Developmental expression of sodium entry pathways in rat nephron

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Schmitt, Roland, David H. Ellison, Nicolette Farman, Bernard C. Rossier, Robert F. Reilly, W. Brian Reeves, Ilse Oberbäumer, Rosemarie Tapp, and Sebastian Bachmann. Developmental expression of sodium entry pathways in rat nephron. Am. J. Physiol. 276 (Renal Physiol. 45): F367–F381, 1999.—During the past several years, sites of expression of ion transport proteins in tubules from adult kidneys have been described and correlated with functional properties. Less information is available concerning sites of expression during tubule morphogenesis, although such expression patterns may be crucial to renal development. In the current studies, patterns of renal axial differentiation were defined by mapping the expression of sodium transport pathways during nephrogenesis in the rat. Combined in situ hybridization and immunohistochemistry were used to localize the Na-Pi cotransporter type 2 (NaPi2), the bumetanide-sensitive Na-K-2Cl cotransporter (NKCC2), the thiazide-sensitive NaCl cotransporter (NCC), the Na/Ca exchanger (NaCa), the epithelial sodium channel (rENaC), and 11β-hydroxysteroid dehydrogenase (11HSD). The onset of expression of these proteins in the nephron has been shown to be expressed by distal nephron cells, together with 11β-hydroxysteroid dehydrogenase type 2 (11HSD) and mineralocorticoid receptors (5, 6, 25, 26). Recent evidence points out that mutations in these genes lead to disorders of sodium and volume homeostasis (37). Mendelian forms of human hyper- or hypotension have been linked, using molecular genetics, to mutations of a number of these genes, including NKCC2, NCC, rENaC, and 11HSD (27, 39).

The kidney plays a central role in fluid and electrolyte homeostasis. Several genes that participate in sodium reabsorption along the nephron have recently been cloned; human mutations of these genes cause alterations in blood pressure (27, 39). These genes encode ion transport proteins such as exchangers (36), cotransporters (16), and ionic channels (7). From an anatomic standpoint, the nephron may be divided into proximal and distal portions. The proximal tubule comprises convoluted and straight portions. The distal tubule comprises the medullary and cortical thick ascending limbs (TAL), the distal convoluted tubule (DCT), the connecting tubule (CNT), and the cortical and medullary collecting ducts (CCD and MCD, respectively). Each of these segments has unique functional properties that have been investigated using physiological techniques (9, 11, 14, 41).

Recent molecular studies on the distribution of sodium transporters have more precisely defined nephron segmentation. The Na/H exchanger (NHE3; Ref. 4) and the Na-Pi cotransporter type 2 (NaPi2; Ref. 10) are both expressed by cells of the proximal tubule. NHE3 contributes importantly to Na and bicarbonate reabsorption, whereas NaPi2 contributes to Na and phosphate reabsorption. Although proximal tubular sodium absorption may adapt to states of volume expansion or contraction, changes in urinary sodium and chloride excretion reflecting variations in salt intake are mainly governed by distal tubular functions. In the ascending limb of the loop of Henle, expression of the electrolyte-sensitive Na-K-2Cl cotransporter (NKCC2) has been found in the TAL, the macula densa, and a portion beyond the macula densa (32). A structurally related thiazide-sensitive NaCl cotransporter (NCC) was localized to the DCT (2, 33, 35). The amiloride-sensitive epithelial sodium channel (rENaC; Ref. 7) has been shown to be expressed by distal nephron cells, together with 11β-hydroxysteroid dehydrogenase type 2 (11HSD) and mineralocorticoid receptors (5, 6, 25, 26). Recent evidence points out that mutations in these genes lead to disorders of sodium and volume homeostasis (37).
During development, the mammalian nephron and collecting duct (CD) system undergo complex, transient patterns of structural differentiation on their way to segmental specialization (12, 30, 31). Despite extensive study, however, it has been difficult to identify developmental regions of transition, especially the junction between the ureteric bud and the nephrogenic blastema. This study examines the expression patterns of sodium transport proteins in the developing rat nephron using high-resolution histochemical techniques. By recording the changing patterns of expression of these proteins in the maturing epithelia, new aspects of the genesis of segmentation of the renal tubule are suggested. The definition of a normal ontogenetic expression of ion transport pathways is thought to provide a basis for the study of transport disorders in later life which may originate during ontogeny.  

**MATERIALS AND METHODS**

**Animals.** For perfusion fixation, Sprague-Dawley rats at postnatal stages of 1, 3, 4, and 8 days were anesthetized with ether. For immunocytochemistry and in situ hybridization, kidneys were perfused via cannulation of the left cardiac ventricle. Perfusion was performed using freshly prepared 3% paraformaldehyde (PFA) in PBS at pH 7.4 for 5 min. To protect the tissues from freezing artifacts, kidneys were subsequently rinsed in situ with a sucrose-PBS solution adjusted to 800 mosmol/kg. Kidneys were then removed, cut into slices, and shock-frozen in liquid nitrogen-cooled isopentane. For fine structural morphology, animals were anesthetized with an intraperitoneal injection of Nembutal (40 mg/kg body wt), and kidneys were retrogradely perfused fixed via cannulation of the abdominal aorta using a solution of 3% glutaraldehyde and 1.5% PFA buffered with PBS, pH 7.4. Kidneys were then removed and processed for Epon embedding.  

Morphology. Semithin sections (1 µm) were cut and stained in Richardson's solution. Slides were viewed in a Leica DMRB microscope. For electron microscopic studies, ultrathin sections were analyzed after contrasting in uranyl acetate and lead citrate. Sections were examined with a Zeiss EM 900 electron microscope.  

In situ hybridization. For generation of a riboprobe to the NCC, a 712-bp partial cDNA fragment of mouse NCC was used (33). A riboprobe to NKCC2 was prepared from a 375-bp partial cDNA fragment of the mouse NKCC2 (32). The NaPi2 probe was prepared from a cDNA fragment that was generated by RT-PCR comprising the sequence from position 1561 to 2355 of rat NaPi2; the identity of the cDNA probe was verified by sequencing. An NAD-dependent 11HSD probe was generated from a 248-nucleotide cDNA fragment of rat kidney 11HSD (5). These fragments were all subcloned into the EcoRI site of pBluescript KS+ vector (Stratagene, La Jolla, CA). To generate sense and antisense riboprobes for 11HSD, the vector was linearized either by Hind III or by BamHI, respectively. For generation of NCC, NaPi2, and NKCC2 riboprobes, PCR fragments comprising the respective insert and specific transcription promoters were synthesized using vector-specific complementary oligonucleotides. RNA probes were synthesized and labeled by in vitro transcription using digoxigenin (DIG)-labeled UTP and T3 or T7 RNA polymerases (Boehringer, Mannheim, Germany) to obtain either sense (control) or antisense transcripts, respectively.  

In situ hybridization on 7-µm cryostat sections was essentially done as described (32). Briefly, DIG-labeled probes were used at concentrations of 2 to 8 ng/ml of hybridization mixture. Hybridization was performed at 40°C for 18 h. Slides were washed at 40°C for 30 min in 1× SSC containing 50% formamide, followed by two washes for 30 min in 0.4× SSC containing 50% formamide and two subsequent washes for 30 min in 0.1× SSC containing 50% formamide. Subsequently, slides were rinsed twice in 0.5× SSC at room temperature for 10 min, followed by a rinse in 0.2× SSC for 10 min and another two rinses in buffer I (100 mM Tris·HCl and 150 mM NaCl at pH 7.5). After quenching of nonspecific antibody binding sites with blocking medium (2% normal sheep serum, 0.5% BSA, 3% Triton X-100 in buffer 1), sheep anti-DIG-alkaline phosphatase conjugate (diluted 1:500 in blocking medium) was administered to the sections. After removal of excess antibody, the substrate (nitroblue tetrazolium with 5-bromo-4-chloro-3-indolyl phosphate) for alkaline phosphatase was added.  

In situ hybridization for detection of rENaC mRNA was performed with radiolabeled riboprobes. To prepare riboprobes, the 3'-untranslated region of the β- and γ-subunits of rENaC (corresponding to nucleotides 2150 to 2463 for the β-subunit, and 2470 to 2911 for the γ-subunit) were subcloned into pBluescript KS+ vector and linearized with BamHI I or Kpn I. Probes were synthesized using a T3/T7 in vitro RNA synthesis kit (Promega) in the presence of 35S-labeled UTP (Amersham). Hybridization was essentially done as described (13). In brief, cryostat sections were postfixed in 4% PFA, treated with proteinase K, acetylated, covered with the hybridization mixture, and incubated overnight at 50°C. For simultaneous detection of NKCC2 and NaPi2 mRNA, the two cRNA probes were combined in the same hybridization mix and applied to the same section. Washing was done with 5× SSC and 10 mM dithiotreitol (DTT) at 50°C for 30 min, followed by a high-stringency wash in 50% formamide, 2× SSC, and 0.1 M DTT at 65°C for 20 min, then several washes in NaCl-Tris-EDTA (0.5 M NaCl, 5 mM EDTA, and 10 mM Tris·HCl, pH 7.4) at 37°C. After subsequent RNase treatment, sections were rinsed in 0.1× SSC for 15 min, dehydrated, and dried. For autoradiographic detection, slides were dipped in Kodak NTB2 photographic emulsion (Kodak, Rochester, NY), dried, and exposed at −20°C for 2 wk. Prior to viewing, slides were counterstained with hematoxylin-eosin.  

Generally, control experiments were done using sense probes for the respective cRNA probes; throughout all experiments, the controls yielded uniformly negative results.  

**Immunocytochemistry.** For immunolabeling, polyclonal antibodies, which had been characterized previously (5, 13, 25, 36), were used. Antibody to NCC was generated against a fusion protein containing the entire NH2-terminal tail of mouse NCC (5). Antibody to 11HSD was generated against a fusion protein corresponding to human 11HSD (25). For generation of antibodies to the α-, β-, and γ-subunit of rENaC, fusion proteins from the NH2-terminal end of α-rENaC (amino acids E10 to F77), from the COOH-terminal end of β-rENaC (amino acids G559 to E636), and from the COOH-terminus of γ-rENaC (amino acids A570 to L650) were used (13). The strongest immunoreactive signal in tissue sections was obtained with anti-β antibody. All these antibodies were generated in rabbits. Antibody against the Na/Ca exchanger (NaCa) was generated in guinea pig against a fusion protein of rabbit NaCa (36). Antibodies to NaCa and rENaC were used at a dilution of 1:100 in PBS, antibody to NCC was used at a dilution of 1:200, and antibody to 11HSD was used at a dilution of 1:200–1:1,000. Antibody to band 3 was used as a control marker for type A intercalated cells (type A IC,
Fig. 1. Overview showing in situ hybridization with digoxigenin (DIG)-labeled riboprobe for Na-K-2Cl cotransporter type 2 (NKCC2) (left), Na-Pi cotransporter type 2 (NaPi2) (middle), and Na-Cl cotransporter (NCC) (right) on kidneys of postnatal day 3 (a–c) and postnatal day 8 (a’–c’). Developmental labeling patterns for NKCC2, for NaPi2, and for NCC are presented. Signal for all probes is absent from subcapsular nephrogenic zone on day 3 (arrowheads). Signals for both NKCC2 and NaPi2 (a and b) in the outer cortex close to nephrogenic zone are stronger than NCC signal (c) on day 3. On day 8, cortex has become larger and formation of new nephrons is completed so that signals are present in tubule profiles directly beneath the renal capsule (a’–c’). Cortical-medullary boundary is indicated by broken lines in 8-day-old kidneys. Magnification, ×110.
RESULTS

Identification of nephrogenic stages. In rat, the centrifugal pattern of fetal nephrogenesis is continued after birth for approximately 6 days so that all the distinct nephrogenic stages may be studied in the kidneys of newborn rats. The development of the nephron has been divided into five clearly distinguishable stages (24). Stage I corresponds to the renal vesicle, stage II to the S-shaped body. Stages I and II are located within the nephrogenic zone, which is the region of nephron induction directly beneath the renal capsule. In stage III, the glomerulus is oval or spherical and the typical glomerular capillary loops are formed. The glomerular visceral epithelial cells are still closely apposed to each other with narrow intercellular spaces. At this stage, the first signs of epithelial segmentation of the tubule become obvious at the ultrastructural level. The loop of Henle does not yet descend past the medullary pole of the glomerulus. In stage IV, the glomerulus has become larger and capillary loops are more numerous than in the preceding stage. The primitive loop of Henle has by then descended beyond the medullary pole of the glomerulus but still lacks a tubular lumen. Later in stage IV, the “primitive loop” transforms into the “immature loop” of Henle with a patent lumen (30). At this level, the proximal tubular epithelium carries the typical brush border, and all the segments defined in the adult nephron can be recognized at the gross structural level in stage IV. In stage V, the nephron is approaching mature morphology.

Expression of NKCC2. In the mature nephron, NKCC2 is expressed by cells of the TAL including the macula densa and a short post-macula segment (32). During early postnatal stages (days 1, 3, and 4), NKCC2 mRNA was absent from the nephrogenic zone directly beneath the renal capsule (Fig. 1a). On postnatal day 8, when formation of new nephrons is completed, NKCC2 expression was present in subcapsular tubule segments (Fig. 1a’). The first cells expressing NKCC2 were found in juxtaglomerular position of stage III nephrons. The direct contact of these cells to the vascular pole of the corresponding glomerulus suggests that they are future macula densa cells (Fig. 2a). With elongation of the loop past the medullary pole of the glomerulus, the intensity of NKCC2 expression in the macula densa region increased and extended into the ascending limb upstream to the future flow direction (Fig. 2b). At this level, the glomerulus was at an early capillary loop stage (Fig. 2d). Ultrastructurally, the prospective macula densa cells already showed particular features of the mature state such as basal and lateral membrane foldings (Fig. 2e), whereas the cells of the immature ascending limb did not yet exhibit characteristic membrane specializations (Fig. 2g). Apical junctional complexes and luminal microvilli were present in cells of the future macula densa region, even though a tubular lumen was not yet patent (Fig. 2f). During further maturation, the NKCC2 signal was found to extend in an upstream direction until expression was found along the entire ascending limb with the signal onset at the transition from the descending limb. The point of transition was located proximal to the bend of the loop of Henle in all newly formed immature loops. Since there is no thin ascending limb in the immature loop, the bend itself throughout expressed

Fig. 2. Early development of the loop of Henle. a: In situ hybridization demonstrates NKCC2 expression during late stage III in cells of the prospective macula densa region. AL and DL, ascending and descending limb of the loop, respectively. b: In early stage IV, NKCC2 signal extends further toward the bend of the loop; expression ends only a few cells beyond the immature macula densa (glomerulus is indicated by dots). c: Double application of NKCC2 and NaPi2 probes shows weak expression of NaPi2 in early proximal convolutions (PC), when NKCC2 signal is already clearly present in the future macula densa region. Offset of NaPi2 signal at the transition of PC into DL and the onset of NKCC2 signal within the AL are indicated by arrowheads. d: Ultrastructural morphology of developing loop of Henle in early stage IV (note indicated areas of enlargement for e, f, and g). e–g: Ultrastructural development of the thick ascending limb (TAL) and macula densa. Basolateral membrane specialization in the immature macula densa. Folding of the plasma membrane is obvious at the lateral (arrowheads) and basolateral membrane (arrow). f: Tubular lumen still consists of a narrow luminal cleft (arrowheads), whereas apical junctional complexes are already clearly developed (tight junctions indicated by arrows). g: Absence of basolateral membrane specialization in an immature AL cell. Magnifications: ×520 (a), ×480 (b and c), ×580 (d), ×71,000 (f), and ×11,100 (e and g).
NKCC2 (Fig. 3a). At the distal end of the TAL, expression of NKCC2 consistently ended a few cells beyond the macula densa at the site of transition to the future DCT.

Expression of NaPi2. To gain insight into concomitant maturation of the proximal tubule and in particular the descending limb of the developing loop, we investigated the developmental expression of NaPi2, which has been shown to be expressed along the proximal tubule in the adult nephron (10). Nephrons were showing first signs of NaPi2 when they were still located in the vicinity to the nephrogenic zone (Fig. 1b). In these stage III nephrons, NaPi2 mRNA appeared first in proximal convolutions, and signal ended at the transition to the descending limb of the primitive loop of Henle. Figure 2c shows the concomitant presence of (weak) NaPi2 mRNA signal in proximal convolutions and (stronger) NKCC2 mRNA signal in the macula densa region of the same nephron in early stage IV. In subsequent stages, NaPi2 did not occur in the descending limb until advanced NKCC2 expression in the corresponding ascending limb was found, which indicates a delayed maturation of the proximal straight tubule compared with the TAL. The complete structural maturation of the proximal nephron was achieved when NaPi2 signal had reached the renal capsule; at this time point, the entire proximal nephron was positively labeled for NaPi2 (Fig. 1b'). Whereas in the adult, NaPi2 expression showed a corticomedullary gradient with increasing signal intensity toward the medulla, such a gradient during ontogeny did not appear until day 8. In subcapsular region, however, NaPi2 expression was strong on day 8 as well (Fig. 1b').

Expression of NCC. In the mature nephron, NCC is expressed by cells along the entire DCT starting beyond the NKCC2-expressing post-macula segment of the TAL and ending at the transition into the CNT (33). A subsegmentation of the mature DCT has been described with a proximal portion (DCT1) containing exclusively NCC-expressing cells, and a distal portion (DCT2) revealing a hybrid pattern of both DCT and CNT specificities, i.e., expression of NCC as a marker of the DCT and concomitant presence of the NaCa (33) or calbindin (28) which typically label the CNT.

Initial expression of NCC occurred distally in DCT segments of early stage IV nephrons; these nephrons were already located deeper within the neonatal cortex than those nephrons exhibiting first expression of NKCC2 and NaPi2 (Fig. 1c). In advanced stage IV, NCC mRNA was present along the entire DCT, beginning distally of the ascending limb of the immature loop (Fig. 3c). At the distal pole of the DCT, signal was extending into longer portions ultimately joining the CNT. At this stage, the loop of Henle presented a patent tubular lumen, whereas the epithelium of the TAL was still composed of cuboidal cells lacking interdigitation and basal striation, both typical elements of the mature state (Fig. 3d). Generally, the distal portions of the distal convolutions showed stronger NCC mRNA expression than did the proximal ones, indicating that NCC expression is initiated at the distal end of the nascent DCT. To strengthen this observation, immunolabeling of NCC was combined with NKCC2 in situ hybridization; in stage III or beginning stage IV nephrons, a portion beyond the NKCC2-expressing post-macula segment was still NCC negative (Fig. 3, b and b'). Therefore the existence of an NCC-unreactive DCT portion between the TAL and NCC-positive distal DCT portions is likely to occur during a restricted period of development. When NCC immunoreactivity (IR) was arising in the initial DCT in later stage IV, partial overlap of luminal NCC IR and NKCC2 mRNA signal was observed (Fig. 3, e and e'). By contrast, in more mature nephrons, the proximal onset of NCC IR was abrupt and showed no overlap with NKCC2. Distally, early NCC expression frequently reached the point of junction with the arcades, and double labeling with NaCa identified those portions as DCT2 segments. In more mature stages, the tubular portions properly joining the arcades were assuming a CNT-specific character with absence of NCC signal. This was particularly evident in sites where consecutive CNT generations were joining to form an arcade (Fig. 4, a and a', and c and c').

Expression of NaCa. In the mature nephron, NaCa has been localized to the DCT2 and entire CNT (5, 36). During development, NaCa was first localized in the CNT of early stage IV nephrons. At this time, cells located in the youngest part of the CNT directly beneath the renal capsule were lacking NaCa IR (Fig. 4b), whereas they were already expressing 11HSD and rENaC (see below). NaCa IR was subsequently extending into this CNT portion signal and concomitantly extending into the distal DCT (Fig. 4, a and a'). As in the adult, NaCa IR was localized at the basal pole of the NaCa-expressing cells in all observed stages.

Expression of 11HSD. In the adult rat nephron, 11HSD has been localized along the DCT2 segment and the CNT and in the CD, and decreasing intensity of 11HSD IR from cortex to medulla has been described (5). During nephron maturation, 11HSD was first present in stage III CNTs that were still lacking IR for NaCa, whereas in early stage IV, NaCa IR colocalized with 11HSD mRNA signal in the CNT. At this time, onset of 11HSD expression was located in convoluted segments, which were concomitantly showing NCC IR but were not showing NaCa IR (Fig. 5, b and b'). In later stages, after the extension of NaCa IR into the DCT, onset of 11HSD expression was colocalized with the onset of NaCa IR in NCC-expressing DCT2 segments. Distally, 11HSD IR and mRNA expression extended toward the point of junction between maturing CNTs and CD ampullae while the expression of NaCa was declining proximal to this point; the ampulla was 11HSD negative (Fig. 5, c and c'). 11HSD signal was strongly present in the MCD in kidneys of day 1, 3, and 4 and was diminishing toward the outer cortical portion of the CD (Fig. 5a). Later in the 8-day-old animal, the entire CNT expressed 11HSD mRNA (Fig. 5a'), and 11HSD IR was equally present. At this stage, signal intensity was decreasing from CCD to inner MCD.
Fig. 3. Expression patterns for NKCC2 and NCC.  

a: In situ hybridization reveals NKCC2 expression all along the ascending limb (AL) including the bend of advanced stage IV loops of Henle; DL, descending limb. 

b and b': In early stage IV nephrons, NKCC2 mRNA signal extends well into the AL when there is still no NCC immunoreactivity (IR) in the initial portion of the distal convoluted tubule (DCT1), as revealed by double labeling immunohistochemistry (solid bars indicate the boundary between the NKCC2-expressing post-macula segment and the beginning of the immature DCT). 

c: In more advanced stage IV nephrons, weak signal for NCC mRNA is found along distal convolutions between bars. 

d: Morphologically, there is a prominent lumen at this stage (stage IV), and the AL of the loop of Henle consists of cuboidal cells that are lacking interdigitation and basal striation (semithin section).  

e and e': Onset of DCT is indicated by bars in late stage IV nephron; NCC IR is present in initial DCT and extends into the NKCC2-expressing post-macula segment, as demonstrated by double labeling technique (arrowhead in e'). Dots show outline of glomeruli. Magnifications: ×520 (a, b, d, and e) and ×400 (c).
Expression of rENaC. Previous studies in the mature rat kidney have demonstrated rENaC expression by in situ hybridization and immunocytochemistry in cells of the DCT, the CNT, the CCD, and the outer MCD, but not in cells of the inner MCD (13). IR and mRNA expression for rENaC were first detected in CNTs of stage III nephrons that were not yet exhibiting NaCa IR and were located within short distance to the renal capsule (see Fig. 7b). In nephrogenic stage IV and throughout later maturation, proximal onset of IR for rENaC and NaCa coincided in DCT segments as demonstrated by double labeling experiments (Fig. 6, a and a’). Proximally in the DCT, intensity of rENaC IR was low and increased progressively toward portions located further downstream. Occasionally, onset of rENaC IR was localized further distally so that tubule segments showing NaCa IR alone were also identified. As demonstrated by immunohistochemistry on consecutive sections, significant overlap of NCC IR and rENaC IR was present along the DCT and proximal CNT in stage IV nephrons (Fig. 6, a and a’). Later, overlap of both signals was restricted to the DCT2 segment, which was variable in length (Fig. 6, b and b’).

Throughout development, intercalated cells (IC) located in CNT segments were lacking rENaC and NaCa IR (Fig. 6, c and c’). Identity of IC was confirmed based on morphological criteria and by combined application with antibody to band 3, marker of the type A subpopu-
Expression of IC in the CNT (29a; not shown). Expression of rENaC mRNA in arcade-forming CNTs extended as far as to the junction with the ampulla (Fig. 7a'). Cells in this terminal portion revealed an immature structure (Fig. 7a''). The ampulla itself was only occasionally showing weak rENaC expression. In the CD, rENaC mRNA and IR were progressively increasing in intensity from the medullary part and decreases toward outer cortex (nephrogenic zone is indicated by arrowheads), whereas on day 8, signal intensity has become weaker in medullary CD and stronger in cortical CD. b−b': 11HSD mRNA can be found in convoluted tubule segments (**) showing IR for NCC (b') but not for NaCa (b'') in the region beneath the nephrogenic zone (triple labeling technique). c−c': CNT joining the CD on day 4 (double labeling immunofluorescence and interference contrast optics). In the CNT, 11HSD (c) extends further distally into the immature CNT than does NaCa (c'). In the CD, 11HSD IR is weak and diminishes before its transition into the ampulla (arrowhead). S, subcapsular S-shaped body. Magnifications: ×110 (a and a'), ×520 (b−b'), and ×400 (c−c').

Fig. 5. Expression of 11β-hydroxysteroid dehydrogenase type 2 (11HSD) during kidney development. Overview of 11HSD expression on day 3 (a) and on day 8 (a') as revealed by in situ hybridization (a and a'). In CNTs, 11HSD expression is equally strong on days 3 and 8. Expression in collecting ducts (CDs) on day 3 is intensive in the medullary part and decreases toward outer cortex (nephrogenic zone is indicated by arrowheads), whereas on day 8, signal intensity has become weaker in medullary CD and stronger in cortical CD. b−b': 11HSD mRNA can be found in convoluted tubule segments (**) showing IR for NCC (b') but not for NaCa (b'') in the region beneath the nephrogenic zone (triple labeling technique). c−c': CNT joining the CD on day 4 (double labeling immunofluorescence and interference contrast optics). In the CNT, 11HSD (c) extends further distally into the immature CNT than does NaCa (c'). In the CD, 11HSD IR is weak and diminishes before its transition into the ampulla (arrowhead). S, subcapsular S-shaped body. Magnifications: ×110 (a and a'), ×520 (b−b'), and ×400 (c−c').
ampulla toward the MCD. Signal was stronger in inner MCD on days 1, 3, and 4 (Fig. 6, d and e) compared with the CCD, whereas on day 8, inner medullary signal intensity was slightly diminished (Fig. 6f).

Generally, probes used for labeling of the different rENaC subunits recognized the same tubular structures, and there was no indication of a differential expression of varying combinations of subunits.

**DISCUSSION**

Distinct transport pathways mediate salt and volume reabsorption by discrete segments of the mammalian nephron and CD (9, 13, 14, 37). The mature axial organization of transport pathways reflects a complex process of differentiation that serves both developing animal and the adult. From early in development, these epithelia adapt to function synergistically for the conservation of the bulk of glomerular ultrafiltrate (19). Yet unique developmental expression patterns of transport proteins might suggest functions that are distinct from those observed in mature nephrons. In the present experiments, we have investigated the expression of gene products involved in salt transport along the distal tubule before, during, and after the onset of filtration in the rat kidney.

Loop of Henle. TAL. Transcription of NKCC2 was observed in stage III nephrons, prior to the onset of glomerular filtration. The earliest signal was expressed in the region of the future macula densa; it then extended proximally, toward the bend of the primitive loop. Comparative analysis of the maturation of the proximal tubule indicated that NaPi2 expression began in proximal convolutions before later extending into the straight portion of the proximal tubule. NKCC2 expression into the ascending limb preceded that of NaPi2 into the descending limb. Although the two limbs of the developing loop of Henle seem to mature in parallel as judged from their morphological appearance and parallel maturation of lysosomal enzyme activities (30), the current results suggest precocious maturation of the ascending limb with regard to epithelial ion transport. In the immature loop, NKCC2 expression was detected along the entire TAL as soon as the tubular lumen had become patent. A similar spatial pattern of NKCC2 expression has been reported in the developing mouse nephron, with expression observed in the TAL in post-S-shape stages (21). The early expression of NKCC2 mRNA in the loop of Henle may not reflect functional maturity, since physiological studies have shown a low reabsorptive capacity for this segment in early postnatal stages (18). Yet, the strong NKCC2 signal at the developing macula densa and TAL of stage III nephrons does suggest that the transporter plays some functional role. The observed ultrastructural specialization of the macula densa at this stage, compared with neighboring TAL cells (see Fig. 2), would be consistent with an early functional differentiation of this epithelium, a phenomenon that has also been reported in human kidney (12).

In the adult, strong expression of NKCC2 is maintained in the macula densa (32), where it participates importantly in the luminal sensing of tubular NaCl concentration as a crucial step in tubuloglomerular signaling (38). It is possible that the function of the macula densa, mediation of tubuloglomerular feedback and renin secretion (38), contributes in some unknown manner to development of the juxtaglomerular apparatus or the loop of Henle. Supporting evidence for such a view comes from the observation of glomerular maldevelopment in a case of neonatal Bartter’s syndrome (44). Mutations of the gene encoding NKCC2 have recently been recognized as causing some cases of the neonatal form of this disease (39). Defective expression of NKCC2 in the macula densa during development may thus also be related to the hypertrophy of the juxtaglomerular apparatus and the overexpression of renin, which typically occur in Bartter’s syndrome (3, 42).

DCT. In the mature nephron, NCC expression begins abruptly at the transition from the TAL to the DCT and ends at the transition from DCT to CNT. In the developing nephron, NCC expression first appears within the distal portion of the DCT. Expression gradually extends into the post-macula segment of the TAL in late stage IV. After this stage, the transition from TAL to DCT is characterized by a short segment of NKCC2 and NCC overlap. Only later, as the nephron approaches maturity, does this overlap disappear. From the earliest stages of expression, NCC is clearly restricted to the apical cell pole, as described for the mature DCT (5, 28).

In the adult kidney, the epithelium of the segment linking midcortical and juxtamedullary DCTs to arcades has the morphological characteristics of a CNT (22). During maturation however, the epithelium of this transitional segment manifests characteristics that are intermediate between DCT and CNT patterns, at the structural level (12, 31). The current results indicate that the intermediate morphological features of...
the developing nephron also reflect intermediate molecular features, as well. Maturing distal convolutions that joined to form arcades frequently express both NaCa and NCC, a characteristic of the distal portion of the DCT, the DCT2, in the adult (33). During further maturation, cells that express only NaCa (not NCC) interpose between the DCT and the boundary to the arcade (see Fig. 4). Plasticity in the length of the DCT appears to be maintained in the adult kidney since in rat, an increase in the distal tubular salt load has been shown to lead to a significant increase in DNA synthesis suggesting elongation of the tubule (30).

Enhanced sodium transport capacity in the developing DCT has been postulated based on experiments documenting an inappropriate response to salt loading during ontogeny (1). This has been proposed to result from either a precocious functional maturation of the DCT or from enhanced mineralocorticoid levels that rise significantly during postnatal development (17). We have shown previously that roughly half of the DCT expresses NCC together with NaCa, rENaC, and 11HSD, whereas the other half expresses NCC but not NaCa, rENaC, and 11HSD; this agrees with functional results indicating that sodium transport by the "early" distal tubule is mediated by the thiazide-sensitive sodium-chloride cotransporter, whereas transport by the "late" distal tubule is in part electrogenic and amiloride sensitive (9, 14). Further evidence for coexpression of NCC and rENaC is derived from studies using an immortalized mouse DCT cell line. These cells demonstrate both electroneutral and electrogenic sodium transport (8).

CNT. The morphology of nascent CNTs forming arcades allows the distinction of a more mature "stem" portion comprising a major (proximal) part of the arcade and a distally adjoining "neck" portion composed of immature cells (31). NaCa IR was observed in the stem portion, whereas it was lacking in the neck region. In contrast, rENaC and 11HSD expression were expressed very early in the neck region, in the absence of NaCa. The early onset of components active in mineralocorticoid-sensitive sodium reabsorption may well have functional relevance in the CNT already during ontogeny. Supportive evidence comes from autoradiographic studies showing expression of the mineralocorticoid receptor in the maturing rabbit (15) and mouse CNT and CCD (unpublished results, N. Farman). Likewise, Kalinyak and colleagues (23) have found a threefold higher level of mineralocorticoid receptor mRNA expression in neonatal compared with adult rat. Although responsiveness of the kidney to mineralocorticoids was shown to be diminished in the postnatal rat (40), a specific effect of aldosterone on sodium reabsorption was apparent; in contrast, administration of the glucocorticoid corticosterone was without effect (40). Coupled with the current results, these results suggest that the metabolic enzyme 11HSD is already functional during development of the nephron.

The embryological origin of the CNT is not clear; some investigators have suggested that it derives from the ureteric bud, whereas others have suggested its origin is from the nephrogenic blastema (22, 31, 34). A third alternative is that it represents an epithelial hybrid, with shared properties induced by the apposition. In favor of a distal origin, the CNT contains IC and expresses rENaC and 11HSD, as does the CD. In favor of a proximal origin, however, the CNT expresses the calcium-regulating proteins NaCa and calbindin (28), which are not expressed by the CD. During development the neck zone of the arcade linking CNT and CCD expresses rENaC and 11HSD in the absence of NaCa so that it appears possible that the CNT develops from the CD by continued mitotic activity at the site of junction with the nephrogenic blastema. On the other hand, the transitional zone linking the developing CNT and DCT expresses NaCa and high levels of NCC, a protein that is characteristic of epithelia derived from nephrogenic blastema. For this reason, the current molecular data suggest that the CNT arises as a product of mutual induction from adjoining segments leading to a unique hybrid epithelium.

Ampulla and CD. In most of the mature ampullae probed for rENaC and 11HSD, the epithelium was unreactive. The developing CD, however, strongly expressed rENaC from the outermost cortical portion to the medulla. Signal for 11HSD was weak in the CD but strong in the MCD early in development. As development progressed, 11HSD expression became equally strong in cortical and MCD, and finally became predominant in the cortex during maturity. In the immature state, the lack of 11HSD expression in outer CCD, which expresses high levels of rENaC and probably also the mineralocorticoid receptor (5, 15), suggests that glucocorticoids could act as sodium-retaining steroids during early postnatal life. In a more general sense, expression of 11HSD during fetal life has been reported from a variety of tissues, and a crucial role for this enzyme has been proposed in protecting maturing organs from elevated levels of glucocorticoids (6, 26). However, our results have shown that expression of 11HSD is spatially restricted, indicating that a general need for tissue protection via this enzyme is unlikely.

Fig. 7. a–a': Localization of β-rENaC mRNA and 11HSD IR in distal CNT segments of analogous stages, and corresponding morphology on a semithin section is shown on day 4 (a'); S, subcapsular S-shaped body. Expression of rENaC (a) extends up to the point of transition from CNT to CD ampulla (A), whereas expression of 11HSD (a') ends a few cells proximal to this point (peroxidase antiperoxidase immunohistochemistry). Bars indicate analogous sites of transition to the ampullae. b: Expression of γ-rENaC is found to be strong in early CNT and less intense in the outer CCD. c: Synopsis of the localization of the investigated transporters in key stages of the developing nephron (modified after Osathanondh and Potter, Ref. 34). c': Physiological roles of the transporters and of 11HSD. Proteins expressed in the nephron are distinguished by different types of hatchings and by different colors; colocalization of different signals is indicated by overlaid hatching, and overlap of NCC and rENaC is indicated by intermingling of colors. Magnifications: ×520 (a) and ×400 (c).
The inner MCD is known to reabsorb sodium in a mineralocorticoid- and amiloride-sensitive electrogenic manner (11, 20, 41). Duc and associates (13), however, failed to detect rENaC mRNA expression and IR in the inner MCD of mature rats. In contrast, Volk et al. (43) found rENaC activity in inner MCD cells, grown in culture. They also reported detecting all three rENaC subunits by Northern blot of rat kidney inner medulla, but the expression levels were dramatically lower than those in cortex and outer medulla. The present findings provide one explanation for these results. High level rENaC mRNA expression and IR was observed in postnatal stages on days 1, 3, 4, and 8, but it was diminished or absent at maturity. This suggests that adult rENaC expression levels are very low and may escape histochemical detection, whereas expression during development of the inner MCD is robust. The high level rENaC expression observed in cultured MCD cells may reflect a dedifferentiation of cells toward a more immature phenotype.

In conclusion, the present observations reveal a distinct pattern of cell specialization in different nephrogenic stages. NaiPi2, NKCC2, NCC, NaCa, 11HSD, and rENaC are all expressed in post-S-shape stages, indicating that microanatomic differentiation of the developing nephron and CD system to some degree precedes tubular cell specialization. During further maturation, NaCl transport proteins are expressed at the early juxtaglomerular apparatus, the nascent loop, and primitive distal convolutions prior to filtrate absorption, suggesting that they play a role in mediating early cellular development, such as the regulation of gene expression, cell differentiation, or cell proliferation. The early expression of 11HSD in the maturing CNT may indicate a particular role for mineralocorticoid hormone-specific effects at the interface between the nephron and CD system. Future evaluation of targeted disruption of the described proteins could further elucidate their effects on renal morphogenesis using the rodent kidney as a model.

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