy. Catalase activity appeared to be present as demonstrated by staining with diaminobenzidine, indicating that at least for this enzyme the peroxisomes were not deficient or leaky. Moreover, the Mpv17 protein seemed not to influence the proliferation of peroxisomes (Figure 5.3) which could be induced in rodents by

hypolipidaemic drugs such as bezafibrate (Reddy and Lalwani, 1983). We therefore concluded that Mpv17 is not a peroxisome biogenesis factor, but rather part of a pathway localised in peroxisomes, that is important for normal kidney function in mice.

Physiological parameters in ZS and the potential role of Mpv17

In order to elucidate a possible involvement of Mpv17 in the development of one of the physiological abnormalities in ZS, the single phenotypical alterations of the disease were investigated in the Mpv17 mouse. This is important since ZS patients display a kidney phenotype that is potentially caused by one of the altered physiological parameters in these peroxisomal disorders and therefore possibly linked to Mpv17 function. We therefore tested mutant and non-mutant mice for several major markers of peroxisomal deficiencies such as the C22/C26 ratio, plasmalogen (ether lipid) synthesis, bile acid synthesis, and catalase activity in the homozygous Mpv17 mice and controls.

An increased **C26/C22 ratio** is indicative of a defective peroxisomal β-oxidation pathway of very long chain fatty acids (Lazarow and Moser, 1989). The enzymes of the peroxisomal fatty acid catabolism have a preference for very long chain fatty acids (those with chain lengths of 24 or more carbons) that cannot be degraded by mitochondrial enzymes, while smaller ones are readily oxidised in mitochondria and to a lesser extent in peroxisomes. This, however, leads in case of a defective peroxisomal system to the accumulation of C26 fatty acids relative to those with shorter C22 carbon chains and an increase in the C26/C22 ratio in serum. The accumulation of these very long chain fatty acids is also found when only single enzymes of the β-oxidation pathway downstream of the actual shortening reaction of the carbon chain, such as in thiolase deficiency, are defective due to saturation of the upstream enzymes with intermediates. The C26/C22 ratio in serum was, however, found to be the same in Mpv17 homozygotes and control mice. (Measurements were performed in the laboratory of Kinderklinik Universität Heidelberg).

Plasmalogens constitute some 5 to 20 % of the phospholipids in most mammalian cell membranes and are precursors of platelet-activating factor (PAF). The first two steps of the ether lipid synthesis that begins

with dihydroxyacetone phosphate (DHAP) are catalysed by membrane bound enzymes whose active sites face the luminal side of the peroxisomes. The subsequent reactions take place on the cytosolic side of peroxisomes and finally in the endoplasmic reticulum. ZS patients or patients deficient for ether lipid synthesis (RCDP) display markedly decreased erythrocyte plasmalogen levels and also lower levels of PAF as measured in polymorphonuclear leukocytes (van den Bosch et al., 1993). In Mpv17 mice no decrease in the level of plasmalogens could be detected. (The measurements were performed by Prof. A. Roscher, Munich).

Bile acid synthesis can also be regarded as the catabolism of cholesterol, and was considered another site of potential involvement of the Mpv17 protein, since the Mpv17 mice show an increased serum cholesterol level. Coenzyme A-conjugated bile acids, that are excreted in the bile are required for digestion and absorption of lipids in the intestine, because of their amphiphilic character and detergent-like properties. The first stage in cholesterol break-down, the rate limiting step in bile acid synthesis, is 7α-hydroxylation. It is catalysed by a P-450 cytochrome that is located in the endoplasmic reticulum, where all other steps of the cholesterol catabolism down to 3α, 7α-dihydroxy-5β-cholestanoic acid (DHCA) and 3α, 7α, 12α-trihydroxy-5β-cholestanoic acid (THCA) also occur. The highest capacity for the subsequent oxidation of the side chains of these two compounds and the formation of the bile acids cholic acid and chenodeoxycholic acid was, however, shown to reside within the peroxisomal fraction (Pederson and Gustafson, 1980).

Hence, one could suggest that the lack of Mpv17 may interfere with these last steps in the degradation of cholesterol to bile acids, resulting finally in elevated serum cholesterol levels or the accumulation of bile acid intermediates that have the same deleterious effects as cholesterol.

In this context it is interesting to note that some research groups have reported that hypercholesterolemia is at least an aggravating factor in the progression of initial glomerular injury to glomerulosclerosis (reviewed in Diamond and Karnovsky, 1992). Animal experiments even suggest a causative role of high cholesterol levels for morphologic changes in the aorta and kidney. Normal laboratory rats fed diets supplemented with 3% cholesterol or cholic acid developed

hypercholesteremia within 4 weeks that was accompanied by segmental and/or global glomerulosclerosis (Peric-Golia and Peric-Golia, 1983). We therefore measured whether we could detect any accumulation of intermediates of bile acid synthesis in the gall bladder contents from homozygous Mpv17 mice in comparison to wild type mice by HPLC. However, no differences in the distribution of bile acid precursors could be detected, indicating a fully functional bile acid synthesis in the Mpv17 homozygous mice. (Measurements were performed by Prof. Stiehl, Heidelberg).

An alternative way to explain the high serum cholesterol level in the Mpv17 mice would be increased biosynthesis of cholesterol. It has been found that the rate limiting enzyme of this pathway is located in peroxisomes and that the organelle accounts for about 30% of total de novo synthesis of cholesterol (Keller et al., 1986). Deregulation of this mechanism in the Mpv17 mouse, however, has not yet been looked at.

Catalase activity was found to be bound to peroxisomes in Mpv17 mice (Figure 5.2) as mentioned above revealing a seemingly intact machinery for the decomposition of H_2O_2.

In summary no abnormalities concerning the peroxisomal functions oxidation of very long chain fatty acids, plasmalogen generation, bile acid biosynthesis, and catalase function were found in the Mpv17 homozygous mouse.

After measuring the major physiological parameters of peroxisomal functions we assume that the Mpv17 protein has a yet unidentified peroxisomal function. Alternatively, a defect in Mpv17 expression may result in different biochemical phenotypes in mice and man. It can also not be ruled out, that the Mpv17 gene has no important function in humans, and that the Mpv17 gene product fulfils a function in a mouse specific metabolic pathway. A precedent for a specific rodent-specific peroxisomal function is the enzyme urate oxidase which converts uric acid to allantoin. In humans this enzyme is missing and the consequence is the susceptibility of this species to urate deposites leading to gout. In rodents, urate oxidase is a major constituent of the peroxisomal paracristalline core readily detected by electron microscopy in the dense region of the organelle´ s matrix. An animal model for gout has been developed by disruption of the urate oxidase gene in mice, rendering the mutated animals susceptible to this disease (Wu et al., 1994). These mice

developed hyperuricemia and renal stones resembling the human condition, although in mice the onset of the urate nephropathy occured much earlier and was more severe. This finding indicates that urate oxidase plays a vital role in the clearance of uric acid in mice, while most humans do not normally develop gout except in conjunction with other unknown factors. The differences between this phenotype and the Mpv17 defect also makes a direct involvement of Mpv17 in this pathway unlikely.

The minor functions of peroxisomes are not likely candidates for an involvement of Mpv17 since the peroxisomal disorders from group B and C that have a single or several impaired peroxisomal functions share no phenotypical similarities with the Mpv17 disease (Lazarow and Moser, 1989).

As stated at the beginning of this chapter renal cysts were observed in most of the ZS patients who were studied pathologically. These cysts vary from glomerular microcysts to large cortical cysts of glomerular and tubular origin (Lazarow and Moser, 1989). Athough the Mpv17 and ZS renal phenotypes are certainly not identical, one could suggest that a potential loss of Mpv17 function in Zellweger patients is due to the mislocation and degradation of the Mpv17 protein could lead to the kidney phenotype, and that the young age, these patient die at, prevents the onset of the glomerulosclerosis. However, in order to address this question, it is neccessary to elucidate the precise function of the Mpv17 gene product and then to check for an impairment of this function in ZS.

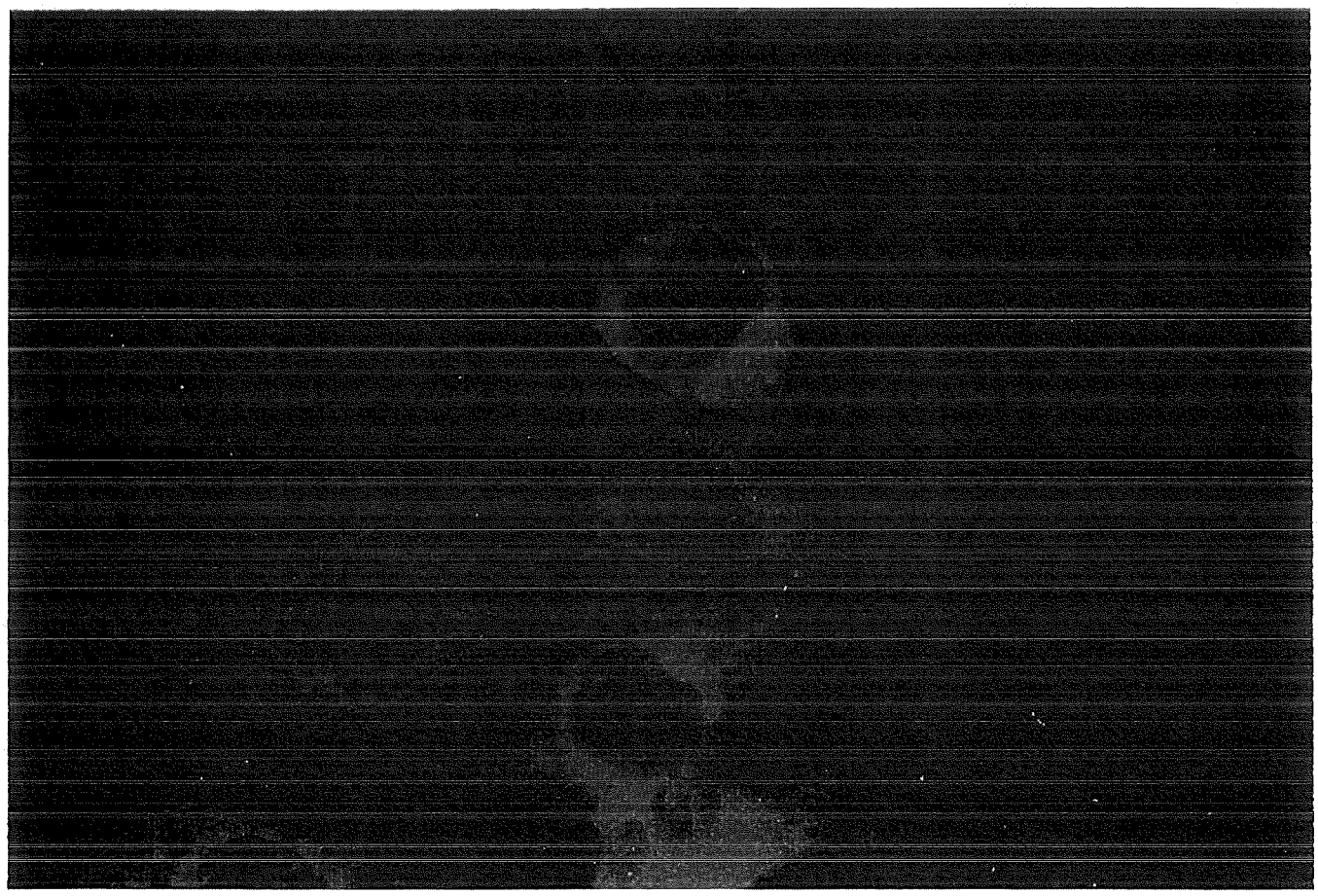
Cellular localisation of the Mpv17 protein in the kidney

Northern blots of various mouse tissue RNAs (Weiher et al., 1990) and in situ analysis (Mika Karasawa, unpublished results) has revealed a ubiquitous expression of Mpv17. These results raised the question of whether the renal phenotype in Mpv17 homozygous mice is a defect originating in the kidney or glomerulus itself, or is a secondary effect caused by a different impaired metabolic function. Since no other apparent phenotypic alterations in organs other than the kidney were observed, it is possible that the glomerulosclerosis is due to an initial renal dysfunction. The puzzle, however, of how the loss of a widely expressed gene can cause such a single-organ phenotype, remained. It

could finally not be ruled out that other tissues were affected in a more subtle manner, thereby escaping detection.
In order to elucidate the sites of Mpv17 expression in the kidney we used the anti-Mpv17 monoclonal antibody 3D8 on a normal human kidney section. It revealed expression in the glomerular epithelial cells (Figure 5.4), which have a critical role in the development of glomerulosclerosis. Peroxisomes were also reported to be present in these cells (Zaar, 1992 and references therein) which is in accordance with the intracellular localisation of the Mpv17 protein. A lack of the Mpv17 gene product could lead to an altered cell metabolism and cell integrity. Kerjaschki (1994) summarises the cellular events required for the morphological changes which manifest in the fusion of the podocytes´ foot processes, that were studied in rat glomeruli treated with a polycationic solution. These included a complex pattern of phosphorylation of intracellular proteins (Kurihara et al., 1992) and the need for a supply of energy and of calcium (Kerjaschki, 1978). An involvement of Mpv17 in these processes is possible, but remains to be shown.

Potential redox function of Mpv17

Highly reactive oxygen species (ROS), potentially inflicting damage on proteins and leading to lipid peroxidation, have been shown to be associated with glomerulosclerosis (Wardle, 1992). Since peroxisomes are a major site of production and decomposition of such radicals we addressed a potential involvement of Mpv17 in these processes.
Though catalase has been shown to be bound to peroxisomes in Mpv17 mice (Figure 4.3), we have so far not demonstrated this for the peroxisomal Cu, Zn superoxide dismutase. Alternatively, Mpv17 could be involved in a pathway that generates ROS, giving rise to a change in the cellular redox state.



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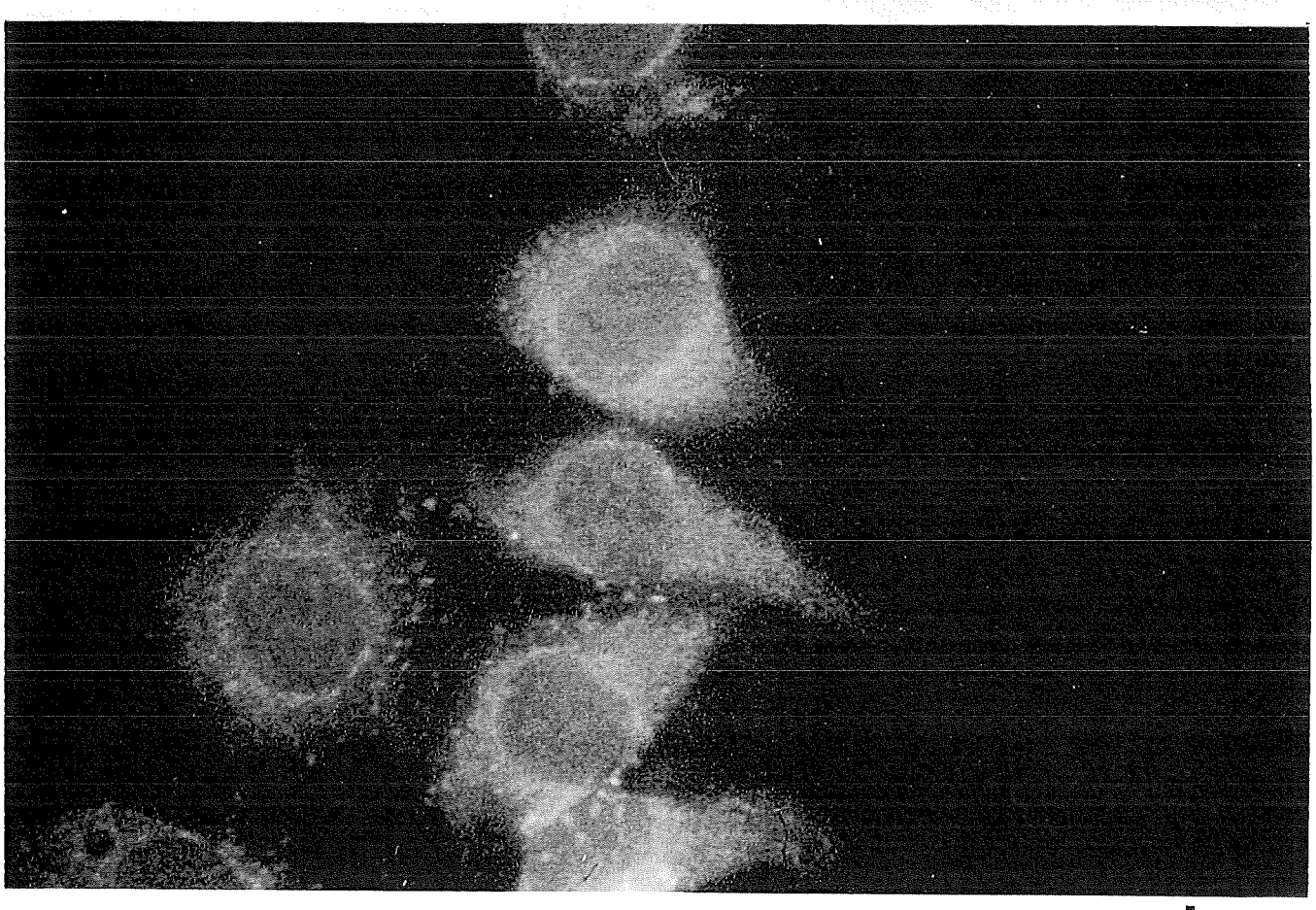

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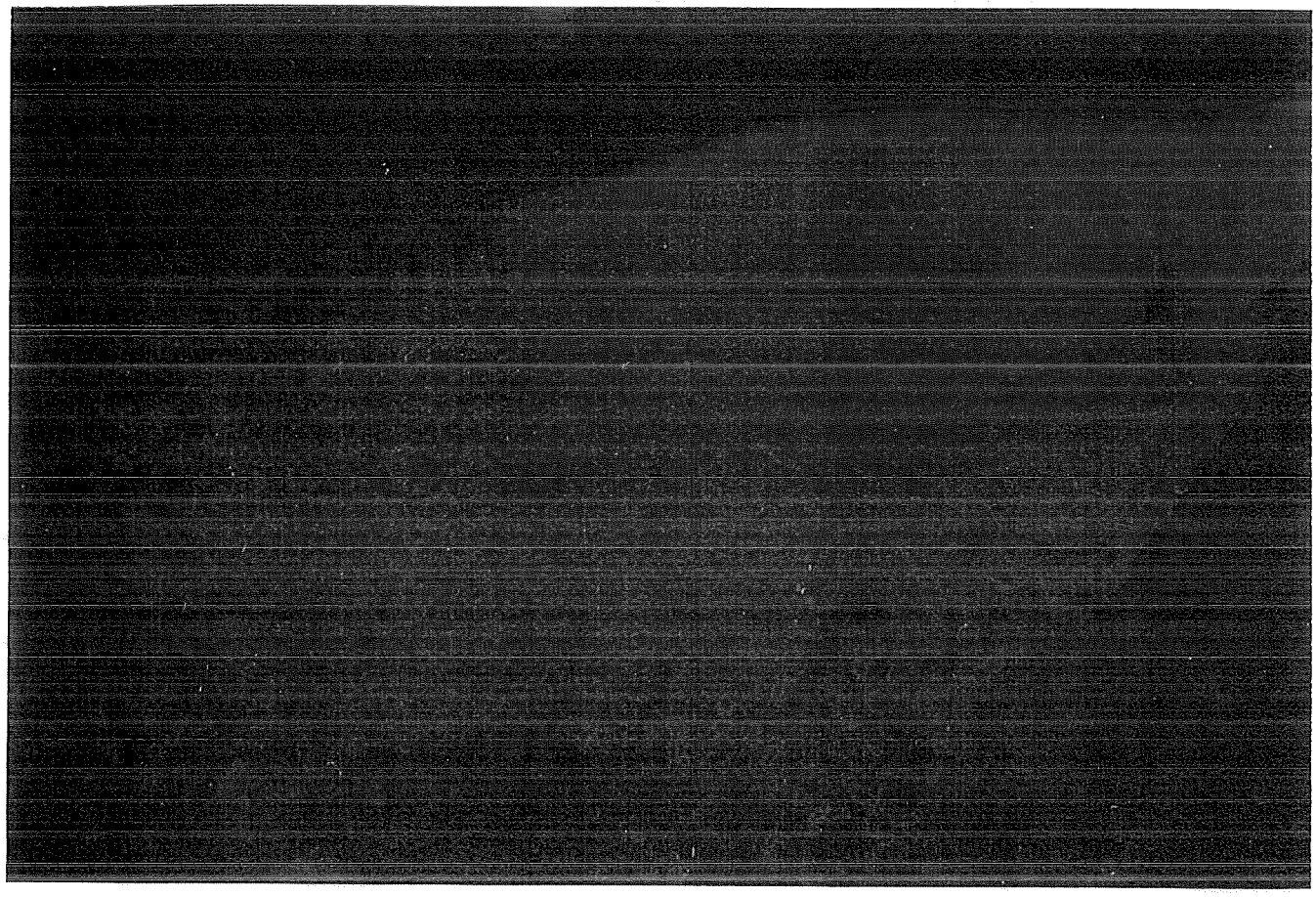

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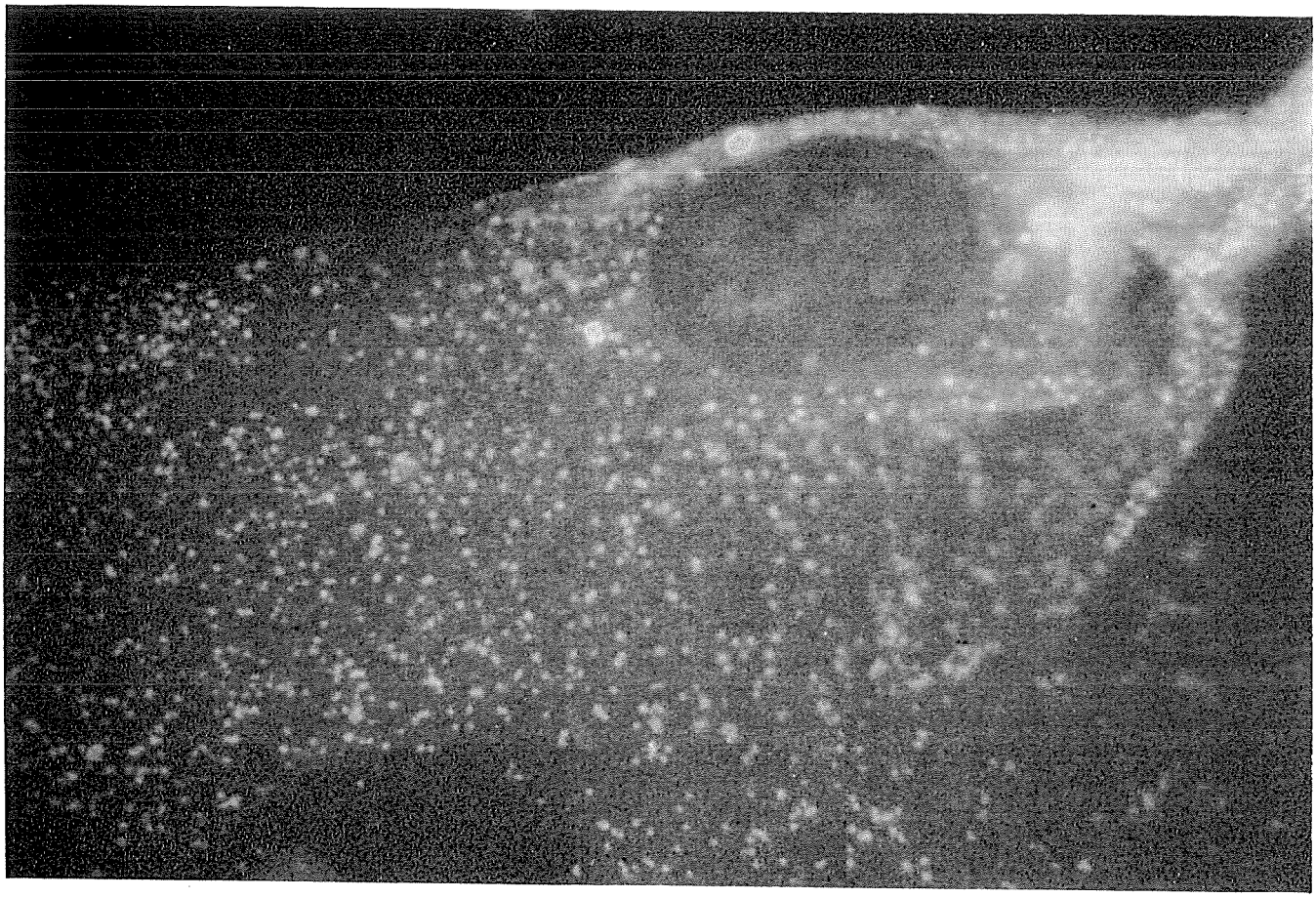

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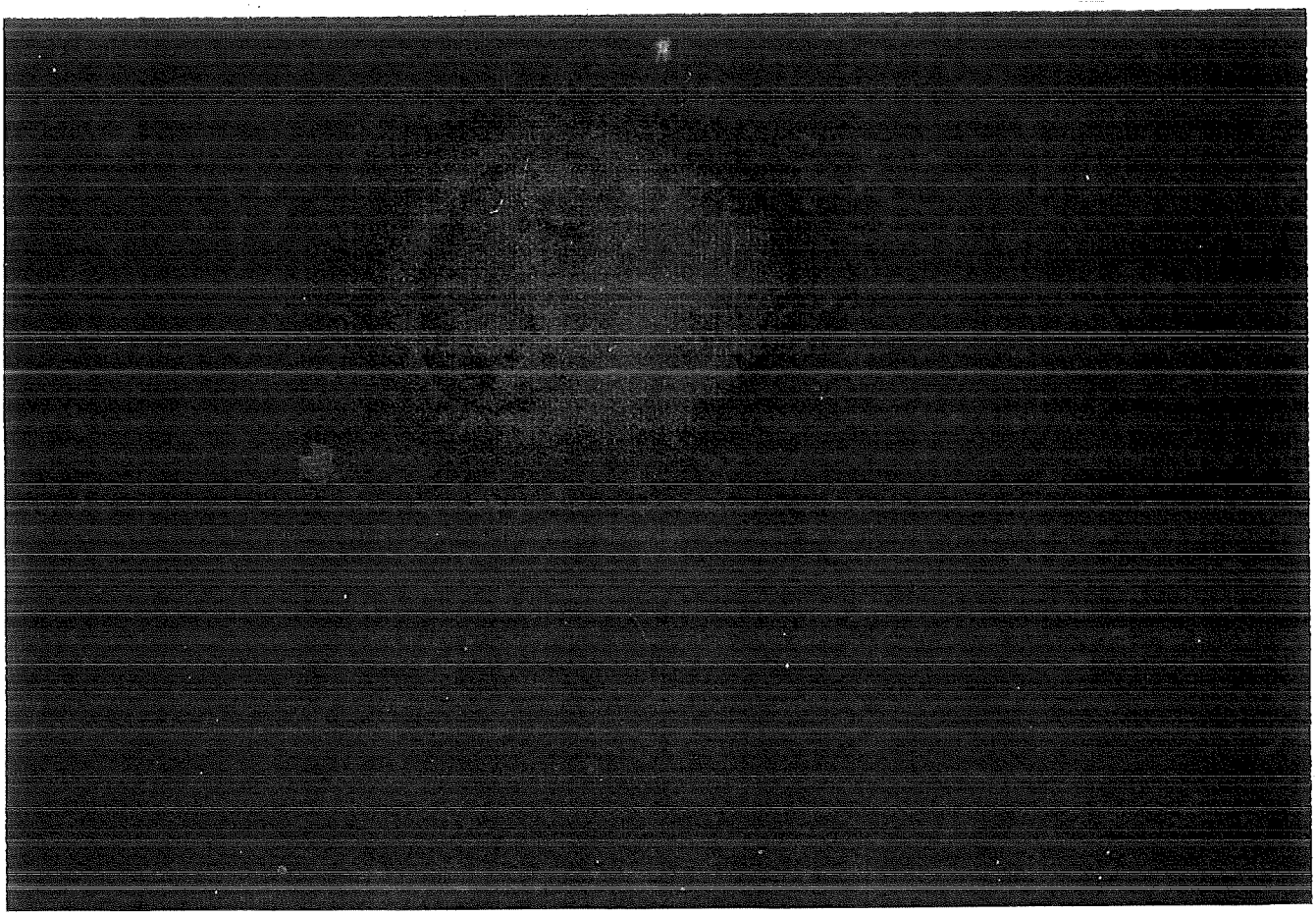

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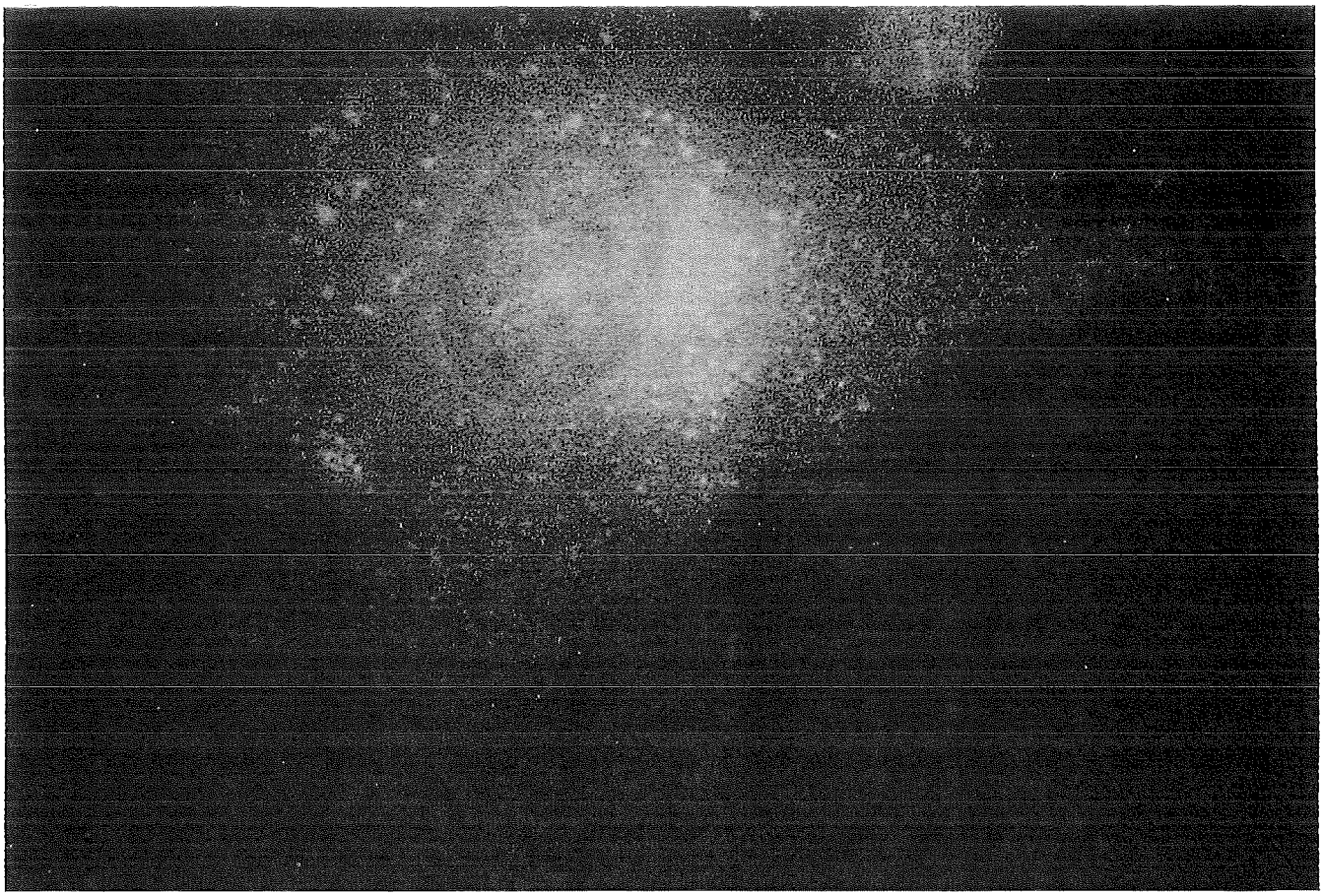

f

Figure 5.1 Colocalisation of Mpv17 protein and catalase in peroxisomes. Immunofluorescence studies were performed on murine fibroblasts: 3T3 cells (**a, b**), primary fibroblasts from Mpv17 heterozygous (**c, d**) and homozygous (**e, f**) mice were analysed. Mpv17 protein was visualized by a sheep anti GST-Mpv17 antiserum (**a**) and a monoclonal anti Mpv17 antibody (**c, e**) decorated with TRITC labelled secondary antibodies. Catalase was stained by a rabbit anti-bovine-catalase antibody, which was decorated with a FITC labelled goat anti-sheep IgG antibody (**b, d, f**).

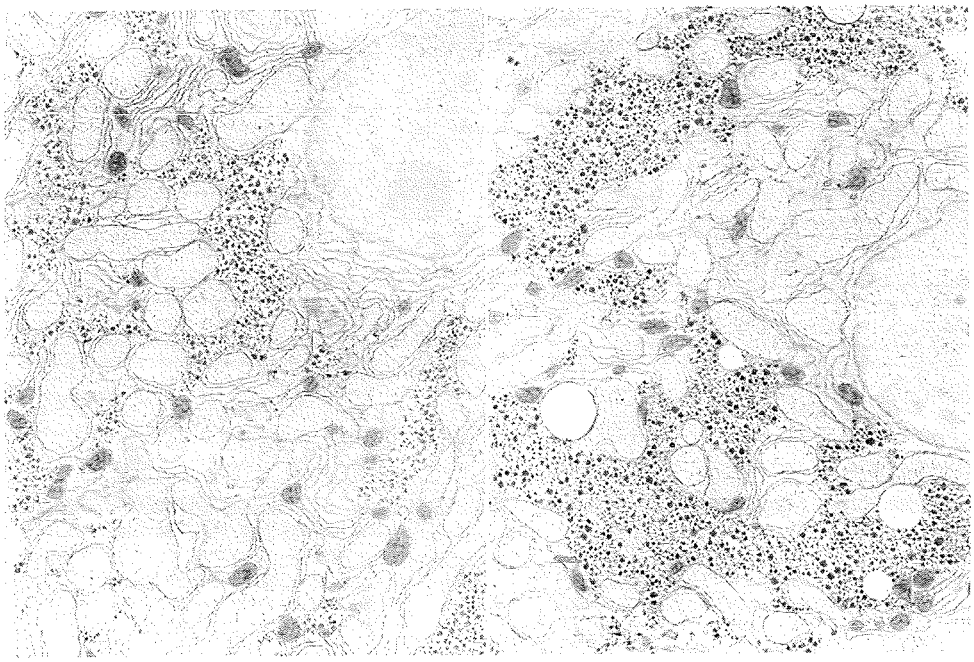

Figure 5.2 Electron microscopy of periportal hepatocytes from a 45 day old Mpv17 mouse (left) and a control mouse (right). The peroxisomes are stained by diaminobenzidine due to their catalase activity (Gorgas, 1985). Magnification : 11000 fold. (Kindly provided by Prof.Karin Gorgas, Heidelberg).

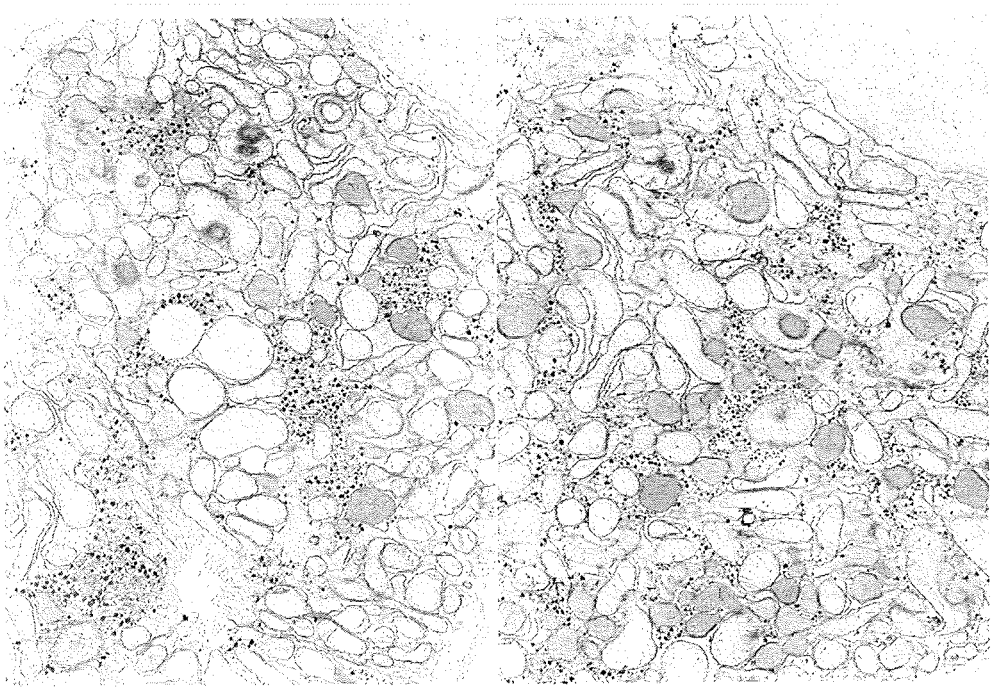

Figure 5.3 Electron microscopy of periportal hepatocytes from an Mpv17 mouse (left) and a control mouse (right) treated with bezafibrate (kindly provided by Boehringer, Mannheim), a hypolipidemic drug that gives rise to an increase in the number of peroxisomes in rodents. The daily dosis for two weeks of bezafibrate, applied orally, was 10mg/kg/bodyweight. (The electron micrographs were kindly provided by Prof.Karin Gorgas, Heidelberg).

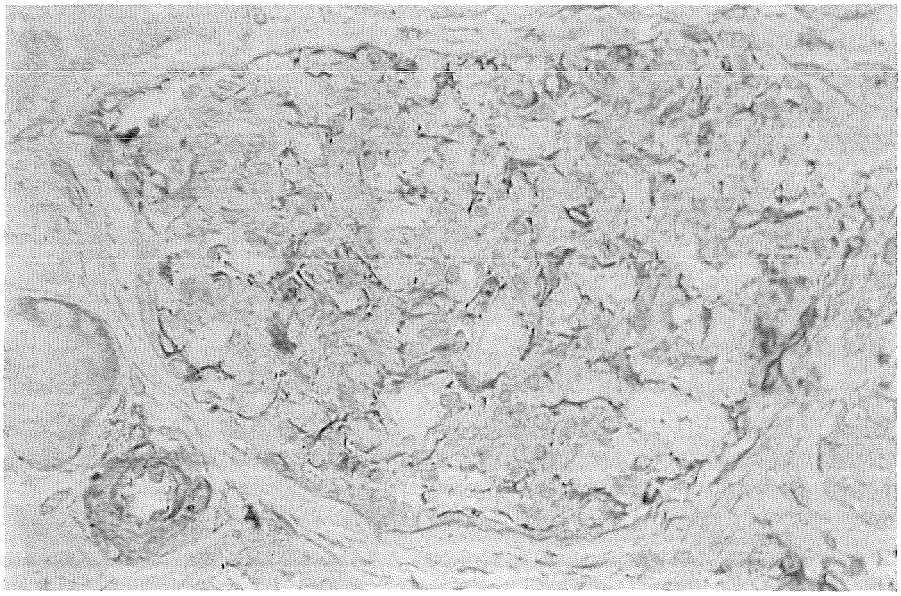

Figure 5.4 Immunohistochemistry of a frozen section from a normal human kidney using the anti-Mpv17 3D8 antibody. A glomerulus is depicted, in which the podocytes are stained by the 3D8 antibody. The primary 3D8 is decorated by a peroxidase-conjugated anti-mouse antidody revealing a brown colour. The cell nuclei are counter stained in blue with hematoxilin.

expression is the first step in the development of glomerulosclerosis in this animal model.

Although an elevated MMP-2 protein expression could be detected in homozygous mice, it is not clear whether it reflects the overall MMP-2 proteinase-activity, as MMPs are subjected to postranslational regulation on different levels. In particular, the regulation by TIMP-2 that is known to bind and supposed to inhibit MMP-2, has not been investigated in our system yet. Nevertheless, these results reveal new insights into the putative role of MMP-2 in human glomerular injury.

Chapter 6. Function of the Mpv17 protein

After we could not detect any abnormalities in the major peroxisomal functions in the Mpv17 mouse such as oxidation of very long chain fatty acids, plasmalogen synthesis, cholesterol metabolism, and catalase activity, the role of the peroxisomal protein Mpv17 in the generation and detoxification of reactive oxygen species was investigated. This was prompted by experimental evidence of an association of renal defects and extra- and intracelluar redox status (Wardle, 1992). Furthermore, a link between Ros mediated cellular injury and kidney disease suggetsed by a report of a mouse mutant in which the bcl-2 gene has been inactivated by insertion (Veis et al., 1993). Bcl-2 protein, an inhibitor of apoptosis, has been shown to play a role in protection against H_2O_2 and lipid peroxidation (Hockenbery et al., 1993). Interestingly, the null mutation for bcl-2 in mice also exhibits a phenotype in the kidney. Although the pathology of kidney disease in these mice is different from that in the Mpv17 mice, this suggests that proper function of this organ critically depends on an intricate regulation of radical metabolism.

ROS and peroxisomes

Peroxisomes possess a variety of enzymes (oxidases) that reduce O_2 to H_2O_2. Hydrogen peroxide is a toxic compound and needs to be decomposed. Catalase converts two molecules of H_2O_2 into two molecules of H_2O and one molecule O_2. Various oxidases also decompose H_2O_2 via another pathway. In the latter case one molecule H_2O_2 gives rise to two molecules of H_2O using ethanol, methanol, formaldehyde, formic acid, or nitrite ions as the hydrogen donor instead of a second hydrogen peroxide (de Duve et al., 1966). More recent experiments (Keller et al., 1991; Wanders et al., 1992; Dhaunsi et al., 1992) have shown the presence of Cu, Zn superoxide dismutase in human and also rat peroxisomes. Superoxide dismutase converts two superoxide anions (O_2^-) into one molecule of H_2O_2 and one molecule of O_2. The highly reactive superoxide anion is a by-product of oxygen reduction in mitochondria and endoplasmic reticulum (Cross and Jones, 1991). A peroxisomal source for O_2^- is the reaction from xanthine to urate catalysed by xanthine oxidase (XO). With the presence of superoxide dismutase, peroxisomes host an effective defense mechanism

against superoxide. It is therefore likely that other sources of peroxisomal superoxide production exist that remain to be identified (Mannaerts and Veldhoven, 1993).

ROS and kidney disease

The capacity of peroxisomes to produce and decompose radicals is interesting in the context of kidney defects, since numerous studies (reviewed in Nath et al., 1989; Shah et al., 1991) imply an important role for reactive oxygen species in mediating injury in several models for renal disease. These include glycerol induced injury (Guidet and Shah, 1989), ischemia-reperfusion injury (McCoy et al., 1988; Nath et al., 1990), gentamicin nephropathy (Walker and Shah 1987), and neutrophil-dependent glomerular injury (Shah et al., 1987; Yoshioka and Ichikawa, 1989). Hydrogen peroxide as such is also toxic, when administered to the intact kidney (Yoshioka et al., 1990) or the isolated perfused kidney (Linas et al., 1987), and inflicts cellular injury at multiple cellular sites either directly or through the generation of more reactive intermediates (Grisham et al., 1986). Moreover, it was found that substances capable of reducing H_2O_2 to water (e.g. pyruvate) protect against renal injury (Salahudeen et al., 1991). The scavenging effects could be shown in vitro by attenuation of H_2O_2-injury to cultured kidney cells and in vivo models by limiting renal damage, indicated by reduction of proteinuria (Salahudeen et al., 1991). Most interestingly, Neale et al. (1993) could identify visceral glomerular epithelial cells as the site of ROS production in an induced rat model for the human nephropathy Passive Heymann Nephritis (PHN).

PHN is a membranous glomerulonephritis similar to minimal change disease and therefore resembles the Mpv17 phenotype to some extent. By immuno-histochemical examination of kidneys from rats with induced PHN and control animals, cytochrome b_{558}, a part of the non-mitochondrial multicomponent NADPH-oxidase enzyme complex, was detected mainly in glomerular epithelial cells and to a lesser extent in mesangial and endothelial cells. The NADPH-oxidase complex is a superoxide generating system that was first discovered in neutrophils and found to be involved in the respiratory burst, which is a response to microbial challenge. However, the enzyme had been detected in many other cell types such as human mesangial cells (Radeke et al., 1991) and

fibroblasts (Meier et al., 1989). In the PHN-rats, the level of the cytochrome b_{558} representing the NADPH oxidase system was found to be markedly elevated in the podocytes, indicating a potential increase in the production of ROS by these cells. This could establish the initial glomerular injury leading to the kidney disease. In the glomeruli of proteinuric PHN rats increased amounts of hydrogen peroxide could indeed be detected by the histochemical cerium precipitation method particularly in the GBM (Neale et al., 1993). These results strongly suggest, that the activation of the NADPH oxidase is the reason for the increase in hydrogen peroxides which are easily produced from the initially generated superoxide by dismutation. However, the molecular mechanisms that lead from elevated ROS levels to proteinuria and the morphological changes of the capillary wall remain to be determined.

ROS and Mpv17

It is intriguing that peroxisomes are a site of production and detoxification of O_2^- radicals and peroxides and that the lack of the peroxisomal protein Mpv17 in mice leads to nephrotic syndrome, which has been shown to be associated with deregulated ROS metabolism. In order to analyse a potential role of Mpv17 in this phenomenon, we investigated mutant and normal cells for intermediates of these pathways. To study the production of ROS in mutant Mpv17 cells we derived primary skin fibroblasts from mutant and non mutant mice and tested them for the ability to produce reactive oxygen.

Cells were loaded with hydroethidine which reacts with intracellular O_2^- radicals and other ROS to form the fluorescent dye ethidium (Rothe and Valet, 1990). The production of intracellular ROS was then measured by FACS analysis. Surprisingly, the result was in contrast to our expectations. While normal mouse primary fibroblasts showed a single peak of labelled cells, cells from homozygous mutant animals displayed a second peak of fluorescence at low intensity (Figure 6.1a, b). This second peak is coincident with that obtained with nonlabelled cells assayed at the same wavelength (data not shown). This indicates that at least in a subpopulation of the primary fibroblasts, lack of Mpv17 expression leads to a complete failure to produce ROS, while in the other subpopulation different mechanisms of ROS production may be in operation.

In order to investigate, if the lack of Mpv17 expression simply interferes with a pathway involved in ROS production or if the Mpv17 protein by itself has a ROS producing activity, we studied ROS production in cells overexpressing the Mpv17 gene product. Thus, 3T3 cells were transfected with a construct constitutively overexpressing high levels of Mpv17 protein under the control of the Rous sarcoma virus (RSV) promoter (RSV1 and RSV7 cells; Zwacka et al. 1994) Untransfected 3T3 cells expressed only very low levels of Mpv17 protein (not shown) and displayed a distribution in which a majority of cells were unlabelled and only a minority of cells were labelled (Figure 6.1c). In contrast, in several independent RSVMpv17 transfectant clones expressing high levels of Mpv17 protein, when grown only one peak representing labelled cells was seen. This is shown for one clone in Figure 6.1d. These results indicate that the Mpv17 gene product itself constitutes a major ROS producing activity in the cell or at least accomplishes a rate limiting step in this process. In summary, the results uncover an new peroxisomal function, involved in the production of ROS, the absence of which intriguingly causes glomerulosclerosis in mice.

Whether these results reflect the in vivo situation in the glomerular cells has yet to be verified. Therefore in situ studies are currently underway in which the redox state of kidney cells in Mpv17 mice, as compared to control mice, will be examined. As Mpv17 protein is mainly found in podocytes it is possible that they are the site where the defect in ROS production is manifested. This raises the question of how the reduced level of reactive oxygen species in glomerular epithelial cells could interfere with the normal function of the kidney and eventually lead to glomerulosclerosis.

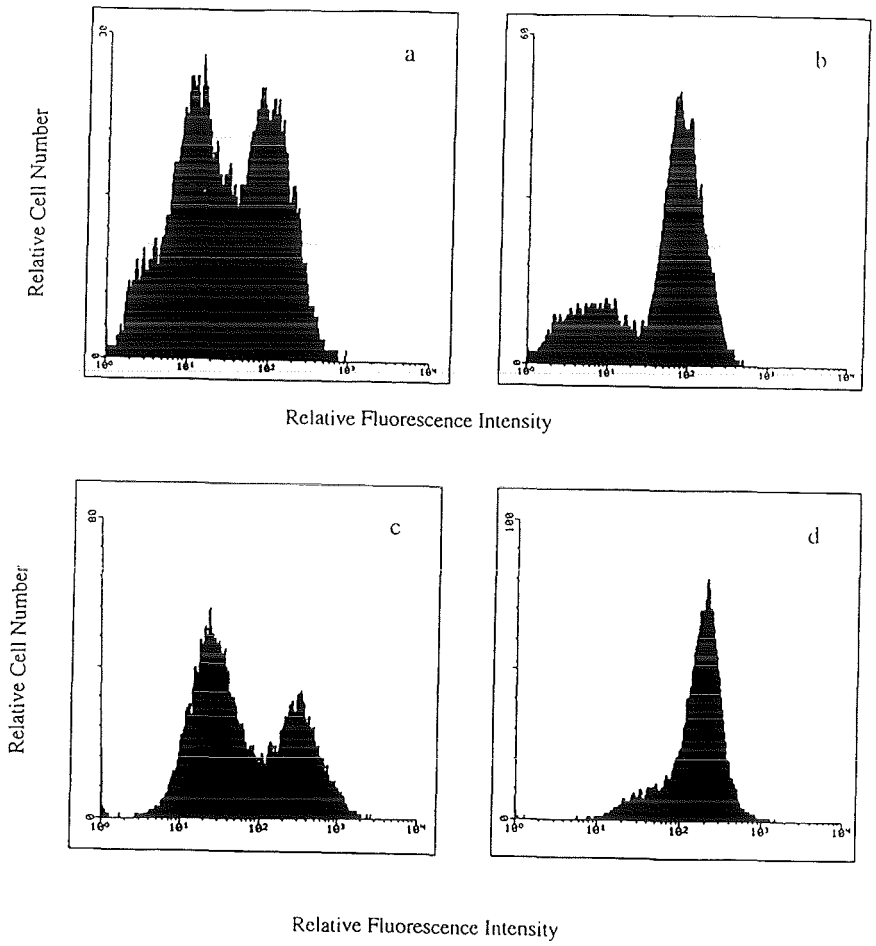

Figure.6.1 Reactive oxygen species produced in primary skin fibroblasts from Mpv17 and control mice. Hydroethidine (HE) histograms each representing 10000 cells after HE staining and analysis by FACS. **a**: Mpv17 homozygous primary fibroblasts, **b**: Mpv17 heterozygous primary fibroblasts **c**: Mouse NIH 3T3 fibroblasts. **d**: RSV7 cells (NIH 3T3 cells transfected with a gene construct expressing the Mpv17 protein of a RSV promoter)

Chapter 7. Implications of Mpv17 in gene regulation.

The Mpv17 protein is involved in the production of ROS and the lack of Mpv17 leads to an intracellular change in the redox potential. So far we have no evidence for a role for increased radicals in glomeruli in Mpv17 mice that could exert direct damage onto the glomerular basement membrane and the cellular components of the glomerulus. In contrast, we found a decreased level of ROS in cells from homozygous animals. We therefore suspected that the changed intracellular redox state which we observed influences the expression of genes important in the formation or the turnover of the GBM.

Podocytes, the major site of expression of Mpv17 in the glomerulus (Figure 5.4) are known to synthesise the components that comprise the glomerular basement membrane such as the different chains of type IV collagen and laminin, as well as the basement membrane heparan sulfate proteoglycan core protein (Glassock et al., 1991). It has been shown that the production of these substrates is significantly increased in glomerulosclerosis (Nakamura et al., 1991; Floege et al., 1991), and is regulated at the transcriptional level (Munaut et al., 1992). In addition, transforming growth factor-β (TGF-β) has been shown to play a role in the development of glomerular defects by regulating the expression of constituents of the GBM, the enzymes involved in matrix degradation and their inhibitors (Border et al., 1993). Moreover, Imai et al. (1993) found glomerular lesions after directing the expression of TGF-β via liposome-mediated gene tranfer to the kidneys of normal laboratory rats. This indicates a direct influence of the growth factor TGF-β on the pathogenesis of glomerular injury. In this particular experimental model the cell staining for type I and III collagen was elevated while the production of type IV collagen was only slightly increased. The increase in ECM deposition is, however, not the only feature and not likely to be the initial event in glomerulosclerosis. Other changes also contribute to the disease, which are more likely to be involved in the primary glomerular defect.

The phenotype of Alport syndrome suggests that events disturbing the integrity of GBM are required to lead to the leakage of macromolecules and blood cells. In Alport syndrome, the mutated collagen IV chains results in disintegration of the basement membrane that allows even

whole erythrocytes to pass through into the glomerular filtrate. More subtle damage to the GBM, though not leading to hematuria, could however result in proteinuria. Enzymes that are involved in the turnover of the membrane and which are able to degrade the single components could give rise to such injuries. This would be possible if their activity was increased beyond the normal levels due to a glomerulus-specific loss of transcriptional and post-translational regulation. The enzymes that are involved in the initial steps of ECM degradation are Matrix metalloproteinases (MMPs). The MMPs are a group of zinc endopeptidases secreted from a variety of cell types as proenzymes, that are activated by cleavage of a propeptide by other proteinases. They are considered to play a central role in extracellular matrix breakdown in various pathological conditions, as well as in normal tissue remodelling and resorption. Many of these enzymes have been shown to be capable of degrading several of the GBM´ s major components. MMP-2 also known as gelatinase A or collagenase IV 72 kD, can readily digest all of these constituents (Murphy et al., 1991) and has also been shown to be expressed in metastasising tumor cells (Brown et al., 1990), indicating that it might be involved in the active proteolytic degradation of the matrix for cell invasion. Moreover, an enhanced expression of MMP-2 was found in puromycin aminonucleoside (PAN) nephrosis, a model for nephrotic syndrome progressing to glomerulosclerosis (Nakamura et al., 1994). In addition, a GBM-degrading metalloproteinase was demonstrated to be secreted by glomerular epithelial cells (Johnson et al., 1992). I therefore started to investigate the possibility that the redox state regulated by Mpv17 is involved in the expression of MMP-2, which could constitute the intial glomerular lesion. This could in return cause the activation and release of growth factors and the accumulation of ECM, which is seen in glomerulosclerosis.

Normal NIH 3T3 cells and those overexpressing the human Mpv17 gene under the control of the RSV promoter (RSV1 and RSV7, Zwacka et al., 1994) were grown to semi-confluency and RNA isolated. Figure 7.1 depicts the Northern analysis of these RNAs probed with the Mpv17 cDNA and an MMP-2 fragment. It reveals a striking reciprocal correlation between the Mpv17 expression and the amount of collagenase IV transcript. RSV1 and RSV7 cells with a constitutively

high level of Mpv17 RNA have no detectable collagenase IV expression, while in 3T3 cells, exhibiting varying degrees of Mpv17 expression for so far unknown reasons, the amount of collagenase varied inversly to the Mpv17 transcript. This suggests that the changes in the redox status, due to Mpv17 expression, result in modulation of collagenase IV transcription or in a faster, selective degradation of its mRNA. In order to demonstrate the involvement of ROS in this process I treated skin fibroblasts from Mpv17 homozygous mice that lack Mpv17 expression and show high collagenase expression, with H_2O_2 according to a protocol outlined in Keyse and Tyrell (1989), RNA was prepared and analysed by Northern blot. (Figure 7.2) depicts that the level of collagenase IV mRNA is indeed reduced by around 50% 2h and 6h after treatment with H_2O_2, potentially mimicking the effect of Mpv17, while 10h later the level of the control RNA, taken instantly after the H_2O_2 treatment, is almost reached again. These results disclose a new link between the intracellular redox potential and the modulation of MMP expression, which is potentially implicated in glomerular disease. (In order to further demonstrate a causal role of Mpv17 in the repression of MMP-2 transcription, homozygous cells are currently being transfected with the RSV-Mpv17 construct). Interestingly, Kohn et al. (1994) have reported that different cell lines exhibit a reduced MMP-2 activity when calcium influx was inhibited, suggesting a role of the protein kinase C (PKC) second messenger signalling pathway in the production of MMP-2. Furthermore, treatment of cells with ROS producing systems (xanthine/xanthine oxidase) resulted in the activation of PKC (Larsson and Cerutti, 1989), establishing a connection between the oxidative status of a cell and reduction in MMP-2, as seen in the Mpv17 overexpressing cells.

Since variations at the transcriptional level do not always reflect actual change in protein activity, and molecular events in cultured fibroblasts cannot just be transferred to the situation in the different cells in the glomerulus, we used an antibody against MMP-2 (kindly provided by Matti Höyhtyä) to check kidney sections from control and Mpv17 homozygotes (Figure 7.3) for changes in the collagenase IV protein level by immunohistochemistry. Figure 7.3 shows that MMP-2 is increased in the glomeruli of the Mpv17 mice. Even if this increase is not dramatic, it supports the hypothesis that enhanced MMP-2

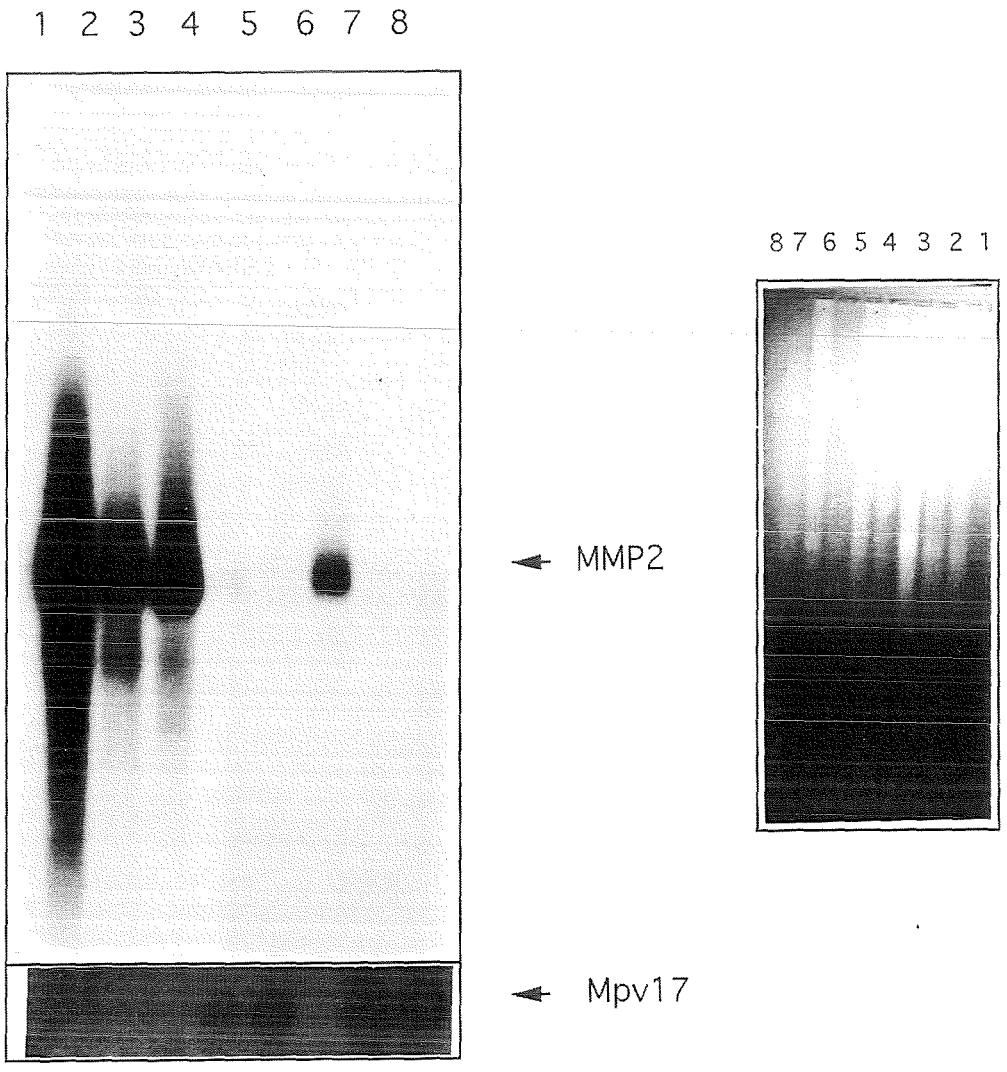

Figure 7.1 Northern blot of fibroblasts expressing reciprocal amounts of Mpv17 and MMP-2 transcripts. Lane 1-3: Poly A^+ RNA from three different immortalised skin fibroblast lines from Mpv17 homozygous mice. Lane 4-6: Poly A^+ RNA from NIH3T3 cells expressing varying amounts of the Mpv17 transcript. Lane 7 and 8: Poly A^+ RNA from RSV1 and RSV7 cells (Zwacka et al., 1994).

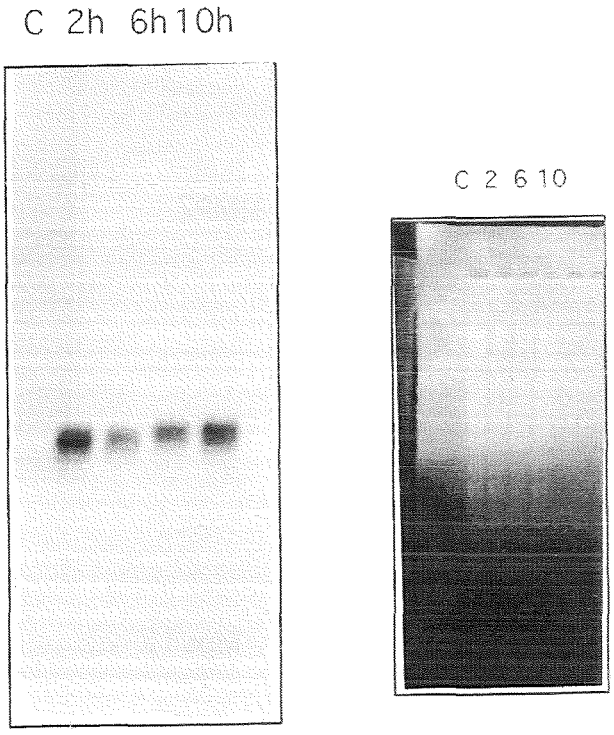

Figure 7.2 Northern Blot of immortilized skin fibroblasts from Mpv17 homozygous animals treated with H_2O_2. The expression of MMP-2 is shown at several time points after the treatment of the cells with H_2O_2 (C=0 minutes).

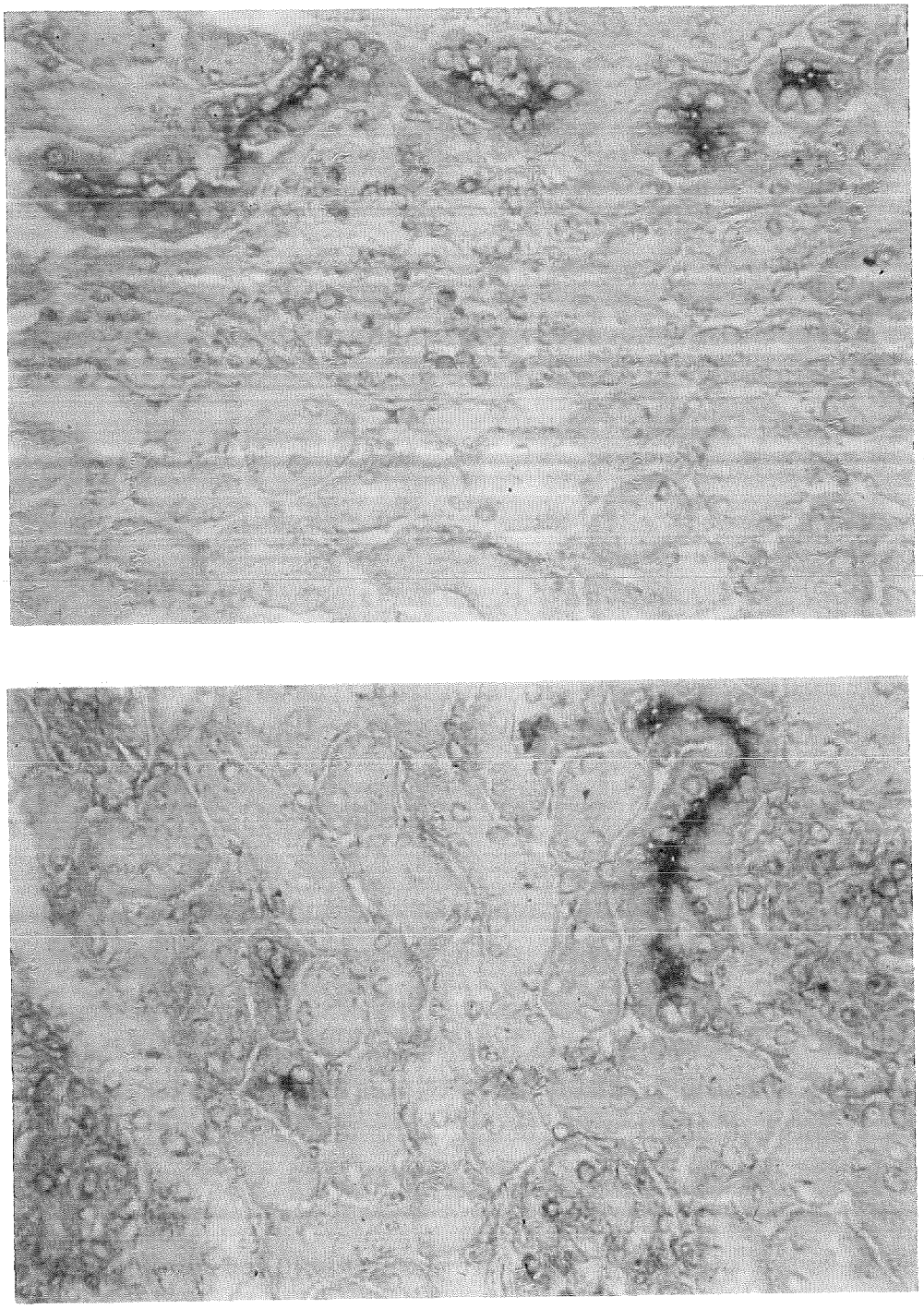

Figure 7.3 Immunohistochemistry of frozen sections from a heterozygous Mpv17 mouse (top) and a homozygous Mpv17 animal (bottom) probed with an anti-human-MMP-2 monoclonal antibody that cross reacts with the mouse protein (kindly provided by Dr. Matti Höyhtyä, Oulu). The primary antidody was detected by the alkaline phosphatase anti-alkaline phosphatase (APAAP) immune complex revealing a pink colour, while cell nuclei are blue due to staining with hematoxilin.

Chapter 8. Rescue of the Mpv17 phenotype by transgenesis

The Mpv17 gene was identified by cloning the pre-integration site of the provirus used for the generation of the mutant mice. The expression of the Mpv17 gene was found to be completely abolished by this integration. The Mpv17 gene is therefore the prime candidate for a recessive disease gene causing glomerulosclerosis. The human homologue, we have identified, isolated, and studied (Karasawa et al., 1993) is highly conserved, indicating an important role in the maintenance of proper kidney function. Moreover, the Mpv17 gene was the only nonrepetitive and conserved region in the vicinity of the proviral integration (Weiher et al., 1990). In accordance with the notion that a provirus only affects regulatory elements or genes close to the insertion it was assumed that the Mpv17 gene, whose loss of function is causal to the disease. However, it could not be ruled out completely that another, possibly more distant gene, besides Mpv17, is affected in the mouse genome by the provirus, thereby actually causing the renal disorder. The only definitive experiment to prove that the silenced Mpv17 gene leads to the kidney disease is the rescue of the phenotype by introducing the wild type gene into the germline or by targeted inactivation of the gene.

Transgenic construct

We decided to take the rescue approach and introduced the human Mpv17 gene under the control of a heterologous promoter as a transgene into the germline of mice. I therefore placed the gene as a cDNA containing one homologous intron of 263bp length (Karasawa et al., 1993) as well as the 3´ untranslated region with its polyA sequence under the control of the mouse metallothioneine I promoter (kindly provided by Dr. Erwin Wagner, IMP, Vienna). The construct carrying the intron between exon 6 and exon 7 had been isolated as an incompletely spliced cDNA in the process of cloning the human Mpv17 cDNA (Karasawa et al., 1993). This molecule was used for the generation of transgenic mice because it has been reported that introns increase the expression of transgenes in general (Brinster et al., 1988; Choi et al., 1991). Secondly it allows the discrimination of amplified cDNA representing transgene transcripts from artefacts due to

contamination of the isolated mRNA with genomic DNA on the basis of the size of the amplification products, when transgene expression is analysed by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). The mouse metallothioneine I promoter (MT-I) contained on a 1.8 kb fragment has been shown to successfully drive the expression of transgenes in kidney and liver (Hamer, 1986). Figure 8.1 depicts the construct used for microinjection. (microinjections were performed by Dr. Johannes Schenkel in our laboratory). This isolated DNA fragment was 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).

Identification of transgenic animals

The mice resulting from microinjections were tested for the integration of the construct DNA by Southern blot analysis of tail DNA. The tail DNA was cleaved with Sac I restriction endonuclease, Southern blotted, and hybridised to the radiolabelled human cDNA fragment. From the initial 10 candidates one (# 670) was found to be positive for a transgene insertion and subjected to further analysis. The transgene was detected as a fragment of 1.2 kb as shown in Figure 8.2 (TG). The second upper transgenic band is probably due to transition fragments that are generated at the border of the transgene integrate by loss of the Sac I site flanking the construct at its 3´ end. Taking this into account, the number of tandem transgene copies in this strain was estimated to be about 15, judging from the intensity of the transgene specific band. Despite stringent hybridisation conditions, the endogenous mouse Mpv 17 locus cross hybridises with the human cDNA as shown in Figure 8.2 (EG). Meanwhile, two other transgenic founders have been generated using the same construct and they are currently being examined. However, none of these three strains displayed any obvious phenotype. Animals originating from the transgene animal 670 were mated into the homozygous Mpv17 background. This is possible because Mpv17 homozygotes are fertile and live long enough to reproduce. The mutant endogeneous allele can be followed in these breedings by use of a polymorphism between the wild type and mutated alleles described in Weiher et al. (1990). The genomic probe, a Sau 3A fragment of about 350 bp, detects a 3 kb fragment in the wild type (WT in Figure 8.2) and

a 6 kb fragment in the mutated allele (MA in Figure 8.2) on Bam HI digested DNA. Thus, heterozygous animals show both the WT and the MA band, whereas hybridistaion only to the MA band indicates homozygosity for the mutation. The initial breeding of the mouse670 with Mpv17 homozygous mice resulted in heterozygotes which were further interbred, and the genotype of the F2 generation was analysed. Homozygous animals also carrying the MT-Mpv17 transgene were again interbred and this new strain was designated MT670.

Survival and histological analysis of MT670

So far, about 50 animals of this particular genotype have been generated, of which none died of or developed signs of nephrotic syndrome. Thus, the transgene obviously affects the survival of the mice. In order to analyse whether the presence of the transgene actually prevents the MT670 mice from developing glomerulosclerosis, several animals were sacrificed at the age of six months and the kidneys were histologically analysed. A standard Periodic-acid Schiff (PAS) staining is shown in Figure 8.3, demonstrating sclerosis in homozygous Mpv17 mice while heterozygous control animals, as well as the MT670 rescue mouse strain, show no abnormalities in their kidneys. Hence, at the level of kidney pathology, successful rescue of the Mpv17 phenotype, depending on the presence of the transgene, could be demonstrated.

Expression of the transgene in kidney

In order to explain the complementation of the Mpv17 phenotype we examined transgene expression in kidneys of MT670 and control animals. Isolated polyA$^+$ RNA from rescue and homozygous animals was subjected to RT-PCR. In addition, control RNAs from the human fibroblast cell line GM 637 and RSV7 cells were analysed. For comparison, the PCR products derived from the human cDNA and the intron-containing construct used for microinjection was examined (Figure 8.4). The primers used in this analysis specifically detect transgene-derived transcripts, because they were chosen from the 5´ and 3´ untranslated regions of the human cDNA, respectively, where the murine and human sequences significantly differ (Karasawa et al., 1993). A product of the expected size of 585 bp representing authentic, fully spliced mRNA was detected in the sample from the MT670 mouse

(lane 6). This corresponds with bands of the same size in samples from GM 637 cells (lane 7), from RSV7 cells (lane 8) and with the PCR product from the fully spliced cDNA construct (lane 9). By contrast, no expression was detected in non-transgenic Mpv17 homozygotes (lane 5) and PCR of the transgenic construct resulted in a bigger product of about the expected size of 848 bp (lane 10). This proves that the amplification products do not result from contaminating genomic DNA in the RNA preparations, as the transgene represents a cDNA in which intron 6 of 263 has not been removed. Amplification of genomic DNA from MT670 mice would therefore result in a larger fragment (848 bp) as shown in the 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 Mpv 17 function in homozygous Mpv17 mice. This functional rescue not only proves that the Mpv17 phenotype 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 man, stressing the potential role of the Mpv17 gene in human kidney disease.

The transgene technology could be used in further experiments to address the question in which tissue the primary injury, leading to the kidney deficiency, might occur. Constructs directing Mpv17 expression to different target tissues could be used for this purpose.

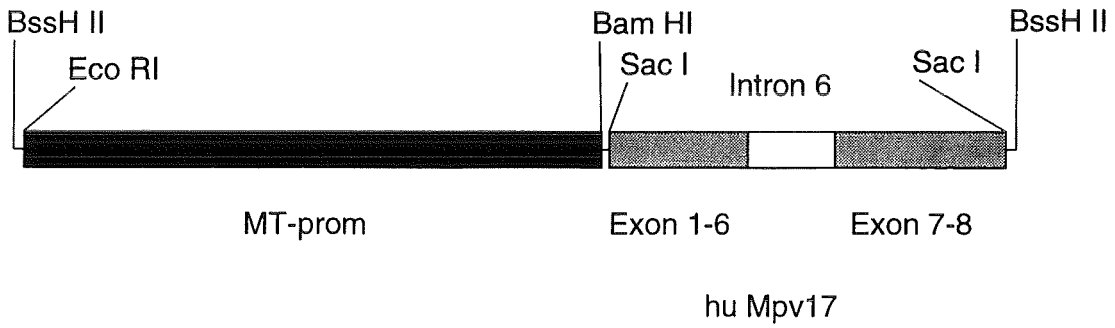

Figure 8.1 The human MT Mpv17 rescue gene construct. The human Mpv17 cDNA gene including intron 6 (Karasawa et al., 1993) is under control of the mouse metallothioneine (MT) promoter. The 3 kb BssHI restriction fragment was used for the generation of transgenic mice.

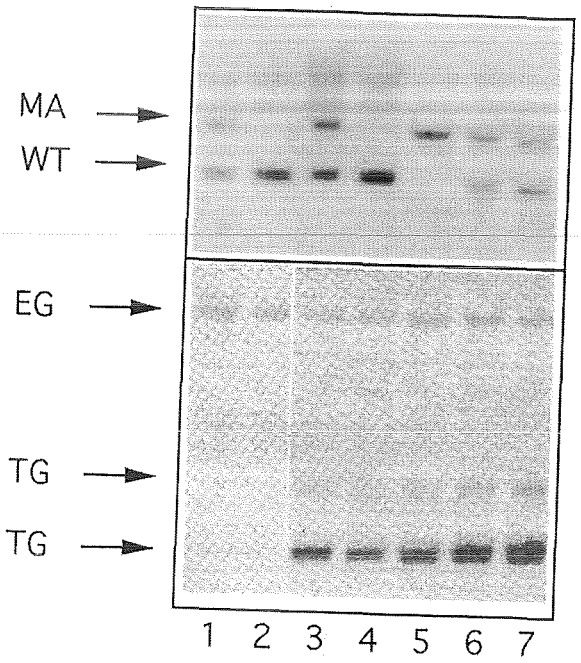

Figure 8.2 Genotyping of transgenic mice. Southern blot analysis of tail DNA of the transgenic mice. Top panel: Endogenous Mpv17 locus: BamH1 cleaved DNA was hybridised with a 350bp Sau 3A fragment diagnostic for the mutation (Weiher et al., 1990); WT: wt Mpv17 allele; MA: mutated Mpv17 allele.
Lower Panel: Presence of the MT-Mpv17 transgene: Sac I digested DNA was hybridised with a fragment representing the human cDNA. TG: transgene fragment. EG: crosshybridising endogenous Mpv17 locus. The kidney histology of mouse 5 (homozygous mutant Mpv17; transgenic for MT-Mpv17) and mouse 2 (heterozygous Mpv17 mouse) are shown in Figure 8.3. The control homozygous mutant animal is not depicted on this Southern blot.

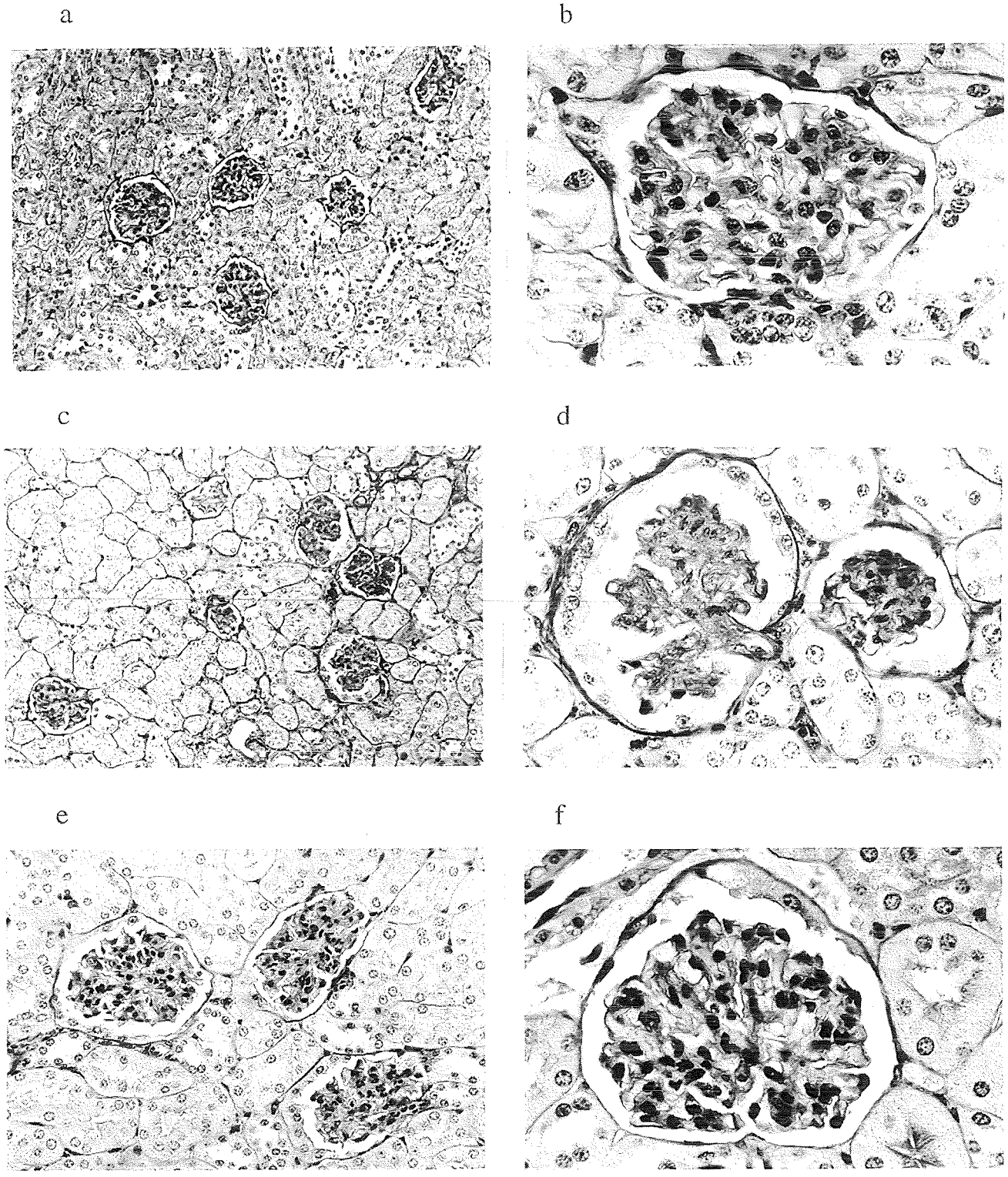

Figure 8.3 Representative histology (PAS stain) of six months-old mice with different Mpv17 genotype. **a** (x140) and **b** (x550): heterozygous Mpv17; **c** (x140) and **d** (x430): homozygous mutant Mpv17 mice demonstrating an early stage of glomerular sclerosis; tubular interstitial changes have not yet developed; **e** (x270) and **f** (x550): strain MT670 (homozygous mutant Mpv 17, transgenic for MT-Mpv17.

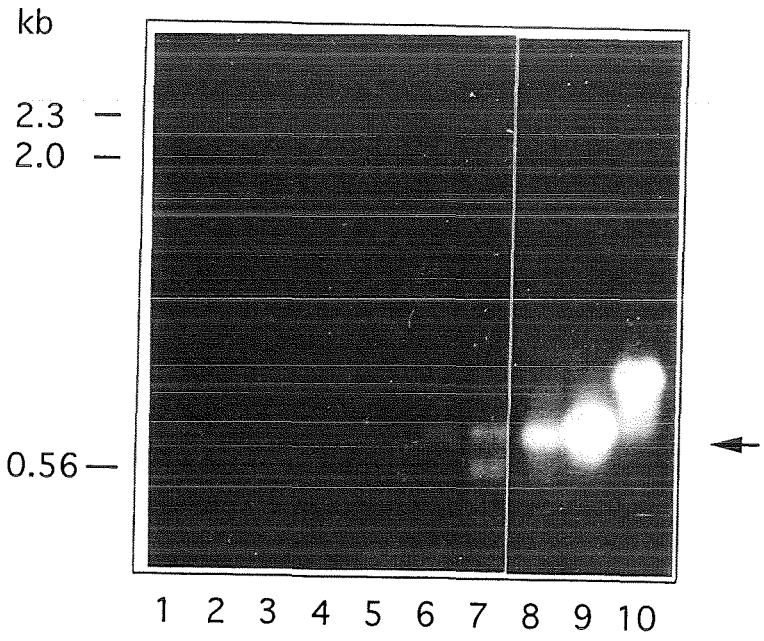

Figure 8.4 Analysis of the transgene expression. Transgene expression was examined using the RT-PCR technique on polyA$^+$ RNA of kidneys from different mice: Lanes 1 and 5: homozygous mutant Mpv17 mouse. Lanes 2 and 6: MT670 mouse (mouse 5 in Fig.2). Lanes 3 and 7: human GM637 fibroblasts. Lane 4 and 8: RSV 7 cells. 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 (Karasawa et al., 1993). Lane 10: amplification on the MT-Mpv17 rescue construct, containing intron 6. For correctly spliced transgene derived mRNA a product of 585bp is expected (lane 9). Unspliced transgene mRNA or contaminating transgene DNA results in a fragment of 848 bp (lane 10).

Chapter 9. Discussion

In contrast to the transgenic mouse lines that serve as animal models for glomerulosclerosis, in which overproduction of a transgene dominantly causes the phenotype (listed in Table 1.2), the Mpv17 strain is a recessive mutant. Thus, loss of function of a gene at the Mpv17 locus causes the disease. An integration of a retroviral genome into a gene at the Mpv17 locus, the Mpv17 gene, suggested that loss of this function was responsible for the development of the phenotype. The isolation and analysis of a human homologue of this gene is described here, and reveals a high conservation of this single copy gene between humans and mice. Besides the structural homology, functional equivalence was also demonstrated, in that the human Mpv17 gene under the control of a heterologous promoter can complement the phenotypic defect of the mutant mice (Schenkel et al., 1995). This proves that the lack of Mpv17 expression in the mutant mice is responsible for the phenotype, and suggests that mutations in the human gene may also lead to inherited glomerulosclerosis in man.

The best characterised and most frequent clinicopathological entity of familial glomerulosclerosis is the Congenital Nephrotic syndrome of the Finnish Type (CNF). We have so far, however, found no mutations of the Mpv17 gene in such patients. Furthermore, our chromosomal localisation of the human gene to chromosome 2 (Karasawa et al., 1993) and the chromosomal assignment of the CNF locus to chromosome 19 by Kestilä et al. (1994a) make a causal role of the Mpv17 gene in CNF unlikely. A potential role for Mpv17 in other inherited kidney diseases is presently under investigation. It is likely that mutations in more than one gene can lead to glomerulosclerosis, and once such genes have been isolated, it will be interesting to study if at least some of their gene products act in a single pathway identified by the Mpv17 mutation.

The Mpv17 gene codes for a protein of 176 amino acids in both humans and mice, harbouring several potential membrane spanning regions. It shows a 54% sequence homology to the peroxisomal membrane protein 22 (pmp22). The comparision between the pmp 22 and Mpv17 membrane-topologic profiles suggests that both are type IIIb membrane proteins. These similarities suggest that these proteins might have an analogous localisation and possibly related functions. To address these

questions, I raised antibodies against the human Mpv17 protein and determined its intracellular localisation by immunofluorescence. The Mpv17 protein does indeed localise to the peroxisome, raising the question whether the Mpv17 protein is involved in human peroxisomal disorders.

The most severe of these peroxisomal diseases, classified as Zellweger syndrome, is characterised by structural defects leading to the loss of function of the whole organelle. I therefore investigated if Mpv17-negative mice are deficient in peroxisome biogenesis. We found, however, that these mice exhibit completely normal peroxisome formation indicating that Mpv17 is not involved in peroxisomal assembly. Considering the peroxisomal localisation of the Mpv17 protein one might expect that glomerulosclerosis would be one of the various disease parameters ZS patients display. However, this is not the case. This apparent contradiction might be explained by the early lethality of Zellweger syndrome, which may not allow for the development of the glomerulosclerosis phenotype. Interestingly, for most Zellweger patients an involvement of the kidney is reported which is described as microcysts (Lazarow and Moser, 1989). Similar pathology is often found in the Mpv17 homozygous mice.

Most other peroxisomal diseases involve defects of single metabolic pathways taking place in this organelle. We therefore screened for the physiological parameters characteristic for theses diseases and found no abnormalities in very long chain fatty acid (VLCFA) degradation, bile acid formation, and plasmalogen synthesis. Again, no differences were seen between mutant and control animals. It is therefore possible that I have identified a new functional link between a peroxisomal defect and a glomerular disease.

One of the most prominent functions of peroxisomes is the production and degradation of reactive oxygen species (ROS), in particular the superoxide anion O_2-. Prompted by numerous reports on the damaging effect of ROS on basement membranes and their role in glomerulosclerosis (Wardle, 1992), I investigated the oxidative status of cells from homozygous Mpv17 and control mice. Much to my surprise, I discovered that the Mpv17 gene product is involved in the production of ROS as homozygous cells show less, not more ROS. Moreover, the

Mpv17 gene itself when transfected into murine fibroblasts exhibits a ROS-producing activity.

The ROS-producing activity of the Mpv17 protein is distinct from other enzymatic mechanisms known to produce ROS in mammals, namely NADPH oxidase and xanthine oxidase (XO, Cross and Jones, 1991). The NADPH oxidase in neutrophils consists of several membrane bound proteins and is responsible for the respiratory burst crucial for phagocytotic defense mechanisms. Mutations in this TPA-inducible system in humans lead to chronic granulomatosis disease (CGD; Smith and Curnutte, 1991, Royer-Pokora et al., 1986). Based on its protein sequence and intracellular localisation, however, Mpv17 must be distinct from this system. Xanthine oxidase (XO) is a peroxisomal enzyme producing O_2- radicals upon oxidation of xanthine to urate. Structurally, Mpv17 is distinct from this enzyme and the phenotype of XO deficiency does not involve the kidney, making a role of Mpv17 in this pathway unlikely. Therefore, although it is not yet formally possible to exclude interactions between Mpv17 and the two ROS-producing pathways, I favour the hypothesis that Mpv17 acts in a yet unidentified oxidation mechanism.

These results prompted me to reconsider the role of reactive oxygen in the Mpv17 system. In contrast to my initial hypothesis, according to which high ROS levels directly impose damage onto the glomerular basement membrane, I now have to postulate that low ROS levels cause regulatory changes within cells which are relevant for kidney damage. The first prerequisite of this postulate would be that Mpv17 expression controls the expression of other genes mediated by the increase of ROS levels. In fact, in stable transfectants of Mpv17 expression vectors I can observe such effects. Most interestingly, the gene for the 72 kD form of type IV collagenase (MMP-2) is strongly expressed in cells lacking functional Mpv17 protein while it is repressed in cells overproducing Mpv17. The MMP-2 protein is of particular interest in the context of glomerulosclerosis, since it has been shown that the development of this disease in experimental animal models is accompanied by enhanced expression of MMP-2 in the glomerulus (Nakamura et al., 1994). MMP-2, as a member of the matrix metalloproteinase family, has a crucial function in the degradation of type IV collagen which is the major component of the glomerular basement membrane (GBM). Thus,

upregulation of this enzyme could potentially inflict damage directly to the GBM. Such initial injury would result in the gradual loss of function of the respective glomerulus. This proposed mechanism represents an attractive explanation of the observed data, including the focal and segmental appearence of the phenotype (Weiher et al., 1990).

This model requires a number of testable prerequisits. First, if the lack of Mpv 17 is important directly at the level of GBM turnover, Mpv17 expression should be detectable in the cells involved in these processes. In fact, I have observed Mpv17 expression in podocytes, the cell type chiefly responsible for GBM synthesis and degradation (Glassock et al., 1991; Johnson et al., 1992). Second, MMP-2 expression is expected to be enhanced in the absence of Mpv17 protein not only in fibroblasts but also in the glomerular epithelial cells. Indeed, such enhanced levels were detected by immunohistochemistry in the glomeruli of Mpv17 homozygotes.

If the Mpv17 gene acts as a general regulator of ROS levels in cells, the question arises, why a failure to express this almost ubiquitously expressed gene causes such a specific phenotype. This seemingly paradoxical phenomenon could be resolved when one consides that many widely expressed genes produce specific `knock out´ phenotypes. The β_2-microglobulin negative mice are an example of such an effect (Zijelstraa et al, 1989). In addition, there may be several overlapping ROS producing functions which are able to complement each other in most cell types. The apparent specificity of the Mpv 17 phenotype would then be the result of a cell type specific lack of redundancy of these regulatory systems.

The Mpv17 mouse model has served as a valuable model to study the molecular mechanisms that can lead to glomerulosclerosis. The isolation of the affected gene has enabled us to reveal a new connection between intracellular ROS levels, gene expression, and the pathology of kidney disease. In the future it should provide a system in which a more detailed elucidation of the glomerulosclerosis pathway can be achieved. This in return could reveal new approaches to the diagnosis and treatment of this disease.

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Danksagung

Die vorliegende Arbeit wurde am Institut für Genetik des Forschungszentrums Karlsruhe durchgeführt. Die Deutsche Forschungsgemeinschaft unterstützte mich durch die Förderung des Mpv17 Projekts.
An erster Stelle möchte ich meinem Doktorvater Dr. Hans Weiher für die Anleitung und Geduld danken.
Den Mitarbeitern des Instituts gehört mein Dank für die Hilfe bei vielen Experimenten, die während meiner Arbeit anfielen und für mich manchmal Neuland bedeuteten.
Insbesondere die Leute aus der Weiher-Gruppe und dem Labor 224 sind hier hervorzuheben.
Christiane Rutenberg danke ich für die technische Unterstützung und für manche angeregte Diskussionen, die diese Arbeit erst ermöglicht haben.
Jonathan Sleeman, Larry Sherman und Richard Jäger haben beim Korrekturlesen eine unschätzbare Hilfe geleistet.
Allen Freunden in Karlsruhe und woanders sowie meinen Eltern danke ich für die Unterstützung in den verschiedensten Lebenslagen.