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Spatiotemporal expression patterns of sialoglycoconjugates during nephron morphogenesis and their regional and cell type-specific distribution in adult rat kidney

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Abstract The expression of $\alpha 2,6$ - and $\alpha 2,3$ -linked sialic acids on *N*-glycans was studied in embryonic, postnatal, and adult rat kidney. Histochemistry and blotting using *Polyporus squamosus* and *Sambucus nigra* lectins for $\alpha 2,6$ -linked sialic acids and the *Maackia amurensis* lectin for $\alpha 2,3$ -linked sialic acids were performed and sialyltransferase activity was assayed. *N*-glycans with $\alpha 2,6$ - and $\alpha 2,3$ -linked sialic acid were differently expressed in the two embryonic anlagen and early stages of nephron. Metanephrogenic mesenchyme was positive for $\alpha 2,3$ -linked sialic acid but not for the $\alpha 2,6$ -linked one, which became detectable initially in the proximal part of S-shaped bodies. Collecting ducts were positive for $\alpha 2,6$ -linked sialic acid, whereas $\alpha 2,3$ -linked sialic acid was restricted to their ampullae. Although positive in embryonic kidney, S1 and S2 of proximal tubules became unreactive for $\alpha 2,3$ -linked sialic acid in postnatal and adult kidneys. In adult kidney, intercalated but not principal cells of collecting ducts were reactive for $\alpha 2,3$ -linked sialic acid. In contrast, $\alpha 2,6$ -linked sialic acids were detected in all cells of adult kidney nephron. Blot analysis revealed a different but steady pattern of bands reactive for $\alpha 2,6$ - and $\alpha 2,3$ -linked sialic acid in embryonic, postnatal, and adult kidney. Activity of $\alpha 2,6$

and $\alpha 2,3$ sialyltransferases was highest in embryonic kidney and decreased over postnatal to adult kidney with the activity of $\alpha 2,6$ sialyltransferase always being three to fourfold that of $\alpha 2,3$ sialyltransferase. Thus, $\alpha 2,6$ - and $\alpha 2,3$ -linked sialic acids are differently expressed in embryonic anlagen and mesenchyme-derived early stages of nephron and show regional and cell type-specific differences in adult kidney.

Keywords Kidney · Kidney development · Sialic acid · Sialyltransferase · Histochemistry · *Polyporus squamosus* lectin · *Maackia amurensis* lectin

Introduction

The kidney belongs to the organs with an exceptional degree of complexity in terms of both morphological organization and function. With regard to the former, three different regions, the cortex and the outer and the inner medulla, can be distinguished because of the presence of different parts of the nephron (Kaissling and Dorup 1995; Kriz and Kaissling 2000). Along the nephron, various cell types can be distinguished fulfilling specific functions in filtering, reabsorption and secretion (Al-Awqati 1996, 2003; Kriz and Kaissling 2000; Pavenstädt et al. 2003). Thus, adult kidney has been subjected to studies on cell type-specific and plasma membrane domain-related expression of *N*- and *O*-glycans present in glycoproteins. For instance, specific expression patterns of sialic acid residues as detected with the *Limax flavus* lectin and of *N*-acetylgalactosamine-terminated oligosaccharides reactive with the *Helix pomatia* lectin were found in the podocyte plasma membrane (Charest and Roth 1985; Roth et al. 1983). Sialoglycoproteins such as podocalyxin and podoplanin have been shown to be involved in establishing and maintaining the peculiar shape of podocytes (Kerjaschki et al. 1984; Matsui et al. 1999; Pavenstädt et al. 2003). Histochemical lectin-binding studies have also revealed a

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remarkable degree of variability in glycocalyx composition of the various epithelia present in the distal and collecting tubules (Brown et al. 1985; Kaneko et al. 1995; LeHir and Dubach 1982; LeHir et al. 1982; Roth and Taatjes 1985; Taatjes et al. 1988; Toma et al. 1999), although the functional importance of these differences remains unknown.

The permanent kidney, in contrast to other organs, develops from two different embryonic anlagen, the metanephrogenic mesenchyme and the epithelial ureteric bud, by interactive processes inducing morphogenesis and differentiation (Horster et al. 1999; Saxén 1987). The blind ends of the sprouting and dichotomously branching ureteric bud induce clustering and condensation of the mesenchymal cells. Through growth and differentiation, they initially form the renal vesicle, which is the first epithelial structure and which further differentiates through the S-shaped body and capillary loop stage into maturing glomeruli. This is accompanied by segmental morphogenesis resulting in the formation of proximal and distal tubules with the loop of Henlé in between, and connecting tubules. Thus, the entire epithelium of the nephron is derived from mesenchyme which undergoes a transition into epithelium, whereas the collecting duct system is primarily epithelia-derived. A variety of studies related to the inductive and morphogenetic events during nephrogenesis revealed spatiotemporal expression patterns of cell adhesion molecules, extracellular matrix components, growth factors, transcription factors, and proto-oncogene encoded receptor tyrosine kinases (Bard et al. 1994; Birchmeier and Birchmeier 1993; Hammerman et al. 1992; Horster et al. 1999; Kumar et al. 1997; Parr and McMahon 1994; Rauscher 1993). Spatiotemporal patterns of lectin-binding sites and of polysialic acids have been observed during the formation and differentiation of the nephron (Holthöfer and Virtanen 1987; Kunz et al. 1984; Lackie et al. 1990; Laitinen et al. 1987; Roth et al. 1987; Toma et al. 2000; Wagner and Roth 1988; Ziak and Roth 1999). The studies on polysialic acids disclosed remarkable correlations between their expression and particular stages of nephron development (Lackie et al. 1990; Roth et al. 1987; Ziak and Roth 1999), which were not as obvious for sialoglycoconjugates terminated by a single sialic acid residue as detected with the *Limax flavus* lectin (Wagner and Roth 1988), *Sambucus nigra* lectin, or *Maackia amurensis* lectin (Sata et al. 1989) and Amaranthin (Toma et al. 2000).

The present study was conducted to analyze the presence and distribution of *N*-glycans terminated in sialic acid either in $\alpha 2,6$ - or in $\alpha 2,3$ -linkage to galactose $\beta 1,4$ *N*-acetylglucosamine during embryonic development and postnatal differentiation of rat kidney and in the adult organ. For this purpose, the *Polyporus squamosus* lectin, highly reactive with the NeuAc $\alpha 2,6$ Gal $\beta 1,4$ GlcNAc trisaccharide sequence (Mo et al. 2000; Zhang et al. 2001), the *Sambucus nigra* lectin reactive with the NeuAc $\alpha 2,6$ Gal/GalNAc disaccharide sequence (Shibuya et al. 1987), and the *Maackia amurensis* lectin reactive with the NeuAc $\alpha 2,3$ Gal $\beta 1,4$ GlcNAc trisaccharide se-

quence (Knibbs et al. 1991; Wang and Cummings 1988) were used for histochemistry and blot analysis. Furthermore, the specific activity of the respective sialyltransferases, β -galactoside $\alpha 2,6$ sialyltransferase (ST6Gal-I) and β -galactoside $\alpha 2,3$ sialyltransferase (ST3Gal-III), was determined in tissue extracts. Although sialic acid linked $\alpha 2,6$ or $\alpha 2,3$ to penultimate galactose of oligosaccharides is commonly found in various organs including the kidney (Kelm and Schauer 1997; Paulson and Colley 1989; Rosenberg 1995), our results show hitherto unrecognized differences in their cellular and regional expression during embryonic development and postnatal kidney differentiation and in the adult rat kidney.

Materials and methods

Reagents

Polyporus squamosus lectin (PSL) was affinity purified as described (Mo et al. 2000). Lectin-digoxigenin conjugates were prepared in the presence of 0.2 M galactose and 0.1% Triton X-100 using a digoxigenin conjugation kit from Roche Molecular Biochemicals (Mannheim, Germany) according to the manufacturer's instructions. Digoxigenin conjugates of *Sambucus nigra* lectin (SNL) and *Maackia amurensis* lectin (MAL) were available from previous studies. Horseradish peroxidase or alkaline phosphatase-conjugated sheep anti-digoxigenin antibodies (Fab fragments), neuraminidase from *Vibrio cholerae*, and *N*-glycanase F were from Roche Molecular Biochemicals, and a 4 nm gold-labeled rabbit anti-horseradish peroxidase antibody was from Immunoresearch Laboratories (West Grove, PA). All other reagents were of analytical grade.

Tissue preparation

Sprague-Dawley rats and pregnant rats were obtained from the Central Animal Facility of the University of Zurich. Kidneys from adult rats were fixed by perfusion from the left cardiac ventricle with freshly prepared 3% formaldehyde–0.1% glutaraldehyde or 4% formaldehyde in Hank's balanced salt solution (pH 7.4, 37°C) for 15 min, followed by immersion fixation at ambient temperature for 1 h 45 min. Kidneys from embryonic day 16, 18, and 20 as well as kidney slices from postnatal days 2, 5, 7, 14, and 21 were fixed by immersion in the above fixatives. Free aldehyde groups were blocked by immersing the tissues in 50 mM NH₄Cl in PBS (10 mM phosphate buffer, 0.15 M NaCl, pH 7.4) for 1 h.

Formaldehyde-fixed tissues were embedded in paraffin according to standard protocol and sections of approximately 5 μ m thickness were prepared. Large pieces from formaldehyde/glutaraldehyde-fixed adult kidney were embedded at progressively lowered temperature (–42°C finally) in Lowicryl K4 M and used to prepare large 0.5–1.0 μ m semithin sections containing cortex and adjacent outer medulla or inner medulla with adjacent outer medulla. Small pieces from kidney cortex and inner medulla were embedded in Lowicryl K4 M for the preparation of ultrathin sections (Roth 1989).

Lectin histochemistry

For light microscopy, a three-step labeling protocol followed by photochemical silver amplification was performed as described in detail previously (Roth et al. 1998; Toma et al. 2001). In brief, deparaffinized and rehydrated paraffin sections or semithin sections of Lowicryl K4 M-embedded tissue were incubated with digoxigenin-conjugated PSL (1.5 μ g/ml), SNL (1.25 μ g/ml), or MAL

(2.5 $\mu\text{g/ml}$) in PBS (pH 7.4) containing 1% BSA, 0.05% Tween 20, and 0.005% Triton X-100 for 2 h at ambient temperature, rinsed in buffer, and incubated with horseradish peroxidase-conjugated sheep anti-digoxigenin Fab (0.3 U/ml) for 1 h. After rinses with buffer, sections were incubated with 4 nm gold-labeled anti-horseradish peroxidase antibody (Roth et al. 1992) adjusted to an absorbance of 0.05 at 525 nm. Finally, silver intensification using silver acetate (Roth et al. 1998) was performed followed by counterstaining with nuclear fast red (paraffin sections). Both paraffin and Lowicryl K4 M semithin sections were also incubated with directly gold-labeled SNL and MAL followed by silver intensification of the lectin-gold labeling (Sata et al. 1989).

For electron microscopy, ultrathin sections were incubated with directly gold-labeled MAL (Sata et al. 1989) or with digoxigenin-conjugated MAL followed by 8 nm gold-labeled anti-digoxigenin antibody (Sata et al. 1990).

The specificity of staining was established by incubating the lectins with varying amounts (0.1 mM to 0.2 M) of either 6' or 3' sialyllactose for 30 min prior to use to stain paraffin sections from kidney.

Furthermore, specificity of lectin staining was corroborated by enzymatic removal of oligosaccharide side chains at the asparagine-*N*-acetylglucosamine core linkage. For this, paraffin sections were treated with 0.1% sodium dodecyl sulfate in TRIS-buffered saline at 70°C for 30 min and washed in NP40 in TRIS-buffered saline. This was followed by *N*-glycanase F (80 U/ml in 0.1 M phosphate buffer, pH 7.4) in a moist chamber for 18 h at 37°C. Afterwards, tissue sections were conditioned with PBS containing 1% BSA, 0.05% Triton X-100, and 0.05% Tween 20 for 20 min at ambient temperature and incubated with the lectins as described above. To enzymatically remove sialic acids, paraffin sections were treated with *Vibrio cholerae* neuraminidase (0.02 U/ml in 50 mM acetate buffer, pH 5.5) for 18 h at 37°C in a moist chamber, washed, conditioned, and incubated with the lectins as described above. To assess the effect of the incubation buffers on the lectin staining, sections were incubated in the respective enzyme buffers only for 18 h at 37°C before lectin labeling.

Mild base treatment to de-*O*-acetylate sialic acids was performed by treating paraffin sections either with 0.5% KOH in 70% ethanol or with 0.1 N NaOH for 20 min at ambient temperature (Reid et al. 1978).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and lectin blotting

Kidneys from embryonic day 20, postnatal days 2, 5, 7, 14, and 21 as well as adult animals were homogenized in PBS (pH 7.4) containing protease inhibitor tablets, and proteins were extracted with 1% Triton X-100 for 1 h at 4°C. Extracts were also prepared from microscopically dissected kidney cortex and inner medulla. The homogenates were centrifuged at 14,000 *g* for 10 min at 4°C and aliquots of the supernatants were used for SDS-PAGE. Lectin blotting was performed as described in detail previously (Zuber et al. 1998). In brief, proteins were denatured in Laemmli buffer and then separated by 3–15% gradient SDS-PAGE (100 $\mu\text{g/lane}$) and transferred to nitrocellulose membranes. Nitrocellulose membranes were incubated with digoxigenin-conjugated PSL (2.5 $\mu\text{g/ml}$ PBS containing 0.05% Tween 20 and 1% BSA) or MAL (5 $\mu\text{g/ml}$ 0.1 M TRIS-HCl, pH 7.5, 0.15 M NaCl containing 0.05% Tween 20 and 1% BSA) for 2 h, washed, and incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (0.25 U/ml TRIS-buffered saline containing Tween 20) for 45 min followed by color reaction.

Sialyltransferase assays

Homogenates from whole kidneys from embryonic day 20, postnatal days 2, 5, 7, 14, and 21 as well as adult whole kidneys were assayed. Furthermore, homogenates from kidney cortex, outer and inner medulla obtained by microscopic dissection were assayed. The activity of the β -galactoside α 2,6 sialyltransferase (ST6Gal-I) and β -

galactoside α 2,3 sialyltransferase (ST3Gal-III) was assessed in identical reaction mixtures with the exception of their specific acceptor substrates which were asialo- α 1-acid glycoprotein and lacto-*N*-tetraose as described in detail previously (Weinstein et al. 1982).

Results

Distribution pattern of α 2,6- and α 2,3-linked sialic acid in embryonic kidneys

Staining for both PSL and MAL indicative of the presence of oligosaccharides terminated in α 2,6- and α 2,3-linked sialic acid, respectively, was detectable in kidneys from embryonic days 16, 18, and 20. In general, the intensity of staining obtained with PSL was lower than that observed with MAL (Figs. 1, 2, 3). Staining patterns for SNL and PSL were identical irrespective of the used techniques. Although PSL staining was observed in all parts of the ureteric bud-derived collecting ducts (*arrows* in Figs. 1B, 2B, 3B), the staining with MAL was restricted to the terminal ampullae of the collecting ducts (*arrows* in Figs. 1D, 2E, 3D). This differential staining pattern was consistently observed in kidneys from embryonic days 16, 18, and 20. The condensed metanephrogenic mesenchyme and the loose mesenchymal stroma were largely unreactive with PSL (Figs. 1B *asterisk*, 2B *asterisk*, 3B). This was in striking contrast to the intense staining observed with MAL in the metanephrogenic mesenchyme of kidneys from embryonic days 16, 18, and 20 (Figs. 1C, D *asterisk*, 2D, E *asterisk*, 3C, D). Additional differences became obvious when the different developmental stages of the glomerulus were considered. Renal vesicles were unreactive with PSL in all embryonic stage kidneys examined (*arrowhead* in Fig. 1B), whereas strong reactivity with MAL was evident in the luminal cell surface and to a lesser degree in the basolateral cell surface of the vesicle epithelia (*arrowhead* in Fig. 1D). The S-shaped bodies showed some PSL reactivity which, however, was restricted to the cells of its proximal part facing the presumptive Bowman's space (*double arrowheads* in Figs. 1B, 2B). In contrast, the entire S-shaped body was stained with MAL (*double arrowheads* in Figs. 1D, 2E, 3D) and the weak staining observed at embryonic day 16 became strong in kidneys from embryonic days 18 and 20. Immature glomeruli which were observed in kidneys from embryonic days 18 and 20 were reactive with both PSL and MAL, in particular the presumptive podocytes (*Gl* in Figs. 2, 3). Likewise, immature proximal tubules observed in kidneys of these embryonic stages were positive for both lectins (Figs. 2C, F *arrows*, 3D *open arrows*). Endothelia of capillaries in the interstitium were faintly positive with PSL (*En* in Fig. 1B) and strongly stained with MAL (*En* in Fig. 1D). This difference in staining intensity was also observed in kidneys from embryonic days 18 and 20 (Figs. 2, 3).

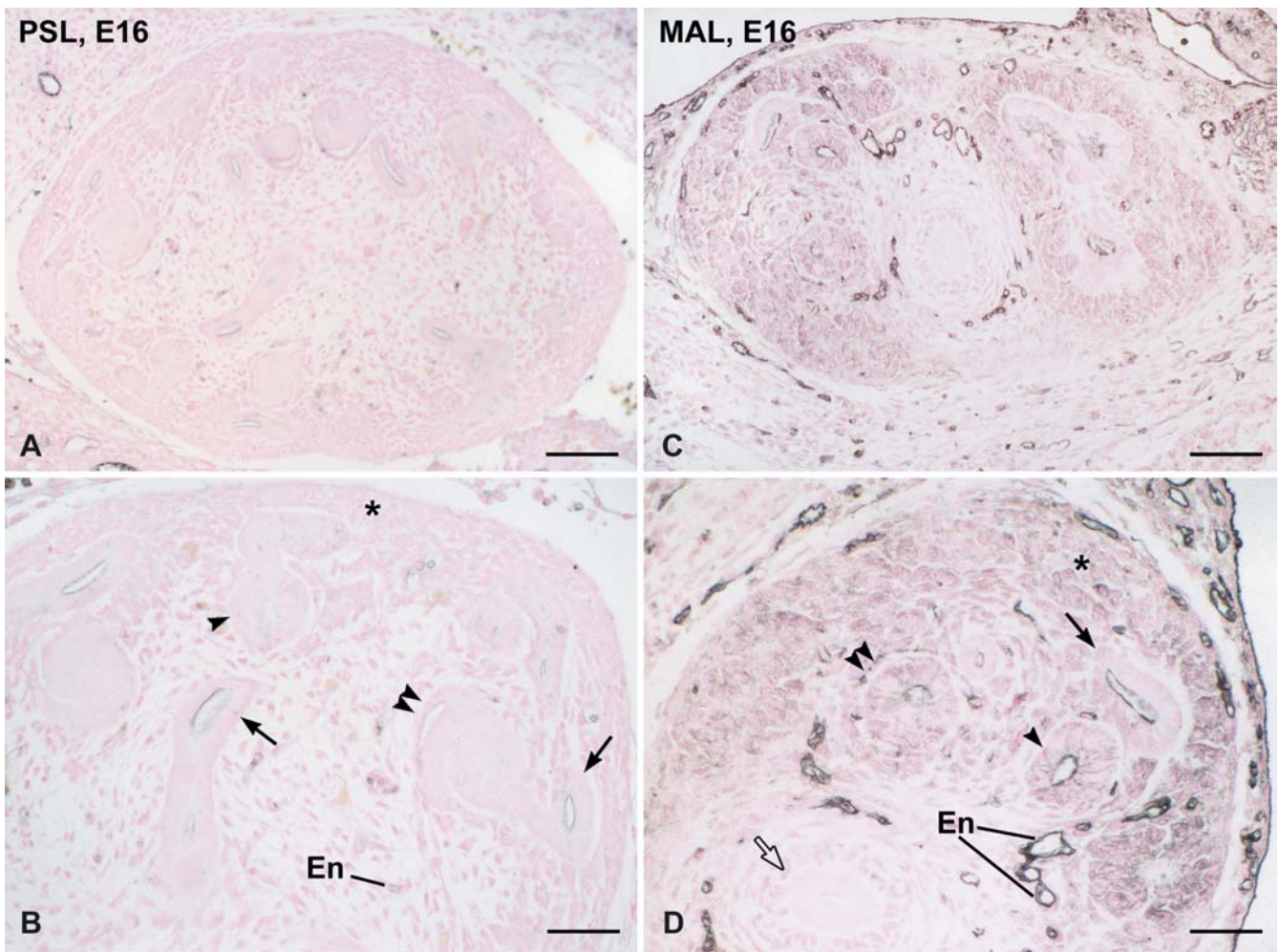


Fig. 1A–D Adjacent sagittal sections of paraffin-embedded rat kidney, embryonic day 16. Silver-intensified lectin–gold staining in this and all other light microscopic illustrations appears in black. **A, B** Staining with *Polyporus squamosus* lectin (PSL) to detect α 2,6-linked sialic acid is observed in ureteric bud-derived collecting ducts and their terminal ampulla (arrows). The condensed metanephrogenic mesenchyme (asterisk), stromal mesenchyme, and renal vesicle (arrowhead) appear unreactive, whereas the crevice of the proximal part of an S-shaped body, which is the first

morphological sign of glomerulus formation, is stained (double arrowheads). **C, D** Staining with *Maackia amurensis* lectin (MAL) to detect α 2,3-linked sialic acid. Of the ureteric bud-derived collecting ducts, only the terminal ampulla is stained (arrow). The condensed metanephrogenic mesenchyme (asterisk), stromal mesenchyme, renal vesicle (arrowhead), and entire S-shaped body (double arrowhead) are labeled. Both PSL and MAL stain endothelia (En in B and D). Bars 90 μ m in A, B; 60 μ m in C, D

In order to exclude that the differences in staining intensity and in distribution pattern were due to *O*-acetylation of sialic acids, tissue sections were subjected to saponification prior to lectin staining. However, no change in intensity and pattern of lectin staining could be observed indicating that the observed differences were not related to *O*-acetylation (not shown).

Distribution pattern of α 2,6- and α 2,3-linked sialic acid in postnatal kidneys

In rat kidney, nephrogenesis continues in the nephrogenic zone until postnatal day 5. We investigated kidneys from postnatal days 2, 5, 7, 14, and 21. The intensity of PSL staining in postnatal kidneys was stronger than that

observed in kidneys from embryonic day 20 with the exception of the nephrogenic zone. At postnatal days 2 (Fig. 4A, B) and 5 (data not shown), the pattern of PSL staining corresponded to the one observed in embryonic day 20 kidneys. Likewise, the MAL staining in the nephrogenic zone was identical to that observed in embryonic kidneys (Fig. 4D). In the underlying cortical region, immature and mature glomeruli were intensely reactive with both PSL (Gl in Fig. 4B, see also E) and MAL (Gl in Fig. 4D, see also F). A most remarkable difference to embryonic kidneys existed with regard to the proximal tubules and this could be observed in all studied postnatal kidney stages (and in adult kidney, see below). All segments of the proximal tubules (S1, S2, S3) were equally and intensely stained with PSL (Fig. 4B, E), whereas the S1 and S2 parts of proximal tubules were

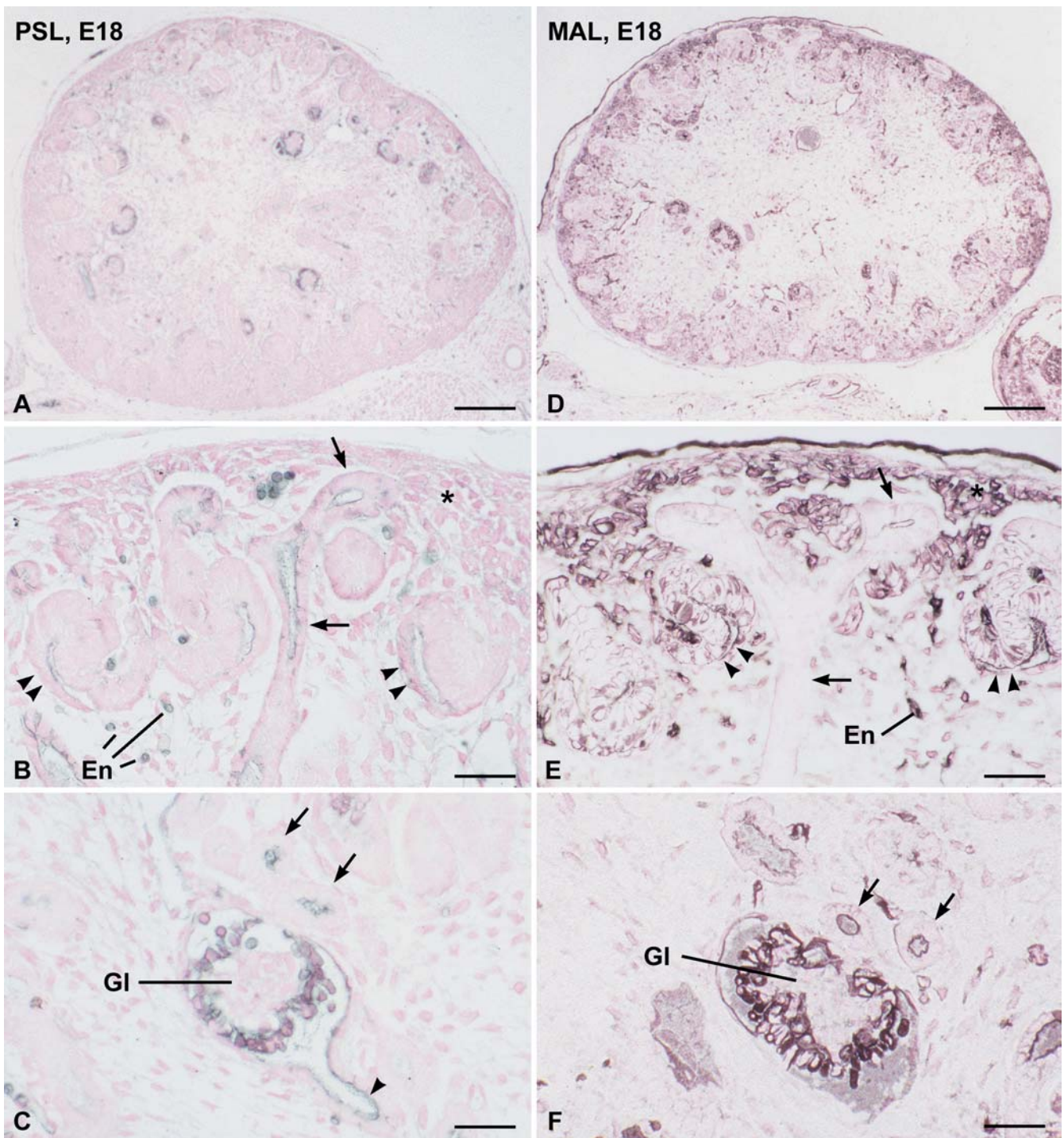


Fig. 2A–F Consecutive sagittal sections of paraffin-embedded rat kidney, embryonic day 18. **A–C** Ureteric bud-derived collecting ducts (*arrows in B*), proximal part of S-shaped bodies (*double arrowheads*), immature glomeruli (*GI*), and immature proximal tubules (*arrowhead and arrows in C*) are positive for $\alpha 2,6$ -linked sialic acid. Condensed metanephrogenic mesenchyme (*asterisk*) and stromal mesenchyme appear unreactive. **D–F** Intense staining

for $\alpha 2,3$ -linked sialic acid is seen in condensed metanephrogenic mesenchyme (*asterisk*), stromal mesenchyme, entire S-shaped bodies (*double arrowheads in E*), immature glomeruli (*GI*), and immature proximal tubules (*arrows in F*). *Arrows in E* point to collecting ducts and staining of the terminal ampullae. *En* Positive endothelium. *Bars* 90 μm in **A, D**; 25 μm in **B, C, E, F**

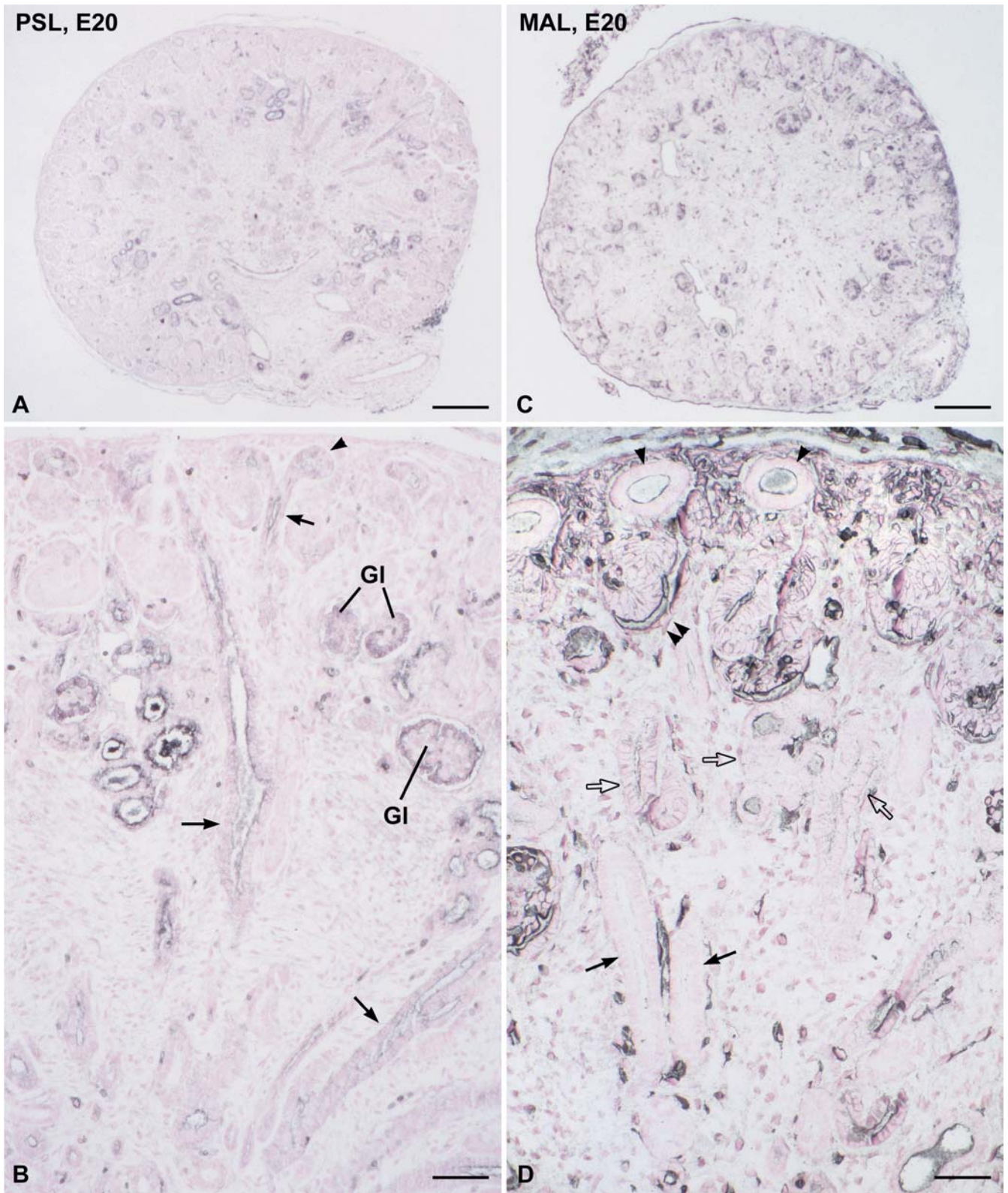


Fig. 3A–D Adjacent sagittal sections of paraffin-embedded rat kidney, embryonic day 20. **A, B** Staining pattern for α 2,6-linked sialic acid corresponds to that of embryonic day 18. Terminal ampulla (*arrowhead*) of collecting ducts (*arrows*), glomeruli (*GI*). **C, D** Staining pattern for α 2,3-linked sialic acid corresponds to that

of embryonic day 18. Positive terminal ampulla (*arrowheads*) of unreactive collecting ducts (*arrows*) are seen as well as positive S-shaped bodies (*double arrowheads*), glomeruli, and immature proximal tubules (*open arrows*). Bars 90 μ m in **A, B**; 20 μ m in **C, D**

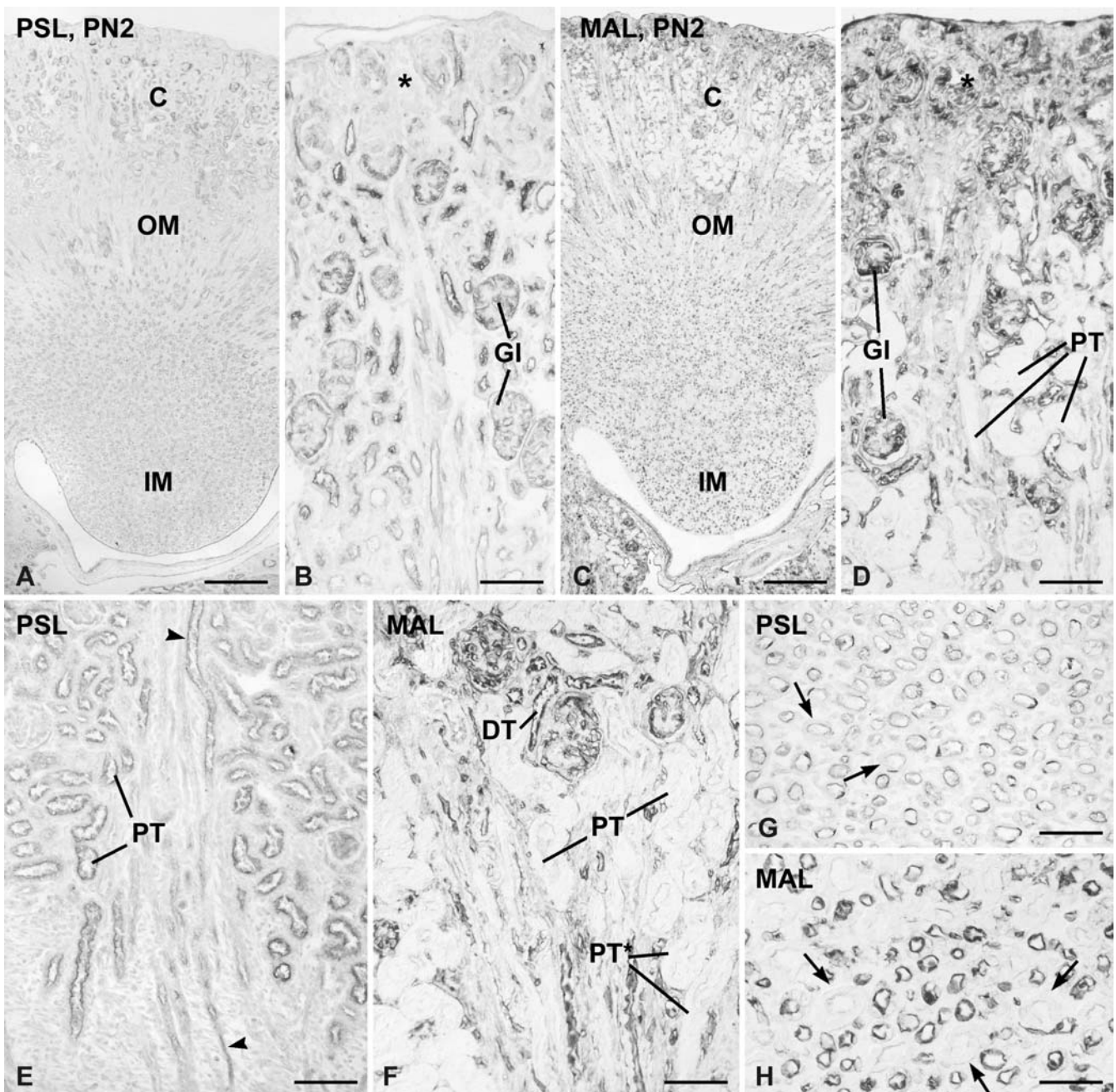


Fig. 4A–H Serial sections of paraffin-embedded rat kidney, postnatal day 2. The general distribution of α 2,6-linked sialic acid in cortex (C), outer (OM), and inner (IM) medulla is shown in A. B is a detail from cortex with nephrogenic zone (asterisk). Glomeruli (Gl) and proximal tubules are positive. In C, the general distribution of α 2,3-linked sialic acid in cortex (C), outer (OM) and inner (IM) medulla is shown. D represents a detail from cortex with positive nephrogenic zone (asterisk) and positive glomeruli (Gl); convoluted proximal tubules (PT) are unreactive. E and F show part of cortex with adjacent outer stripe of outer medulla.

Although convoluted (PT in E) and straight (arrowheads in E) proximal tubules are positive for α 2,6-linked sialic acid; reactivity for α 2,3-linked sialic acid is undetectable in convoluted proximal tubules (PT in F) and only faint staining is detectable in straight proximal tubules (PT*). DT Positive distal tubule. G, H Details of inner medulla showing that all structures are reactive for α 2,6-linked sialic acid (G) and α 2,3-linked sialic acid (H) although the staining for the former is weak. Arrows in G and H point to positive large collecting ducts. Bars 280 μ m in A, C; 60 μ m in B, D, G, H; 45 μ m in E, F

unreactive with MAL (PT in Fig. 4D). Only S3 of proximal tubules were faintly MAL positive (PT* in Fig. 4F). This differential PSL and MAL staining of proximal tubules is illustrated for postnatal day 7 in Fig. 5

and postnatal day 21 in Fig. 6. Thin loops of Henlé and distal tubules as well as intertubular capillaries of the examined postnatal stages were positive with both PSL and MAL (Figs. 4, 5, 6). The stromal cells were largely

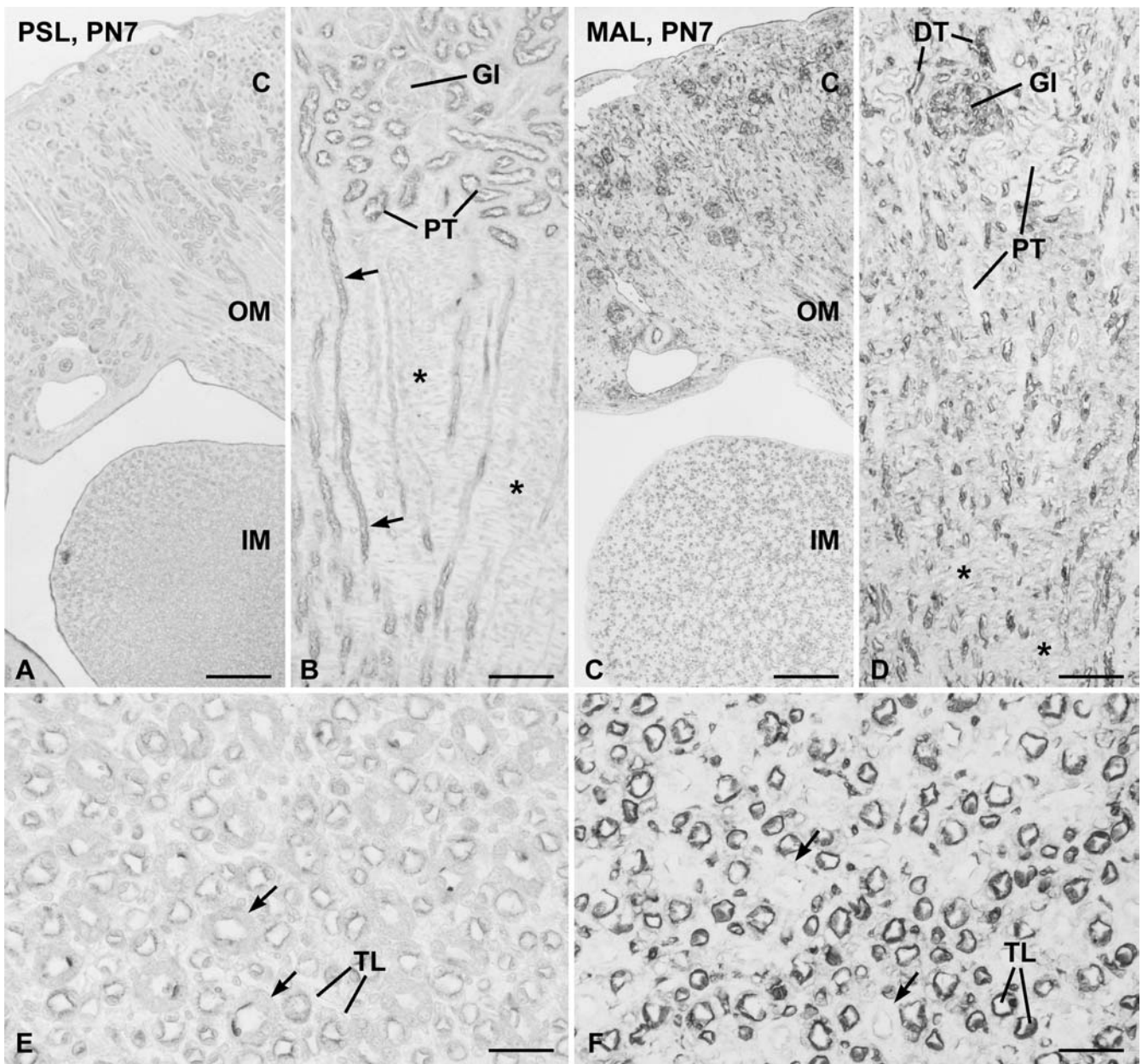


Fig. 5A–F Serial sections of paraffin-embedded rat kidney, post-natal day 7. At low magnification, the distribution of $\alpha 2,6$ -linked sialic acid (A) and of $\alpha 2,3$ -linked sialic acid (C) in cortex (C), outer (OM) and inner (IM) medulla is seen. In the cortex, staining for $\alpha 2,6$ -linked sialic acid (B) is present in glomeruli (Gl) and convoluted (PT) as well as straight (arrows) proximal tubules. Stromal mesenchyme (asterisks) appears unreactive. Although

glomeruli (Gl), distal tubules (DT), and stromal mesenchyme (asterisks) are positive for $\alpha 2,3$ -linked sialic acid (D), proximal tubules (PT) are unreactive. In inner medulla, all structures are weakly positive for $\alpha 2,6$ -linked sialic acid (E) whereas all elements with the exception of large collecting ducts (arrows) were strongly positive for $\alpha 2,3$ -linked sialic acid (F). TL Thin loops. Bars 200 μm in A, C; 45 μm in B, D; 30 μm in E, F

unreactive with PSL (Figs. 4E, 5B) but stained by MAL (Figs. 4F, 5D). Likewise, the collecting ducts of cortex and outer as well as inner medulla were reactive for both PSL and MAL (Figs. 4, 5, 6).

The de-*O*-acetylation of sialic acids by saponification of tissue sections had no influence on intensity and pattern of lectin staining (data not shown).

Distribution pattern of $\alpha 2,6$ - and $\alpha 2,3$ -linked sialic acid in adult kidneys

To achieve the highest spatial resolution and morphological comparability, consecutive serial semithin sections of Lowicryl K4 M-embedded kidney were investigated. Although the staining pattern of proximal tubules for PSL and MAL in adult kidney corresponded to that observed

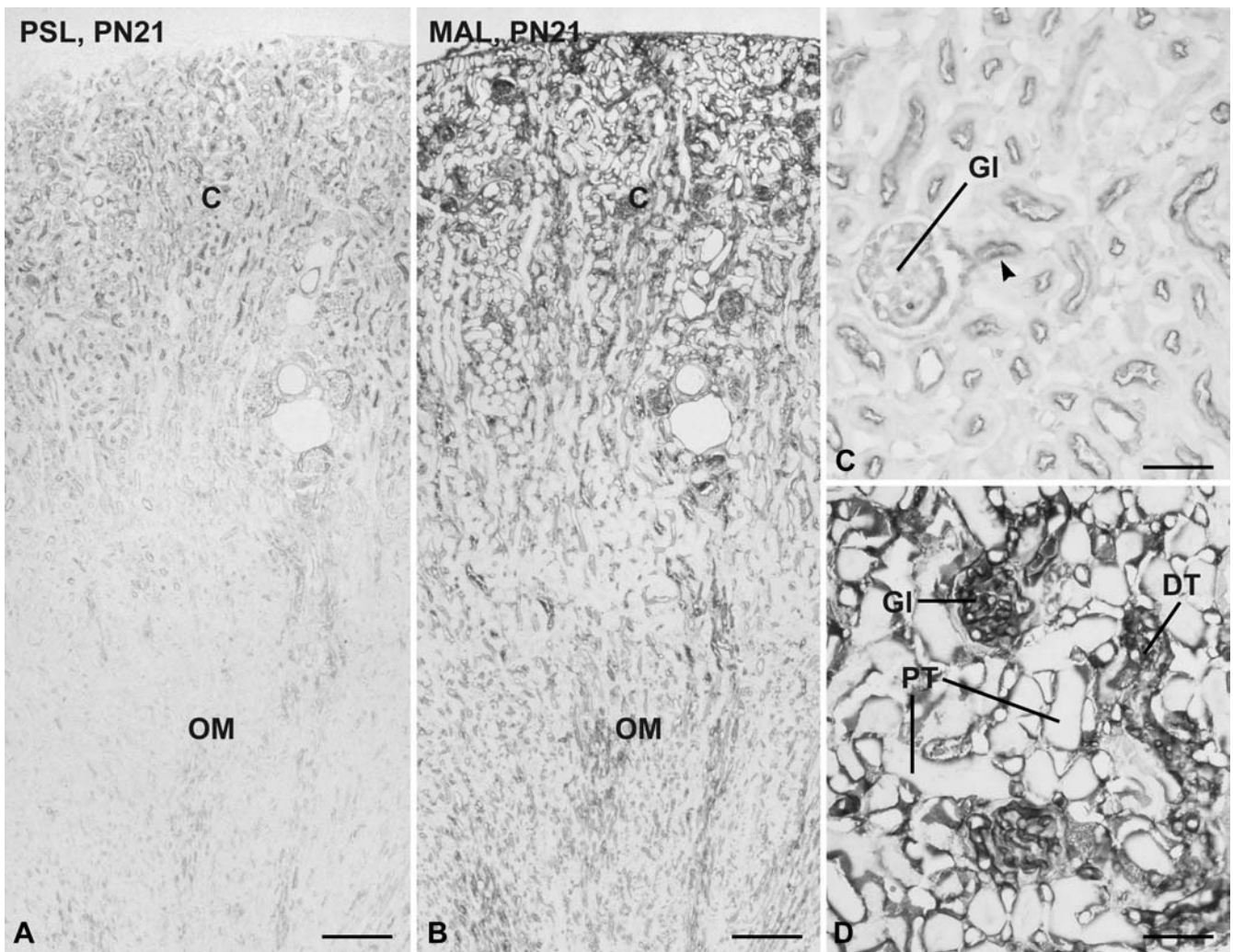


Fig. 6A–D Serial sections of paraffin-embedded rat kidney postnatal day 21. Low magnification showing staining for α 2,6-linked sialic acid (**A**) and α 2,3-linked sialic acid (**B**) in cortex (**C**) and adjacent outer medulla (**OM**). Details from cortex with glomeruli (**Gl**) and proximal tubules (*arrowhead* points to the origin of

proximal tubule) positive for α 2,6-linked sialic acid (**C**). Although glomeruli (**Gl**) and distal tubules (**DT**) are positive for α 2,3-linked sialic acid (**D**), proximal tubules (**PT**) in cortex are unreactive. *Bars* 120 μ m in **A**, **B**; 40 μ m in **C**, **D**

from postnatal day 2 onward, subtle changes in the collecting ducts were noted.

In the cortex, the glomeruli, parietal Bowman's capsule, proximal and distal tubules with the loops of Henlé in between and including the macula densa, cortical collecting ducts, and intertubular capillaries as well as small arteries and veins were positive with PSL (Fig. 7A–C). Proximal tubules stained stronger with PSL than distal tubules and cortical collecting ducts (Fig. 7B). Glomeruli were also positive for MAL but the staining was confined to the endothelia with the podocytes and mesangial cells being unreactive (compare Fig. 7F and C). As already observed in postnatal kidneys, the S1 and S2 segments of proximal tubules were unreactive with MAL (compare Fig. 7E, F and B, C). In contrast, the S3 of proximal tubules was weakly MAL positive (compare Fig. 8E and B). Thin loops were reactive with both lectins (Figs. 8, 10). Distal tubules and collecting ducts in cortex and outer

medulla as well as capillaries and small arteries and veins were stained with MAL (Fig. 7E, F). Although the epithelia lining the collecting ducts in cortex and outer as well as inner medulla were reactive with PSL (Fig. 8B, C), a mosaic of MAL-positive and MAL-negative epithelia in cortical and outer medulla collecting ducts was clearly visible (Figs. 8E, F, 9A–C). By electron microscopy, the intercalated cells were identified as the MAL-positive cells (Fig. 9D). In Fig. 10, a detail of the inner medulla is presented. The large collecting duct principal cells were unreactive with MAL, whereas epithelia of thin loops and capillary endothelia were labeled.

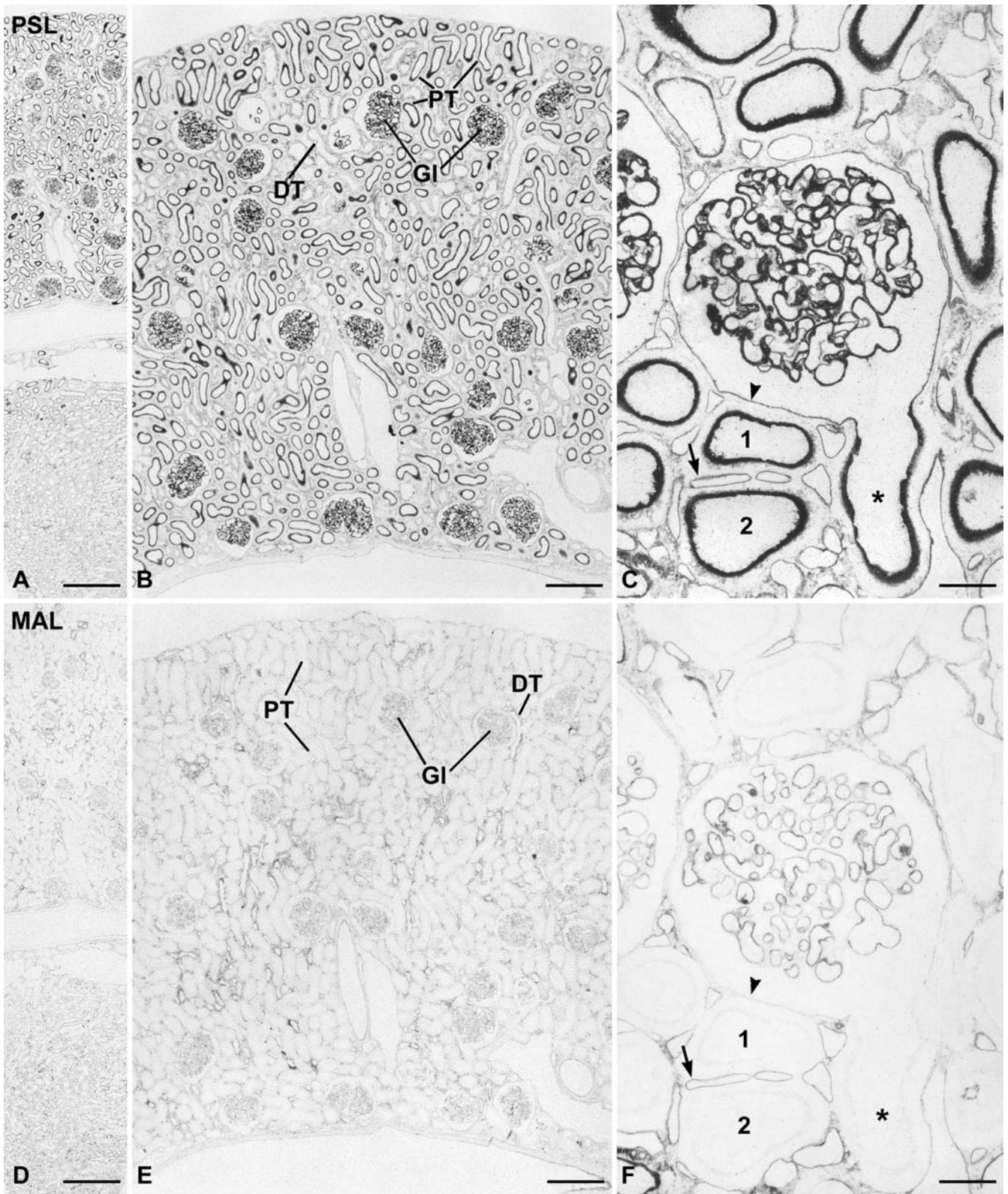


Fig. 7A–F Consecutive serial semithin sections from Lowicryl K4 M-embedded adult rat kidney. General staining pattern for $\alpha 2,6$ -linked sialic acid (A) and of $\alpha 2,3$ -linked sialic acid (D) in cortex and adjacent outer medulla. Glomeruli (GI) as well as proximal (PT) and distal tubules are intensely stained for $\alpha 2,6$ -linked sialic acid (B) whereas staining for $\alpha 2,3$ -linked sialic acid (E) in glomeruli (GI) and distal tubules (DT) is weak and absent in proximal tubules (PT). High power magnification reveals that podocytes, endothelia, and mesangial cells of glomeruli are positive

for $\alpha 2,6$ -linked sialic acid (C) whereas only the endothelia of glomeruli are positive for $\alpha 2,3$ -linked sialic acid (F). The origin of the proximal tubule at the urinary pole (asterisk in C and F) and cross-sectioned profiles of the same segment S1 of proximal tubules (1, 2 in C and F) are reactive for $\alpha 2,6$ -linked sialic acid (C) and not for $\alpha 2,3$ -linked sialic acid (F). Arrowhead Parietal Bowman's capsule, arrow cross-sectioned intertubular capillaries. Bars 50 μm in A, D; 20 μm in B, E; 5 μm in C, F

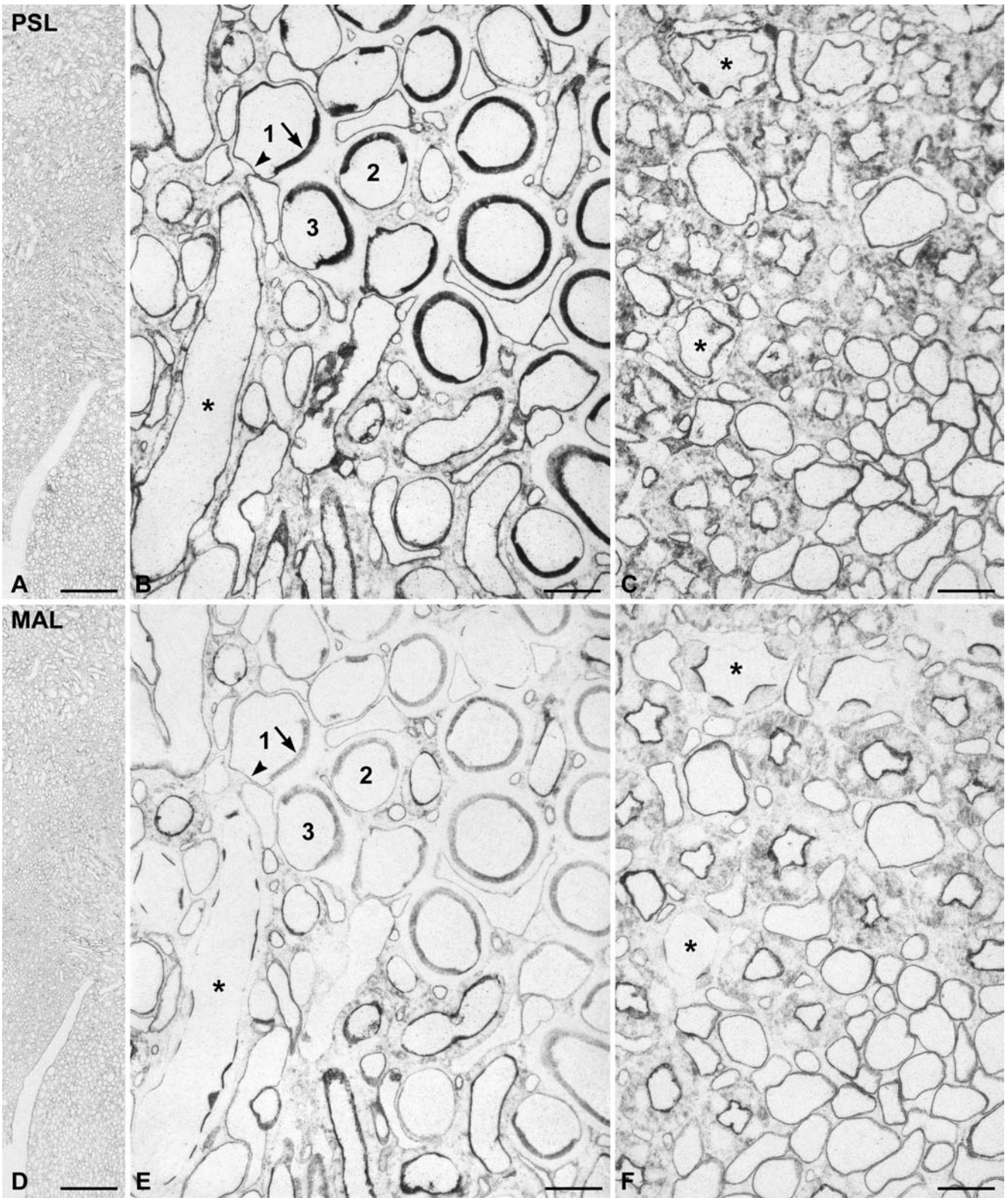


Fig. 8A–F Consecutive serial semithin sections from Lowicryl K4 M-embedded adult rat kidney. General staining pattern for α 2,6-linked sialic acid (**A**) and of α 2,3-linked sialic acid (**D**) in parts of outer and inner medulla. **B**, **E** Cross-sectioned profiles of the same tubules (1–3 in **B** and **E**) showing transition of S3 segment of proximal tubule (arrow in **B** and **E**) in descending thin loop (arrowhead in **B** and **E**) are positive for both α 2,6-linked sialic acid

(**B**) and α 2,3-linked sialic acid (**E**). All epithelia of collecting ducts are positive for α 2,6-linked sialic acid (asterisk in **B** and **C**) whereas the same collecting ducts exhibit a mosaic of cells positive and negative for α 2,3-linked sialic acid (asterisk in **E** and **F**). Loops of Henlé and capillaries are positive for both α 2,6- and α 2,3-linked sialic acid (**C** and **F**). Bars 50 μ m in **A**, **D**; 5 μ m in **B**, **C**, **E**, **F**

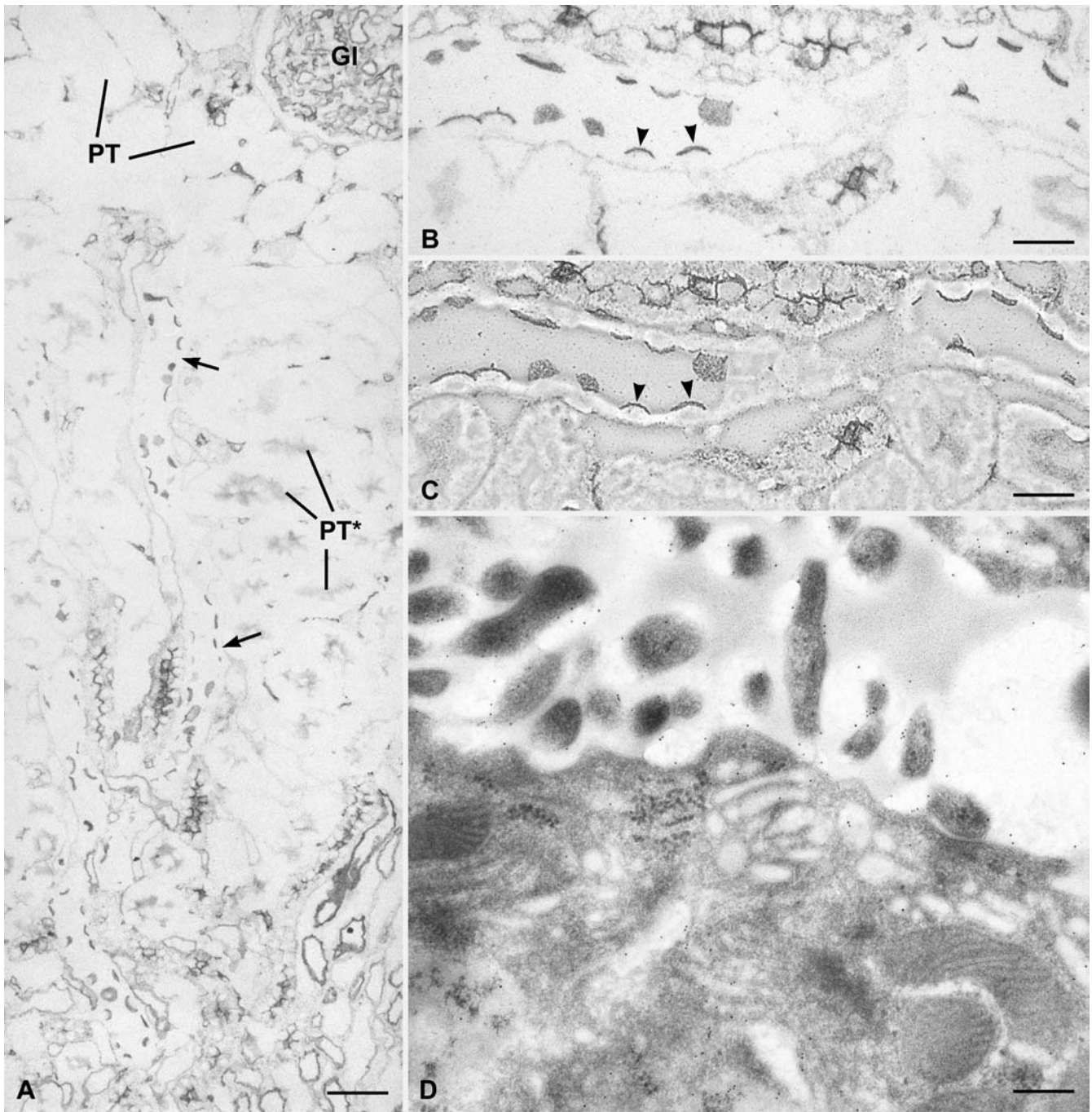


Fig. 9A–D Adult rat kidney, Lowicryl K4 M embedding, detection of α 2,3-linked sialic acid. Detail from cortex and outer medulla with glomerulus (*Gl*) and unreactive S1 and S2 proximal tubules (*PT*) as well as weakly stained S3 proximal tubules (*PT**). Collecting ducts (*arrows* in **A**) with a mosaic of positive

(*arrowheads* in **B** and **C**) and negative cells are seen at higher magnification in **B** and **C**. The positive cells were identified as intercalated cells by electron microscopy and MAL-gold labeling (**D**). *Bars* 15 μ m in **A**; 10 μ m in **B**, **C**; 0.25 μ m in **D**

Sialyltransferase activities in embryonic, postnatal, and adult kidney

A more than threefold difference in specific activity between β -galactoside α 2,6 sialyltransferase (ST6Gal-I) and β -galactoside α 2,3 sialyltransferase (ST3Gal-III) was measured in aliquots of pooled homogenates from

embryonic day 20 kidneys (Fig. 11). Although activity levels of both sialyltransferases decreased postnatally, the one for β -galactoside α 2,6 sialyltransferase was always higher and decreased steadily, whereas the activity levels for β -galactoside α 2,3 sialyltransferase remained steady from postnatal day 5 onward. As compared to embryonic and postnatal kidneys, activity levels for both sialyltrans-

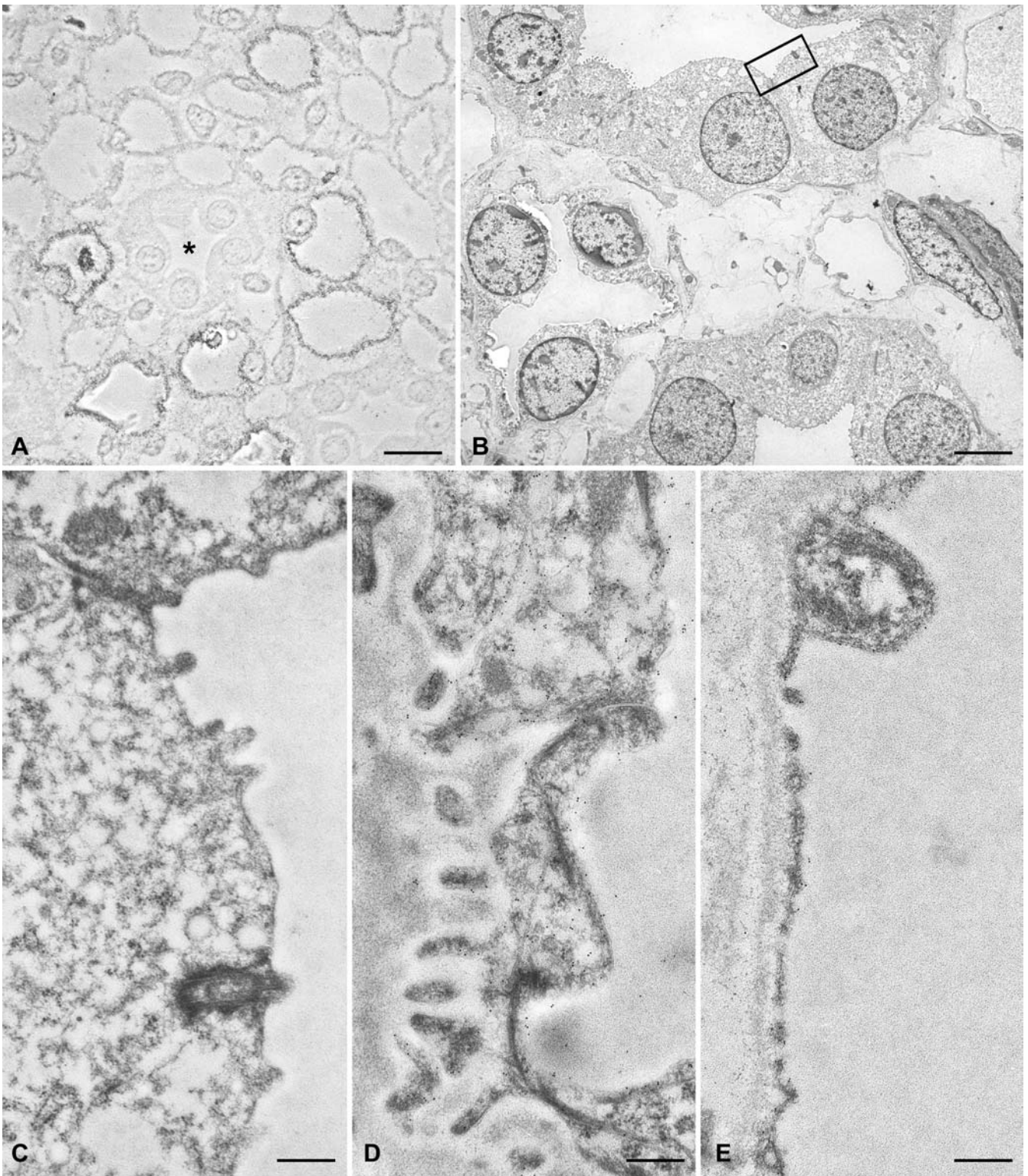


Fig. 10A–E Details from inner medulla of adult rat, Lowicryl K4 M embedding, detection of α 2,3-linked sialic acid. Principal cells of collecting ducts (*asterisk* in **A**) are unreactive and this was confirmed by electron microscopic MAL–gold labeling (**B**, **C**),

whereas gold particle labeling was detectable in thin loops (**D**) and endothelia of capillary (**E**). *Bars* 8 μ m in **A**; 2 μ m in **B**; 0.25 μ m in **C**; 0.3 μ m in **D**, **E**

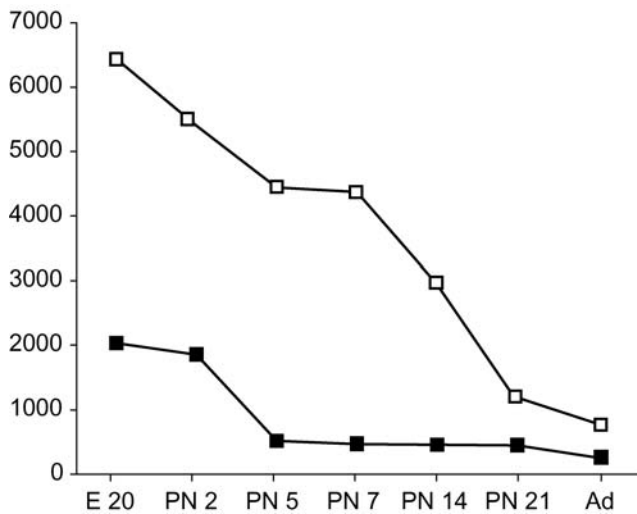


Fig. 11 Enzyme activity levels for β -galactoside α 2,6 sialyltransferase (open squares) and β -galactoside α 2,3 sialyltransferase (filled squares) in embryonic (E), postnatal (PN), and adult (Ad) kidneys. Enzyme activity levels are expressed as picomoles of [14 C] NeuAc transferred per milligram of acceptor substrate per hour

ferases were lowest in adult kidney. The activity for β -galactoside α 2,6 sialyltransferase as compared to that of β -galactoside α 2,3 sialyltransferase was almost fourfold higher in homogenates prepared from whole kidneys (Fig. 11). When microscopically dissected cortex, outer medulla, and inner medulla were assayed separately, activity for both sialyltransferases was lowest in cortex and highest in inner medulla (Fig. 12). Although activity for β -galactoside α 2,6 sialyltransferase as compared to β -galactoside α 2,3 sialyltransferase was higher in all three regions, this difference was most striking in inner medulla.

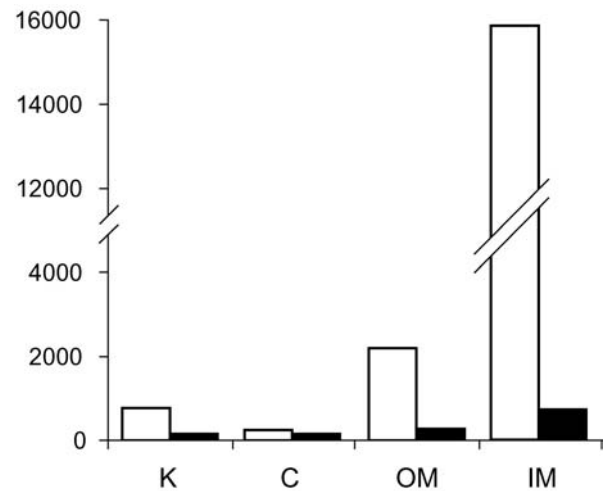


Fig. 12 Enzyme activity levels for β -galactoside α 2,6 sialyltransferase (open columns) and β -galactoside α 2,3 sialyltransferase (filled columns) in whole adult kidney (K), dissected cortex (C), outer medulla (OM), and inner medulla (IM). Enzyme activity levels are expressed as picomoles of [14 C] NeuAc transferred per milligram of acceptor substrate per hour

Lectin blot analysis of embryonic, postnatal, and adult kidney

The aim of this analysis was to establish solely the pattern of PSL- and MAL-reactive glycoproteins and not to identify specific glycoproteins.

Although different from each other, the pattern of reactive bands for PSL and MAL was remarkably steady when homogenates of whole kidneys from embryonic day 20, postnatal days 2–21, and adult kidney were investigated (Fig. 13). Interestingly, only few major reactive bands were detected in the 3–15% gradient gels. A band with an approximate molecular mass of 200 kDa was reactive with both PSL and MAL. Although its reactivity with MAL was observed in embryonic day 20 and all postnatal stages studied as well as in adult kidney,

Fig. 13 Lectin blot analysis of glycoproteins bearing α 2,6-linked sialic acid (α 2,6 NANA) and α 2,3-linked sialic acid (α 2,3 NANA), kidney homogenates resolved in 3–15% SDS-polyacrylamide gels. Lanes 1 Embryonic day 20, lanes 2 postnatal day 2, lanes 3 postnatal day 5, lanes 4 postnatal day 7, lanes 5 postnatal day 14, lanes 6 postnatal day 21, lanes 7 whole adult kidney, lanes 8 adult kidney cortex, lanes 9 adult kidney inner medulla

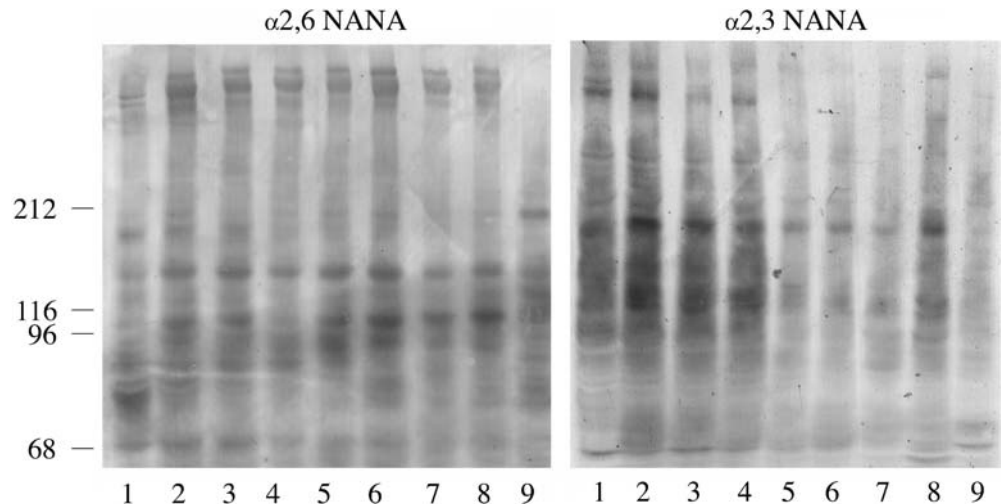
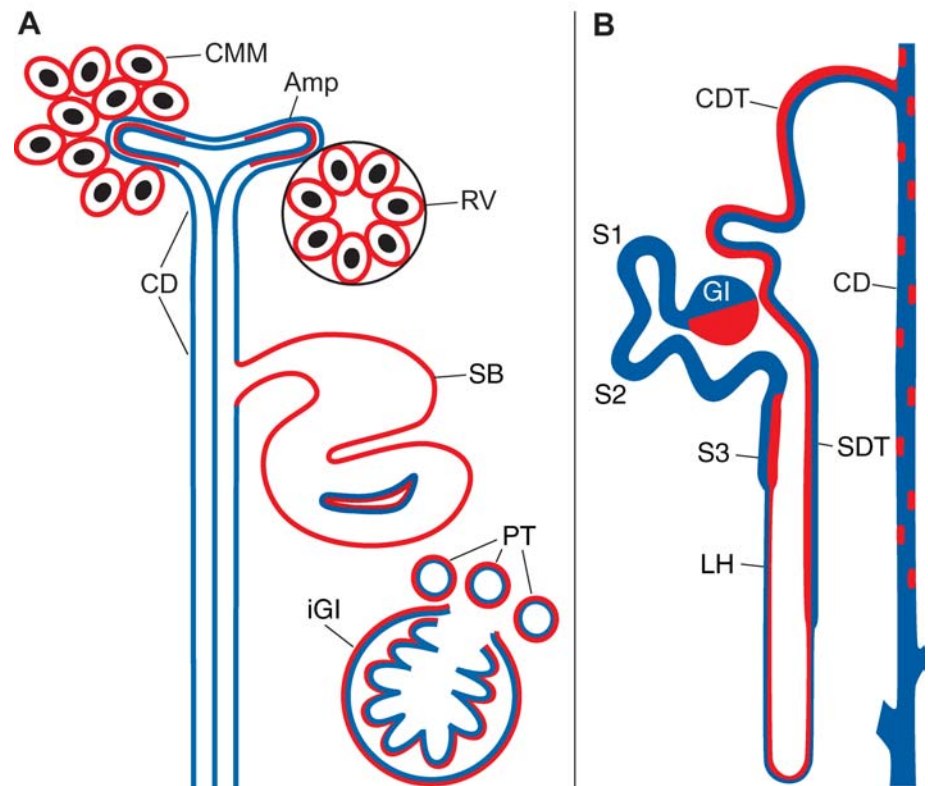


Fig. 14A, B Schematic presentation of the distribution of $\alpha 2,6$ - and $\alpha 2,3$ -linked sialic acids in embryonic and adult rat kidney. **A** Embryonic kidney with condensed metanephrogenic mesenchyme (*CMM*), renal vesicle (*RV*), S-shaped body (*SB*), immature glomerulus (*iGl*), immature proximal tubule (*PT*), collecting duct (*CD*), and ampulla of collecting duct (*Amp*). **B** Adult kidney with glomerulus (*Gl*), S1–S3 segment of proximal tubule (*S1*, *S2*, *S3*), loop of Henlé (*LH*), straight distal tubule (*SDT*), convoluted distal tubule (*CDT*), and collecting duct (*CD*). *Blue* coloration in **A** and **B** indicates presence of $\alpha 2,6$ -linked sialic acid as detected with PSL and SNL, whereas *red* represents $\alpha 2,3$ -linked sialic acid as detected with MAL.



it became undetectable with PSL from postnatal day 7 onward. One of the high molecular mass bands seen in all samples (except for medulla) apparently represents megalin in cortex (Christensen et al. 1995; Farquhar et al. 1995; Kerjaschki and Farquhar 1982), which has previously been shown to carry both $\alpha 2,6$ - and $\alpha 2,3$ -linked sialic acids (Morelle et al. 2000; Ziak et al. 1999). When microscopically dissected cortex and inner medulla of adult kidney were probed with PSL and MAL, remarkable differences were observed between the lectins and the kidney regions investigated (Fig. 13 lanes 8, 9).

Discussion

In the present study, we showed that: (a) *N*-glycans terminated in $\alpha 2,6$ - and $\alpha 2,3$ -linked sialic acid as well as activity for the respective sialyltransferases are detectable in embryonic, postnatal, and adult kidney, (b) that $\alpha 2,6$ - and $\alpha 2,3$ -linked sialic acids are differently expressed in the two embryonic anlagen and in the mesenchyme-derived early stages of nephron morphogenesis (Fig. 14A), and (c) that regional and cell type-specific differences exist in adult kidney (Fig. 14B). Lectin blot analysis revealed steady patterns of bands reactive for $\alpha 2,6$ - and $\alpha 2,3$ -linked sialic acids from embryonic to postnatal and adult kidneys. The activity of both the β -galactoside $\alpha 2,6$ sialyltransferase and β -galactoside $\alpha 2,3$ sialyltransferase declined from embryonic over postnatal to adult kidney. The activity for β -galactoside $\alpha 2,6$

sialyltransferase was always three to fourfold higher than β -galactoside $\alpha 2,3$ sialyltransferase.

The present lectin histochemical results indicate that distinct patterns of expression of $\alpha 2,6$ - and $\alpha 2,3$ -linked sialic acids exist during embryonic metanephron formation and postnatal kidney differentiation. One concerns the metanephrogenic mesenchyme which was reactive with MAL, but not with PSL and SNL, from embryonic day 16 until about postnatal day 5 after which nephrogenesis in rat kidney ceases. This indicates that the condensed metanephrogenic mesenchyme is rich in *N*-glycans bearing $\alpha 2,3$ -linked sialic acids. This was also observed for the loose mesenchyme in embryonic, postnatal, and adult kidney. In contrast, $\alpha 2,6$ -linked sialic acids were absent from this embryonic anlage or existed in amounts too low to be detectable with PSL and SNL. Likewise, the loose mesenchyme was unreactive with PSL and SNL in embryonic, postnatal, and adult kidney. In this regard, the only other studied sialoglycoconjugates were the poly $\alpha 2,8$ -neuraminic acid on NCAM (Lackie et al. 1990; Roth et al. 1987) and the oligo/poly $\alpha 2,8$ -KDN of megalin (Ziak and Roth 1999) which exhibited a mutually exclusive distribution with the poly $\alpha 2,8$ -neuraminic acid being detectable in the metanephrogenic mesenchyme. A local mediator of signaling events during metanephrogenic mesenchyme induction is *Wt1* and together with *Wnt-4* is representative of the very few genes expressed in the mesenchyme but not in the ureteric bud (Armstrong et al. 1993; Bard et al. 1994; Kispert et al. 1998; Kreidberg et al. 1993; Stark et al. 1994).

Another difference in expression of $\alpha 2,6$ - and $\alpha 2,3$ -linked sialic acids concerns the mesenchyme–epithelium transition and glomerulus morphogenesis. All stages of nephron formation were reactive with MAL, as shown previously with the sialic acid specific *Limax flavus* lectin (Wagner and Roth 1988) and for poly $\alpha 2,8$ -neuraminic acid on NCAM (Lackie et al. 1990; Roth et al. 1987). In contrast, the renal vesicle, which represents the first epithelial differentiation, was unreactive with PSL and SNA. The S-shaped body which consists of a proximal and a distal portion gives rise to the glomerulus and tubular part of the nephron, respectively, exhibited PSL and SNL staining only in the presumptive visceral (podocytes) and parietal Bowman's epithelium which coincides with transient labeling by GalNAc-reactive lectins (Laitinen et al. 1987) and the expression of WT-1 (Horster et al. 1999). However, immature proximal tubules occurring with the capillary loop stage were then reactive with both PSL and SNL. This indicates that $\alpha 2,6$ -linked sialic acids occur rather late in early nephrogenesis and initially are cell type restricted. They are, however, maintained during maturation of the tubule epithelium and persist in adult kidney. This contrasts with the MAL reactivity in proximal tubules which is transient during embryonic development and no longer observed in S1 and S2 segments of mature proximal tubules from postnatal day 2 kidneys onward. A similar transient expression of poly $\alpha 2,8$ -neuraminic acid on NCAM was observed in chicken and rat kidney (Lackie et al. 1990; Thiery et al. 1982). With regard to the mechanism of remodeling of the S-shaped body which results in the formation of a double slit, changes in cell–cell contact and adhesiveness have been postulated to be involved (Hilfer and Hilfer 1983; Kiremidjian and Kopac 1972). One might speculate that negatively charged sialic acids are involved in this process (Lackie et al. 1990; Roth et al. 1987; Ziak and Roth 1999; present study).

The ureteric bud and its ramification give rise to the collecting duct system. Both, PSL and SNL staining indicative of the presence of $\alpha 2,6$ -linked sialic acids were detectable in the entire collecting duct system of embryonic kidneys in contrast to the metanephrogenic mesenchyme. Staining persisted in postnatal kidneys and was observed in collecting ducts of adult kidneys as well. However, MAL staining indicative of $\alpha 2,3$ -linked sialic acids was detectable only in the terminal ampullae (tips) of the collecting ducts during embryonic days 16, 18, and 20. This resembled the reported restricted expression of poly $\alpha 2,8$ -neuraminic acid on NCAM (Lackie et al. 1990) and coincides with the expression of *Wnt-11* (Horster et al. 1999).

The observed changing pattern of $\alpha 2,6$ - and $\alpha 2,3$ -linked sialic acids as detected by lectin histochemistry during induction, nephrogenesis, and differentiation in embryonic and postnatal rat kidneys revealed a high degree of cell type-specific expression during these processes. Their functional importance remains to be investigated. Due to the terminal position of sialic acids in glycans at the cell surface, it has been suggested that they

are involved in cell–cell interactions during embryonic differentiation and functional maturation in adult organs. The regulated expression of a Gal $\beta 1,3$ GalNAc $\alpha 2,3$ sialyltransferase in developing thymocytes and during T lymphocyte activation (Gillespie et al. 1993; Priatel et al. 2000) and the control of lymphocyte activation by the Gal $\beta 1,4$ GlcNAc $\alpha 2,6$ sialyltransferase provide appropriate examples (Hennet et al. 1998). However, in Gal $\beta 1,4$ GlcNAc $\alpha 2,6$ sialyltransferase knockout mice, biological defects in addition to those of B and T lymphocytes are unknown at present (Martin et al. 2002).

In adult kidney, all cells of the nephron and the collecting duct system expressed $\alpha 2,6$ -linked sialic acids and this correlates well with previously published data of Gal $\beta 1,4$ GlcNAc $\alpha 2,6$ sialyltransferase immunohistochemistry (Burger et al. 1998; Kaneko et al. 1995) and SNL staining (Kaneko et al. 1995). In contrast, expression of $\alpha 2,3$ -linked sialic acid of *N*-glycans in kidney cortex was much more limited. We previously reported an even more restricted expression of $\alpha 2,3$ -linked sialic acid of *O*-glycans in cortex and parts of medulla (Toma et al. 1999). Based on the intensity of staining and abundance of positive structures, kidney cortex contains $\alpha 2,6$ -linked sialic acid of *N*-glycans in higher amounts than $\alpha 2,3$ -linked sialic acid which is in contrast to most other organs. Based on the same histochemical criteria, the kidney medulla appears to contain similar amounts of $\alpha 2,6$ - and $\alpha 2,3$ -linked sialic acids of *N*-glycans. At the cellular level, other remarkable differences existed. Proximal tubular epithelia in S1 and S2 segments were unreactive with MAL, whereas those in S3 segment were positive for $\alpha 2,3$ -linked sialic acid. To our knowledge, the only other example of such a drastic difference in glycocalyx composition in a single cell type depending on its position in the nephron are the intercalated cells of collecting ducts with regard to reactivity toward the *Helix pomatia* lectin (Brown et al. 1985). Although the principal and intercalated cells of collecting ducts were positive with PSL, SNL, and MAL in all studied embryonic and postnatal stages, in adult kidney, principal cells were reactive with PSL and SNL but not with MAL. Thus, our study revealed that regional and cell type-related expression patterns for $\alpha 2,6$ - and $\alpha 2,3$ -linked sialic acids of *N*-glycans are characteristic features of rat kidney.

By immunocytochemistry, Gal $\beta 1,4$ GlcNAc $\alpha 2,3$ sialyltransferase immunoreactivity was detected in S1 and S2 segments of proximal tubules and in principal cells of collecting ducts (Burger et al. 1998). We were unable to detect the product of this sialyltransferase in these regions. The most plausible explanation could be that this is the result of competition between the β -galactoside $\alpha 2,6$ sialyltransferase and β -galactoside $\alpha 2,3$ sialyltransferase for the common donor substrate. In this regard, we found higher activity levels for Gal $\beta 1,4$ GlcNAc $\alpha 2,6$ sialyltransferase in adult kidney and also in embryonic and postnatal kidneys. However, it may not be appropriate to relate results of the localization at the cellular level to those obtained from homogenates of the entire organ. We consider it unlikely that the undetectability of $\alpha 2,3$ -linked

sialic acids in S1 and S2 segments of proximal tubules and principal cells of collecting ducts is due to a shift from sialylation to fucosylation as reported for postnatal small intestine (Taatjes and Roth 1990), because fucose residues in adult kidney are detectable only in endothelia (Holthöfer et al. 1981; Roth 1983). Based on the lectin blot analysis, we also consider it unlikely that lack of acceptor substrate for the Gal β 1,4 GlcNAc α 2,3 sialyltransferase, which has been shown in another system to be the rate limiting step (Baum et al. 1996), is the cause although this would require the application of techniques providing higher resolution.

In summary, we found that commonly occurring α 2,6- and α 2,3-linked sialic acids are differently expressed in the two embryonic anlagen of kidney, exhibit a dynamic expression pattern during nephron morphogenesis and differentiation, and show regional as well as cell type-specific expression in adult kidney.

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