Differentiation and cell polarity during renal cyst formation in the Han:SPRD (*cy*/+) rat, a model for ADPKD

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[1]*Medical Research Center and* [2]*V. Medical Department, Klinikum Mannheim, University of Heidelberg, D-68167 Mannheim;* [3]*Institute of Anatomy and Cell Biology I, University of Heidelberg, D-69120 Heidelberg, Germany;* [4]*Department of Medicine, Yale University School of Medicine, New Haven, Connecticut 06520; and* [5]*Institute of Physiology, University of Zürich, CH-8057 Zürich, Switzerland*

Obermüller, Nicholas, Norbert Gretz, Wilhelm Kriz, Fokko J. Van Der Woude, Robert F. Reilly, Heini Murer, Jürg Biber, and Ralph Witzgall. Differentiation and cell polarity during renal cyst formation in the Han:SPRD (*cy*/+) rat, a model for ADPKD. *Am. J. Physiol.* 273 (*Renal Physiol.* 42): F357–F371, 1997.—Despite the recent positional cloning of genes responsible for autosomal dominant polycystic kidney disease (ADPKD), the exact pathogenetic mechanisms underlying this disorder are still unclear. To learn more about cyst formation, we investigated cell differentiation and cell polarity in the Han:SPRD (*cy*/+) rat between 21 days and 60 wk of age. At early stages of cyst development, alkaline phosphatase, aquaporin-1, NaSi-1 cotransporter, and Na^+-K^+-adenosinetriphosphatase (Na^+-K^+-ATPase) were expressed normally. Clusterin mRNA was only sparsely expressed at the onset of cystic degeneration and increased thereafter, being highest in noncystic nephron segments. In cyst wall cells, clusterin on the one hand and alkaline phosphatase, aquaporin-1, NaSi-1-cotransporter, and Na^+-K^+-ATPase on the other were expressed in a mutually exclusive fashion. No change in cell polarity could be observed at any stage. Our data therefore argue against a change in cell polarity and against an early arrest in normal tubular development during cyst formation in the Han:SPRD (*cy*/+) rat model of ADPKD but favor the hypothesis that tubular epithelia develop in an orderly fashion and degenerate thereafter.

polycystic kidney disease; aquaporin-1; alkaline phosphatase; sodium-sulfate-1 cotransporter; clusterin

AUTOSOMAL DOMINANT polycystic kidney disease (ADPKD) represents an hereditary form of cystic transformation in the kidney and also in other organs such as the liver and the pancreas. It accounts for ~10% of all cases of end-stage renal failure (14). Several hypotheses have been brought forward to explain the pathogenesis of cyst formation (for recent reviews, see Refs. 5, 14). There is some evidence that indicates that cyst-lining epithelia have undergone a change in cell polarity (1, 6, 25, 39). This would result in an increased vectorial transport of fluid into the lumen of the cyst and ultimately to cystic expansion. Furthermore, it seems likely that at least at some stage of cyst formation an increase in cell proliferation has to occur. This assumption is supported by the development of polycystic kidney disease (PKD) in transgenic animals carrying a c-*myc* or H-*ras* T24 transgene (35, 38). It must also be clear, however, that a high rate of cell proliferation alone will only result in solid tumors. Therefore, cell proliferation has to be accompanied by lumen formation and cystic expansion. The latter may be influenced by the extracellular matrix, which interacts in an intricate fashion with the epithelium. The cyst wall epithelia may secrete proteinases to degrade the extracellular matrix and create room for expansion (23, 33). On the other hand, a defect in the extracellular matrix might even provide a signal for the epithelium to form cysts. Looking at the required complexity in the formation of cysts, it has been hypothesized that the appearance of cystic cells is caused by a differentiation defect. Differentiation defects could be both a cause or an accelerating factor in cyst formation.

During nephrogenesis, the kidney undergoes highly organized morphological and functional changes. In the course of differentiation, the kidney not only acquires its shape but also its functional characteristics, such as ion and fluid transport. Channels, transporters, and enzymes have to be expressed in the correct nephron segment in a highly polarized apical or basolateral fashion. Missorting of these proteins would result in disastrous consequences for the organ and the whole organism. Na^+-K^+-adenosinetriphosphatase (Na^+-K^+-ATPase) is an important protein for the homeostasis of many cells. In tubules of the normal adult kidney, it is located on the basolateral side of the cells, although there have been reports that, in renal cysts, Na^+-K^+-ATPase is missorted to the apical side and might therefore contribute to cyst formation. Very little, however, is known about other markers of cell polarity (1, 6, 25, 39). Aquaporin-1, NaSi-1 cotransporter, and alkaline phosphatase appear late during renal development and are sorted either exclusively (NaSi-1 cotransporter, alkaline phosphatase) or predominantly (aquaporin-1) to the apical membrane (Refs. 8, 11, 32; unpublished observations). With the exception of aquaporin-1, which is also expressed in the descending thin limb, they can only be found in the proximal tubule (20, 22, 32) and therefore serve as excellent parameters of differentiation and cell polarity during cystogenesis in the Han:SPRD (*cy*/+) rat model of ADPKD, in which the cysts develop from the proximal tubule.

High mRNA levels of clusterin, a heterodimeric glycoprotein expressed in many parenchymous tissues, have been reported not only after renal injuries such as acute tubular necrosis and ureteral obstruction but also in genetic renal disease (29). Although its function has not

yet been fully illuminated, it could be shown that clusterin influences cell-cell adhesion in a renal tubular cell line (Ref. 36; for recent reviews, see Refs. 29, 30). During normal renal development, clusterin is expressed transiently after the induction of the metanephrogenic mesenchyme (13, 18). Several studies have described renal clusterin expression in patients with polycystic kidneys (12) or in a murine model of hereditary PKD (18). By performing Northern blot analysis, Cowley and Rupp (10) recently described abnormally high expression of clusterin mRNA in the polycystic kidneys of Han:SPRD (*cy/+*) rats. This observation was made by comparing affected rats to unaffected littermates at different time points ranging from 2 to 24 wk of age. To gain a clearer understanding of the significance of clusterin induction in the kidneys of Han:SPRD (*cy/+*) rats, the exact sites of clusterin mRNA expression must be known.

We therefore examined the expression pattern of clusterin mRNA in polycystic kidneys of Han:SPRD (*cy/+*) rats by the means of high-resolution, nonradioactive in situ hybridization. Together with the analyis of the time-dependent appearance or absence of characteristic apical tubular markers in cysts during early and late stages of cyst development, we intended to answer the question whether changes in the differentiation state of cystic epithelia have to be considered as an early or late defect and whether a change in polarity can be identified in cyst-lining epithelia. The Han:SPRD (*cy/+*) rat model, which displays a slowly progressive form of ADPKD, was initially described by Kaspareit-Rittinghausen et al. (21), followed by a more detailed morphological analysis by others (9, 34). In kidneys from Han:SPRD (*cy/+*) rats, cysts almost exclusively arise in proximal tubules. Because of the gender difference of PKD severity (males are more affected than females), the slow progression and the extrarenal manifestations of cystic disease, this strain currently represents the only suitable rat model of ADPKD for human ADPKD (16).

MATERIALS AND METHODS

Animals. Heterozygous Han:SPRD (*cy/+*) rats as well as unaffected littermates at an age of 21 and 38 days and 60 wk were chosen for analysis. The rats were obtained from the Animal Care Facility in Mannheim maintained as an inbred colony under control of one of the authors (N. Gretz). Animals were allowed free access to standard rat chow (containing 19% protein) and tap water. For optimal conservation of tissue morphology, rats were anesthetized by intraperitoneal injection of Nembutal (pentobarbital sodium, 40 mg/kg body wt) and perfused retrogradely through the distal abdominal aorta. Perfusion was conducted for 3 min at a pressure level of 210 to 220 mmHg with 2% freshly dissolved paraformaldehyde [in phosphate-buffered saline (PBS) pH 7.4] for 3 min and subsequently with a 18% sucrose solution (in PBS and adjusted to 800 mosmol/kgH_2O) for another 3 min at the same pressure level. After removal, kidneys were cut into slices and mounted qickly onto small pieces of styrofoam before being snap frozen in liquid nitrogen-cooled isopentane. Tissues were stored at −80°C until further use.

Preparation of riboprobes. Sense and antisense riboprobes were prepared from a 1.4-kbp rat clusterin cDNA fragment (Ref. 4, kind gift of R. Buttyan, New York, NY). In vitro transcription was carried out according to the protocol supplied by the manufacturer (Boehringer-Mannheim, Mannheim, Germany). For better penetration of the probes during in situ hybridization, the transcripts were partially hydrolyzed to a calculated average length of 250 bases by exposure to an alkaline pH.

In situ hybridization. In situ hybridization was essentially carried out as described previously (24). Hydrolyzed clusterin probe was used at a concentration of 0.8 to 1.5 ng/μl. Hybridization was carried out a temperature of 42°C.

Combined in situ hybridization and immunocytochemistry. To identify the nephron segments that express clusterin mRNA, in situ hybridization with the clusterin riboprobe and immunohistochemical staining using nephron segment-specific antibodies were performed on the same tissue section (24). Each of the antibodies used in the double labeling experiments has been investigated extensively, and specifity has been demonstrated clearly. The following segment-specific antibodies were used: a rabbit polyclonal antibody to NaSi-1 cotransporter (proximal tubule cells, Ref. 22), a rabbit anti-aquaporin-1 antibody (proximal tubule and descending thin limb cells, Ref. 32), a rabbit anti-Tamm Horsfall protein (THP) antibody (thick ascending limb cells, Ref. 19), a mouse monoclonal antibody against the α_1-subunit of Na^+-K^+-ATPase (strongest expression in distal convoluted cells, Refs. 17, 26), a guinea pig polyclonal antibody against Na^+/Ca^{2+} exchanger (connecting tubule cells, Refs. 24, 28), and a rabbit polyclonal antibody against aquaporin-2 (principal cells of collecting ducts, Ref. 31). After posthybridization washes, a mixture of the anti-digoxigenin antibody and one of the segment-specific antibodies, diluted in blocking medium, was applied. The anti-digoxigenin antibody was diluted 1:500, and the other antibodies were diluted 1:30 to 1:100. This mixture was administered to the sections for 2 h at room temperature, followed by an overnight incubation at 4°C in a moist chamber. Slides were then rinsed twice for 5 min in *buffer I*, and, subsequently, specific antibodies were detected using a Cy3-coupled secondary antibody (Dianova, Hamburg, Germany) diluted 1:300 in *buffer I* or fluorescein isothiocyanate-labeled antibody (Sigma, Deisenhofen, Germany) diluted 1:60. After being incubated for 1 h at room temperature, slides were washed twice for 5 min in *buffer I* and subsequently processed as described in the normal in situ hybridization protocol (24).

Single and double antibody immunocytochemistry. Cryostat sections (5–7 μm thick) were air dried for 1 h and blocked for 30 min in 2% bovine serum albumin (in 1× PBS), after which one or two primary antibodies (dilution was 2-fold higher than described above) were applied. The sections were incubated for 2 h at room temperature and then overnight at 4°C. The next morning, sections were rinsed twice for 10 min each in PBS before the secondary antibodies were applied simultaneously (diluted as described above). After a 1-h incubation at room temperature, sections were washed and mounted.

Controls. Control experiments in which the primary antibody was replaced with buffer yielded only background fluorescence. The specifity of the in situ hybridization signal was verified by parallel incubation with antisense and sense riboprobes on alternate sections. Throughout all experiments, sense probes did not produce any detectable signal. Some sections were hybridized without antisense probe, while others were processed without applying the anti-digoxigenin antibody. Both controls yielded completely negative results.

Alkaline phosphatase histochemistry. For identifying the epithelium of all segments of the proximal tubule, the high enzymatic activity of alkaline phosphatase present in the brush border of the proximal tubule was demonstrated histochemically. In brief, cryostat sections were covered with reaction solution (0.3 mM nitro blue tetrazolium chloride and 0.3 mM 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt, in 0.2 M Tris·HCl buffer, pH 9.5). After 5 min, when blue reaction products were strongly visible, sections were immersed for 10 min in bidestilled water, postfixed in 4% paraformaldehyde (in PBS pH 7.4) for 10 min, rinsed again in water, and finally mounted in bicarbonate-buffered glycerol, pH 8.6.

RESULTS

Overview of sites of clusterin and alkaline phosphatase expression. Overviews in Figs. 1–3 show representative kidney sections of Han:SPRD (*cy*/+) rats of ages 21 and 38 days and 60 wk. The regional topography as well as cystic tubular changes were demonstrated by labeling the brush border of proximal tubules through alkaline phosphatase histochemistry (Fig. 1, *B* and *D*; Figs. 2*B* and 3*B*). In situ hybridization to clusterin mRNA was performed on serial sections (Fig. 1, *A* and *C*; Figs. 2*A* and 3*A*).

In a kidney from one 21-day-old animal with only moderate cystic changes, only occasional clusterin mRNA-positive tubular profiles and cysts could be identified (Fig. 1*A*), whereas, in a kidney from a littermate with already pronounced cyst formation, more clusterin mRNA-positive profiles and cysts were seen (Fig. 1*C*). Remarkably, at this age most of the cystically expanded proximal tubules still showed nor-

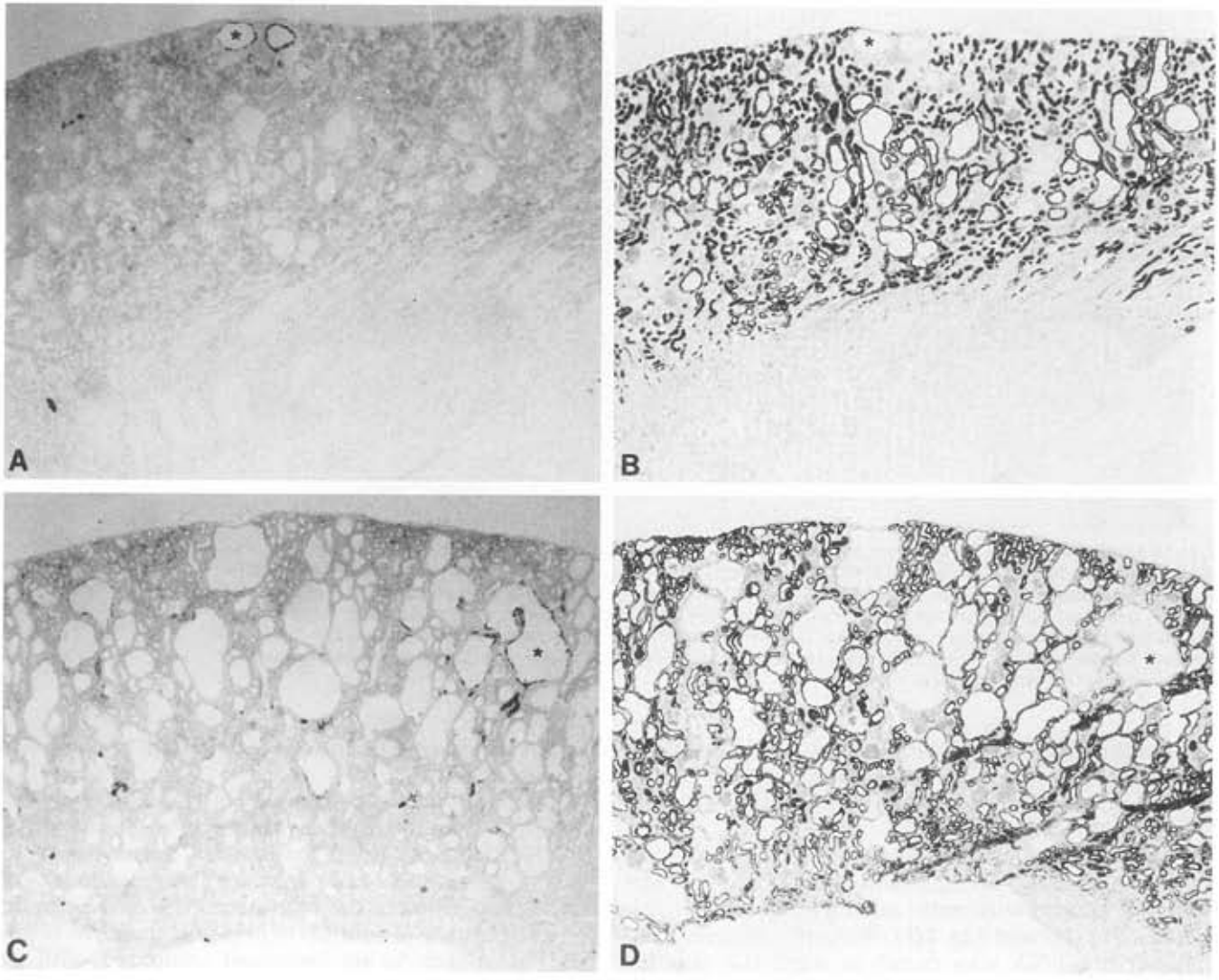


Fig. 1. In situ hybridization for clusterin mRNA (*A* and *C*) and alkaline phosphatase histochemistry (*B* and *D*) on serial kidney sections from two 21-day-old Han:SPRD (*cy*/+) littermates. Cyst formation occurs exclusively in the cortex and outer medulla as demonstrated by alkaline phosphatase histochemistry in proximal tubules (*B* and *D*). In one animal at an early stage (*A* and *B*), only occasional clusterin-positive profiles can be detected, whereas, in another animal (*C* and *D*) with a considerable degree of cyst formation, more clusterin-positive profiles are seen. *Clusterin mRNA-positive cyst-lining cells show no alkaline phosphatase activity. Overviews were restricted to cortex and outer stripe of the outer medulla, because clusterin mRNA expression was not detected in inner stripe or papilla at this magnification (×32).

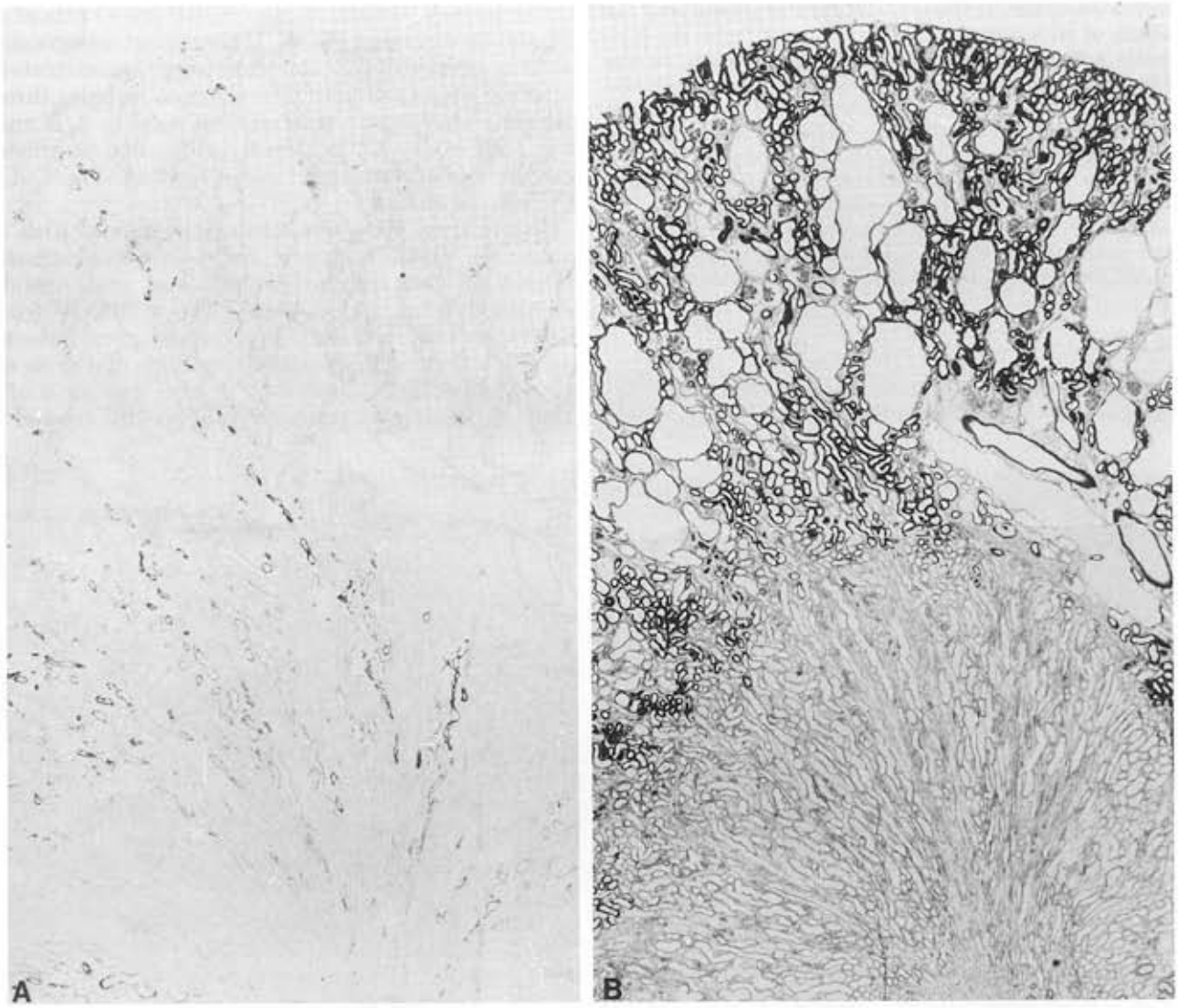


Fig. 2. In situ hybridization for clusterin mRNA (*A*) and alkaline phosphatase histochemistry (*B*) on serial kidney sections from a 38-day-old Han:SPRD (*cy*/+) rat. Although cysts have increased in size, their numbers do not differ dramatically in comparison to the one 21-day-old rat with a higher degree of cystic transformation, and there are still many proximal tubules not cystically transformed. Cysts are confined to the cortex and outer stripe. Many clusterin-positive tubular profiles extend from the cortex to the inner stripe and appear to imitate the outline of nephrons. Border between outer and inner stripe is marked by abrupt end of alkaline phosphatase-positive proximal tubules (*B*). Magnification, ×24.

mal alkaline phosphatase activity (Fig. 1, *B* and *D*). In cysts with clusterin mRNA expression, alkaline phosphatase and clusterin seemed to be mutually exclusive (also see below).

In rats 38 days of age, cystic expansion had progressed only little further compared with the one 21-day-old animal with more advanced cyst formation (compare Fig. 1*D* and Fig. 2*B*). Widespread expression of clusterin mRNA was found in noncystic tubular profiles in the cortex and the outer medulla, although clusterin mRNA was also expressed in cystically enlarged tubules (Fig. 2*A*). In the overview, no hybridization signal could be seen in the inner medulla. Sometimes tubules expressing large amounts of clusterin mRNA seemed to extend from the cortex deep into the inner stripe, suggesting that a large portion of clusterin mRNA expression could be attributed to the thick ascending limb.

Compared with the situation in the 38-day-old animal, gross analysis of a kidney from a 60-wk-old Han:SPRD (*cy*/+) rat demonstrated massive morphological alterations, i.e., giant cyst expansion and fibrotic remodeling of the interstitium. As shown in Fig. 3*A*, the number of tubules expressing clusterin mRNA did not appear to be increased compared with the 38-day-old animal. In the epithelia of giant cysts that extended into the inner stripe, very occasional clusterin mRNA expression was seen (Fig. 3*A*). Alkaline phosphatase activity was not present in these cysts (Fig. 3*B*). In smaller cortical cysts, the exclusive presence of clusterin mRNA and alkaline phosphatase activity in the same cystic profiles could be revealed.

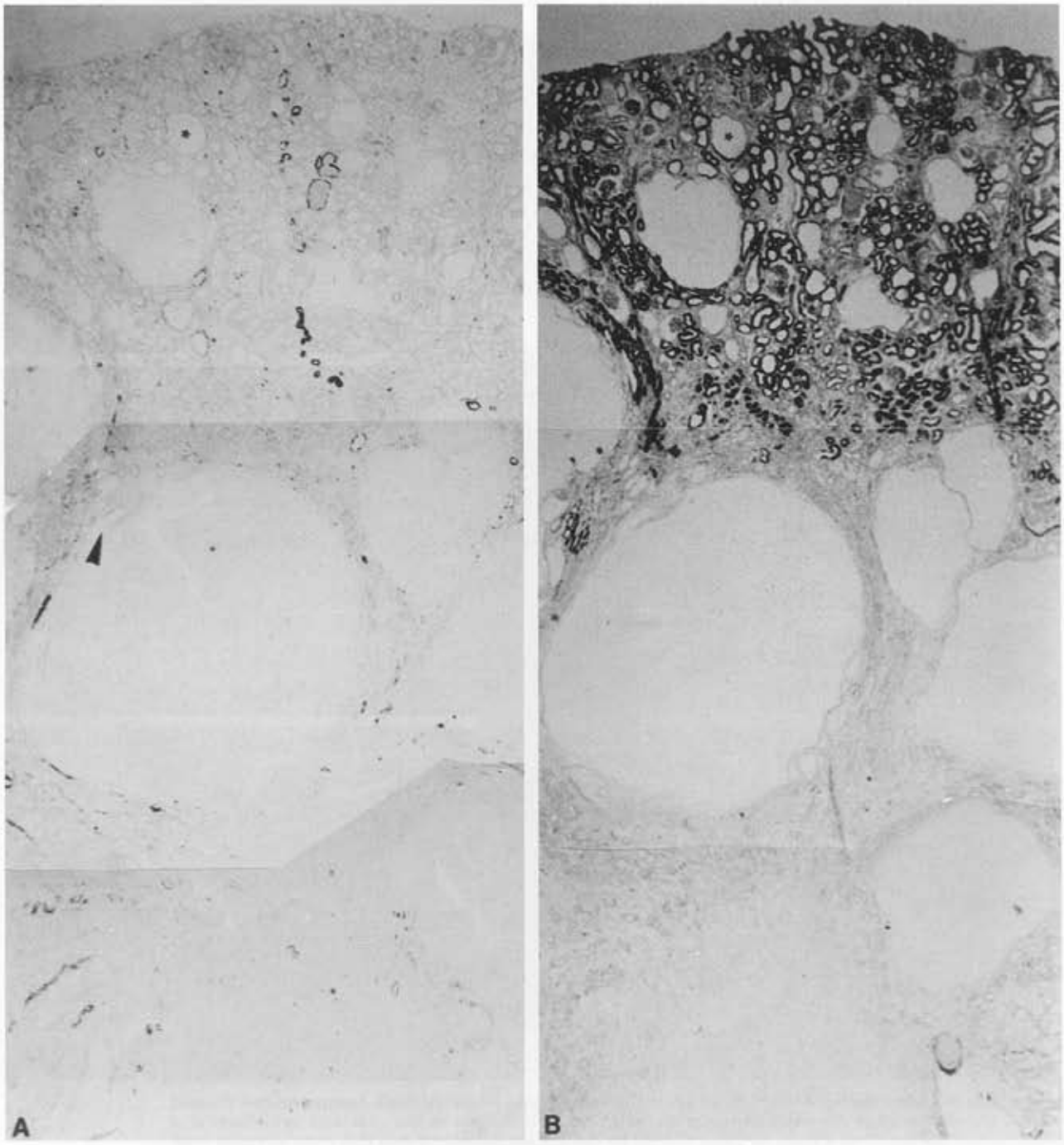


Fig. 3. In situ hybridization for clusterin mRNA (*A*) and alkaline phosphatase histochemistry (*B*) on serial kidney sections from a 60-wk-old Han:SPRD (*cy*/+) rat. Size of some cysts has increased considerably, and cysts can now also be found in inner stripe (*A*, arrowhead points to a site of clusterin expression in one of those giant cysts). Despite a large degree of tissue remodeling, even at this late stage, many cystic and noncystic proximal tubules show an homogeneous staining for alkaline phosphatase. Compared with kidney from 38-day-old Han:SPRD (*cy*/+) rat, no increase in clusterin-positive tubules was noticed. *Cyst with mutually exclusive expression of clusterin mRNA and alkaline phosphatase in cyst-lining cells. Magnification, ×25.

Clusterin mRNA expression could not be discovered at any age in kidney sections of unaffected littermates.

Detailed analysis of expression of aquaporin-1, alkaline phosphatase, NaSi-1 cotransporter, Na^+-K^+-ATPase, and clusterin in cystically transformed proximal tubules. Serial kidney sections from 38-day-old Han:SPRD (*cy*/+) rats subjected to alkaline phosphatase histochemistry (Fig. 4*B*) and in situ hybridization with an antisense clusterin riboprobe (Fig. 4*A*) revealed that considerable amounts of clusterin mRNA were expressed in cysts derived from proximal tubules. In these cysts, cells either were positive for clusterin mRNA or alkaline phosphatase. To confirm that these cysts originated from proximal tubules and also to

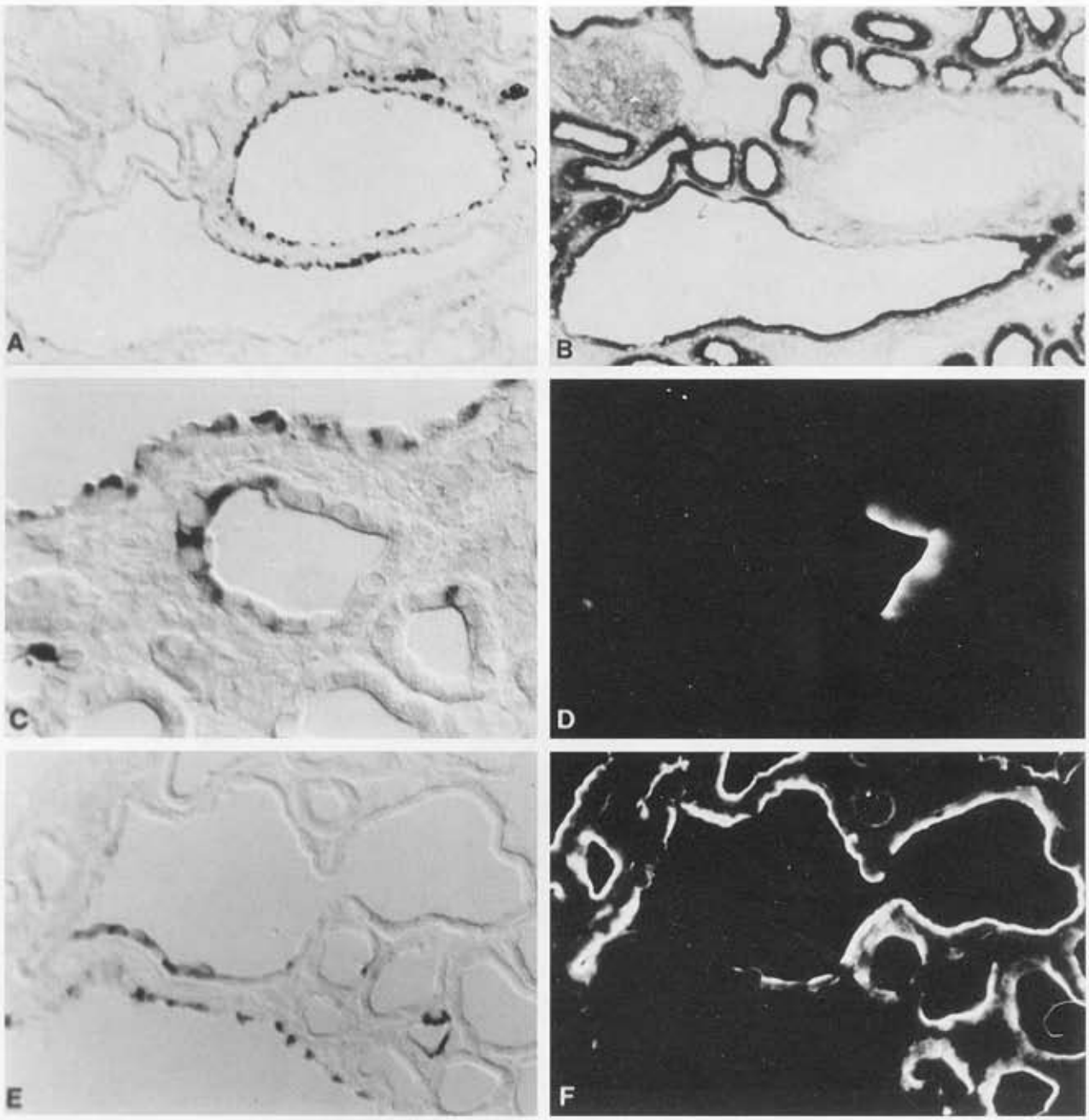
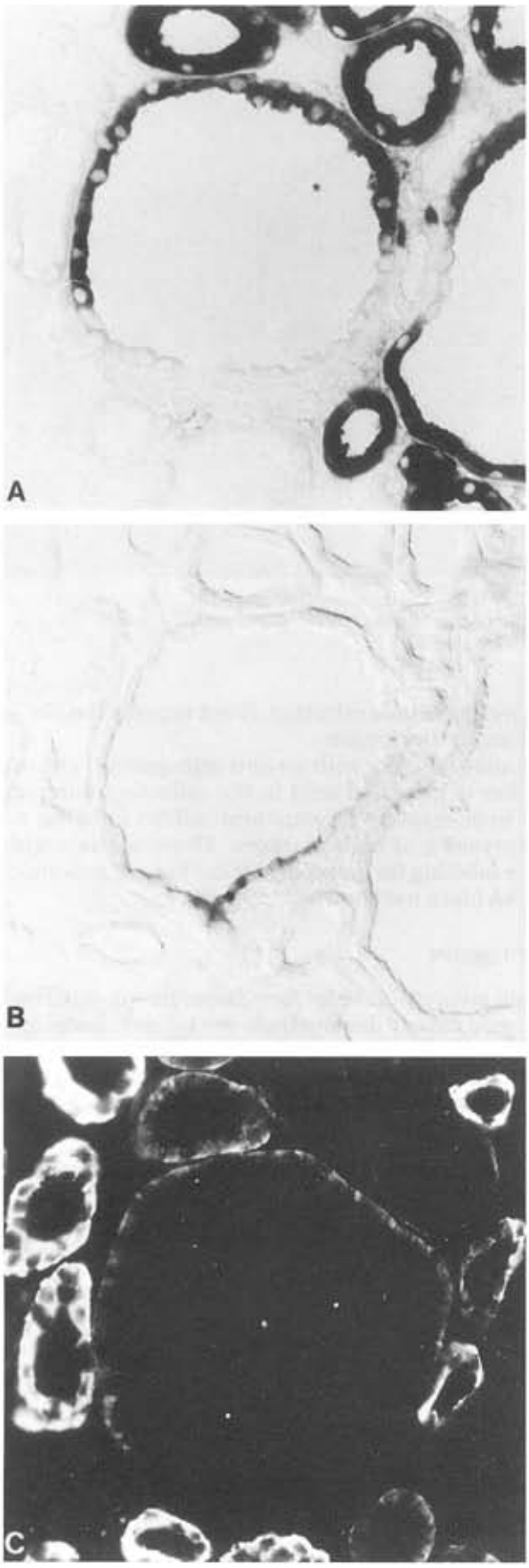


Fig. 4. Clusterin mRNA on the one hand (*A*, *C*, and *E*) and alkaline phosphatase (*B*), NaSi-1 cotransporter (*D*), and aquaporin-1 (*F*) on the other are expressed in a mutually exclusive fashion in the cyst wall epithelium of a 38-day-old Han:SPRD (*cy*/+) rat kidney. By using specific markers, it can be clearly seen that cysts originate from proximal tubules. Clusterin mRNA-positive cells show no alkaline phosphatase activity (serial sections in *A* and *B*) and do not stain with antibodies against NaSi-1 cotransporter and aquaporin-1 (double labeling on the same section in *C* and *D* and *E* and *F*, respectively). Cells that express alkaline phosphatase, NaSi-1 cotransporter, and aquaporin-1 do not express clusterin mRNA. Strong apical expression of alkaline phosphatase, NaSi-1 cotransporter, and aquaporin-1 indicates that no change of cell polarity has occured. Magnifications: *A* and *B*, ×146; *C* and *D*, ×380; *E* and *F*, ×233.

learn more about the characteristics of the cyst wall epithelia, two other markers expressed by proximal tubular cells, NaSi-1 cotransporter and aquaporin-1, were investigated on sections from 38-day-old Han:SPRD (*cy*/+) rats. Cyst-lining cells, stained by the anti-NaSi-1 cotransporter antibody (Fig. 4*D*) and the anti-aquaporin-1 antibody (Fig. 4*F*) did not express clusterin mRNA, whereas clusterin-positive cells were unreactive with both antibodies (Fig. 4, *C* and *E*). NaSi-1 cotransporter was expressed exclusively and aquaporin-1 predominantly apically according to their already described pattern of expression in the fully differentiated proximal tubule. Na^+-K^+-ATPase, which, besides its strong expression in thick ascending limb and distal convoluted tubule, can also be found in proximal tubules (Fig. 5*A*), was clearly located to the



basolateral membrane of cyst-lining epithelia of proximal tubules in kidneys from 38-day-old Han:SPRD (*cy*/+) rats (Fig. 5*C*). Na^+-K^+-ATPase staining had already decreased or completely disappeared in those cells that expressed clusterin mRNA (Fig. 5, *B* and *C*). In no case could an apical missorting of the Na^+-K^+-ATPase be demonstrated (Fig. 6).

To rule out stage-specific changes, we also examined polycystic kidneys from very young and very old Han:SPRD (*cy*/+) rats. Alkaline phosphatase histochemistry (Fig. 7*A*) and staining with antibodies against aquaporin-1 (Fig. 7*B*), Na^+-K^+-ATPase and NaSi-1 cotransporter (data not shown) did not reveal a change of cell polarity in polycystic kidneys from 21-day-old Han:SPRD (*cy*/+) rats. Double labeling experiments with antibodies against NaSi-1 cotransporter and Na^+-K^+-ATPase demonstrated that the regular cell polarity is still maintained even in polycystic kidneys from 60-wk-old Han:SPRD (*cy*/+) rats (Fig. 8).

All markers described above were also investigated on kidney sections from corresponding unaffected Han:SPRD (+/+) rats, where the regular distribution of the polarity markers could be demonstrated (data not shown).

Clusterin expression in other structures of the kidney. A considerable degree of clusterin mRNA was found outside of cysts. Apart from tubular cells, we also observed expression of clusterin mRNA in parietal cells of Bowman's capsule (Fig. 9). Cells of the glomerular tuft were always negative for clusterin mRNA.

Double labeling of a section through the inner stripe with the clusterin antisense probe and the anti-aquaporin-1 antibody revealed the presence of tubular profiles, which coexpressed clusterin (Fig. 10*A*) and aquaporin-1 (Fig. 10*B*), indicating that cells of the descending thin limb are capable of expressing clusterin mRNA.

Many tubular profiles in the cortex and in particular in the inner stripe of the outer medulla expressed clusterin mRNA. Figure 10 illustrates the in situ hybridization for clusterin mRNA (Fig. 10, *C* and *E*) and simultaneous staining with an antibody against THP, a marker for the thick ascending limb (Fig. 10, *D* and *F*). Although there were many profiles staining positive for both clusterin and THP, an overlap of THP immunoreactivity and clusterin mRNA expression in the same profile was not seen. Cells of the thick ascending limb either expressed THP or clusterin but not both. This demonstrates that cells of the thick ascending limb express clusterin, while, at the same time, they cease expressing THP.

Figure 11 presents profiles of the distal convoluted tubule in the cortical labyrinth as defined by strong

Fig. 5. Alkaline phosphatase histochemistry (*A*), clusterin mRNA expression (*B*), and Na^+-K^+-ATPase immunocytochemistry (*C*) on consecutive kidney sections from a 38-day-old Han:SPRD (*cy*/+) rat. Similar to alkaline phosphatase (*A*), Na^+-K^+-ATPase (*C*) is expressed in that portion of a proximal cyst that does not express clusterin mRNA (*B*). Staining with anti-Na^+-K^+-ATPase antibody can only be demonstrated on basolateral membrane of cyst wall epithelia (*C*). Magnification, ×305.

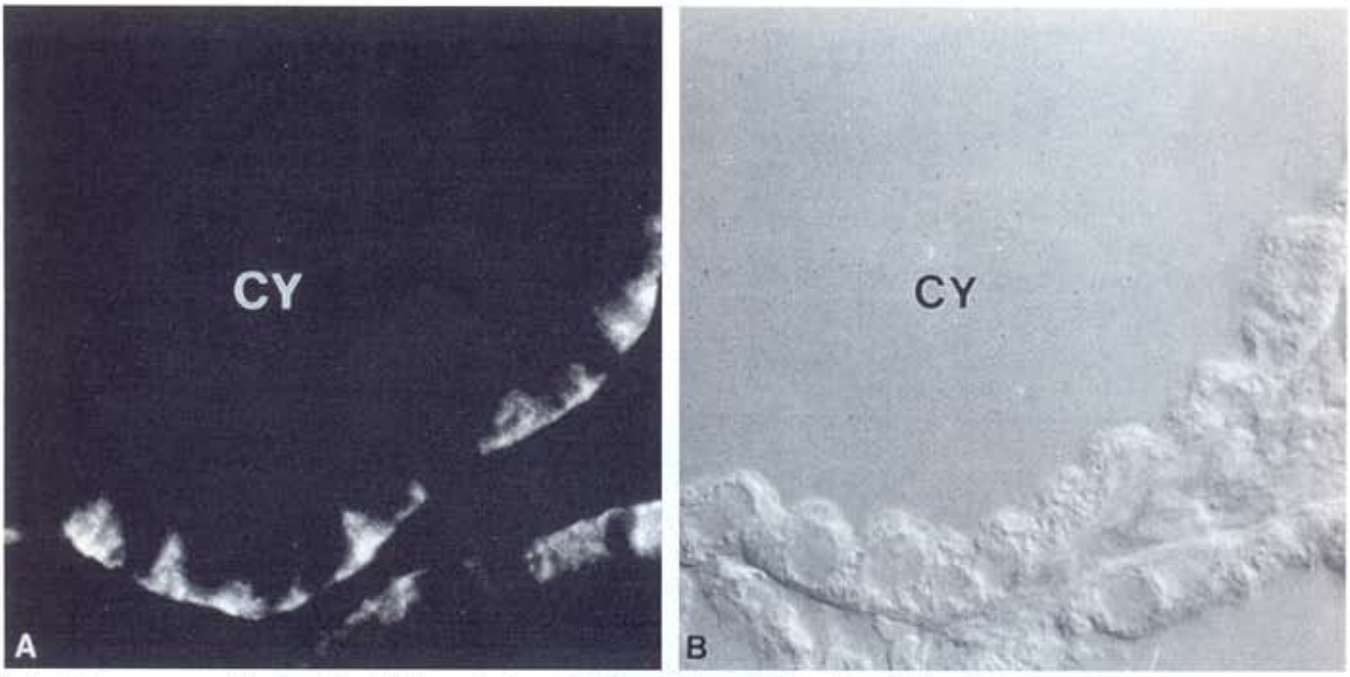


Fig. 6. High-power magnification of cyst-lining cells from a 38-day-old Han:SPRD (*cy*/+) rat kidney. Staining with an antibody against Na^+-K^+-ATPase clearly demonstrates basolateral sorting of this marker (*A*) compared with corresponding interference-phase contrast micrograph (*B*). CY, cyst. Magnification, ×870.

labeling with an anti-Na^+-K^+-ATPase antibody. Although cells exhibiting a strong hybridization signal for clusterin mRNA (Fig. 11*A*) also expressed Na^+-K^+-ATPase (Fig. 11*B*) as seen by double labeling, Na^+-K^+-ATPase immunoreactivity appeared somewhat reduced in clusterin expressing cells compared with the staining in clusterin mRNA-negative tubular cells.

By employing an antibody against the Na^+/Ca^{2+} exchanger that labels connecting tubule cells, we identified connecting tubule profiles (Fig. 11*D*) with prominent staining for clusterin mRNA (Fig. 11*C*). There was, however, a subset of cells in connecting tubules that expressed clusterin but did not react with the antibody against the Na^+/Ca^{2+} exchanger. Those cells may correspond to intercalated cells of connecting tubules, although we cannot rule out that they are connecting tubule cells that do not express the Na^+/Ca^{2+} exchanger any longer.

Double labeling with an anti-aquaporin-2 antibody, a marker of principal cells in the collecting duct, and in situ hybridization for clusterin mRNA failed to reveal coexpression of both markers. Those profiles with intense labeling for aquaporin-2 did not express clusterin mRNA (data not shown).

DISCUSSION

Cell polarity and cyst formation. In our experiments, we could clearly demonstrate the correct apical expression of markers such as alkaline phosphatase, aquaporin-1, and NaSi-1 cotransporter, and the correct basolateral expression of Na^+-K^+-ATPase in the kidneys of

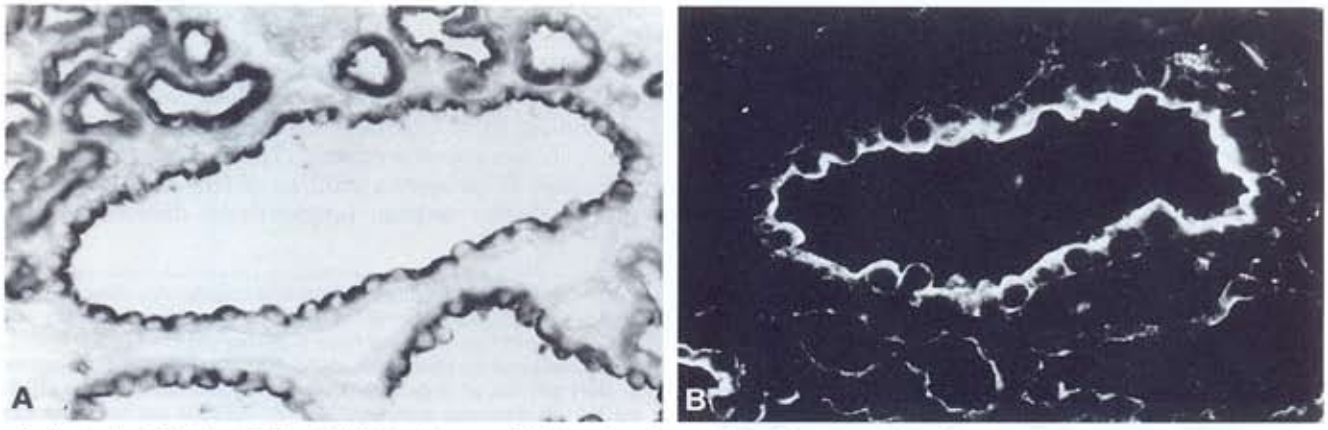


Fig. 7. Analysis of 21-day-old Han:SPRD (*cy*/+) rats. Alkaline phosphatase histochemistry (*A*) and immunohistochemisty with an antibody against aquaporin-1 (*B*) on polycystic kidneys from 21-day-old rats demonstrates correct sorting of those markers in cyst wall cells. Magnification: *A*, ×235; *B*, ×360.

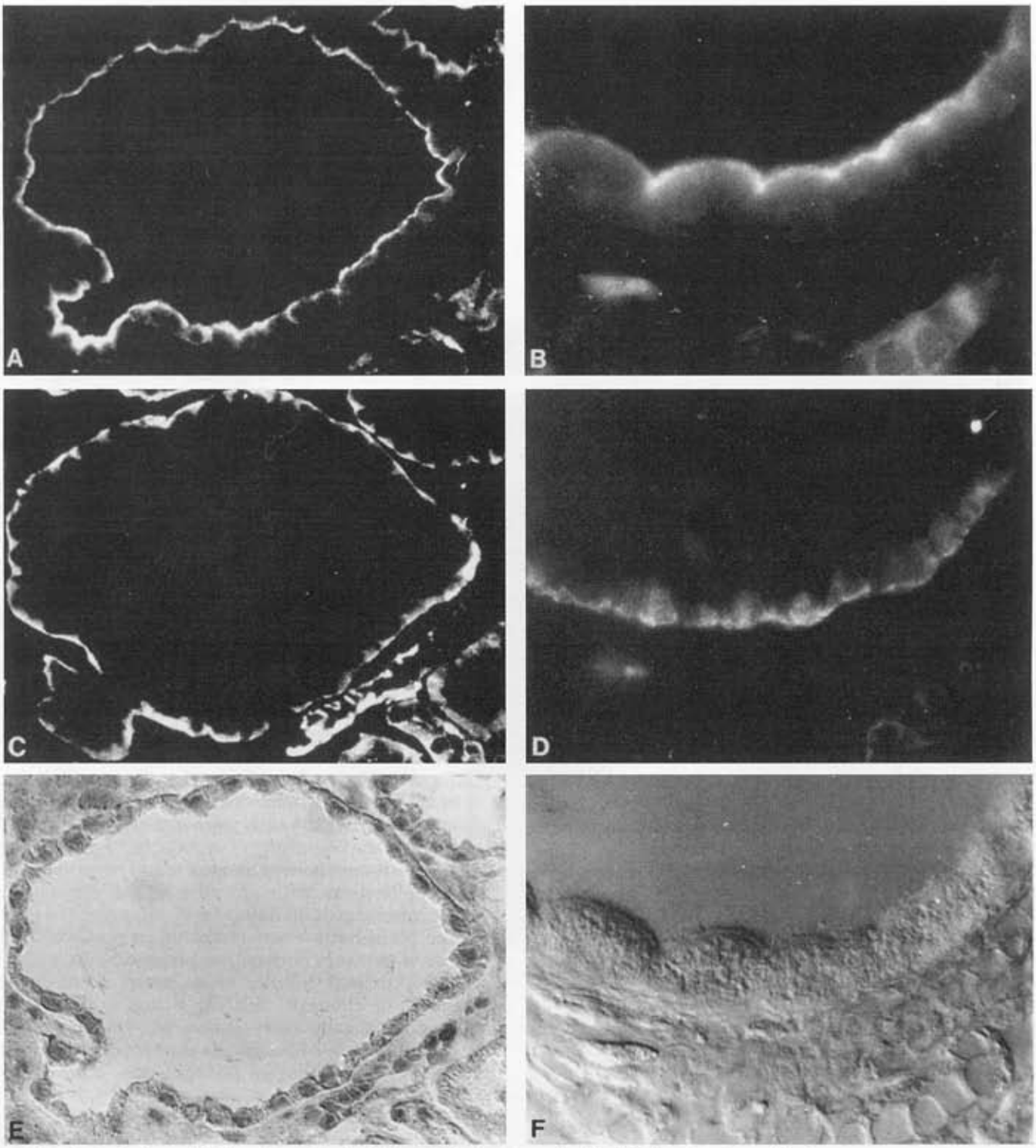


Fig. 8. Analysis of polycystic kidneys from 60-wk-old Han:SPRD (*cy*/+) rats. Double labeling of cystic profiles from a 60-wk-old rat with antibodies against NaSi-1 cotransporter (*A* and *B*, detected with fluorescein isothiocyanate fluorescence) and Na^+-K^+-ATPase (*C* and *D*, detected with Cy3 fluorescence) demonstrates correct apical and basolateral expression, respectively, of either protein (also compare corresponding phase-contrast micrograph of hematoxylin-stained cyst in *E* and interference phase-contrast micrograph in *F*). Correct sorting of NaSi-1 cotransporter and of Na^+-K^+-ATPase is also shown at a higher magnification in another cyst (compare *A* with *B* and *C* with *D*, respectively). Magnifications: *A*, *C*, and *E*, ×460; *B*, *D*, and *F*, ×960.

Han:SPRD (*cy*/+) rats. However, in the course of cyst formation a complete loss of aquaporin-1, alkaline phosphatase, NaSi-1 cotransporter and Na^+-K^+-ATPase in cyst wall epithelia could be observed, indicating a less differentiated state. This was accompanied by the appearance of considerable levels of clusterin mRNA in these cyst wall cells. Our results therefore indicate that, in cyst-lining cells of Han:SPRD (*cy*/+) rat kid-

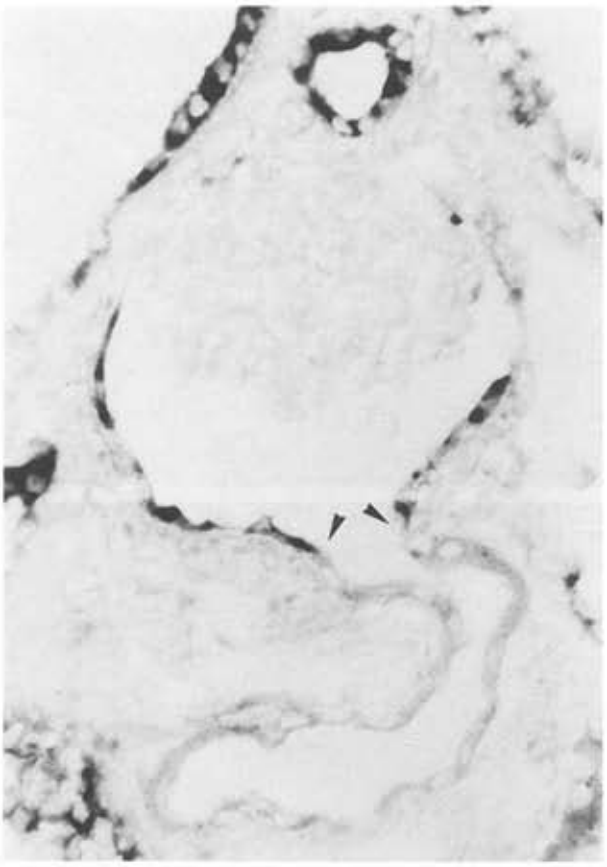


Fig. 9. Clusterin mRNA expression in parietal cells of Bowman's capsule. As indicated by arrowheads, a sharp demarcation between clusterin-positive parietal cells and clusterin mRNA-negative, noncystic S1 segment can be seen in a kidney from a 38-day-old Han:SPRD (cy/+) rat. Cells of glomerular tuft do not express clusterin mRNA. Magnification, ×391.

neys, cell polarity is maintained both in young and old animals, at least as indicated by the markers used in our study, and argue against the hypothesis that a reversal of cell polarity plays a major role in the initiation and progression of cystic disease. Further evidence against a change in cell polarity leading to increased fluid transport into the tubular lumen, higher intraluminal pressure levels, and therefore to cyst formation also comes from a recent analysis of cysts in the Han:SPRD (cy/+) rat, which demonstrates higher pressure levels only in obstructed cysts, whereas nonobstructed cysts yielded normal pressure values (37).

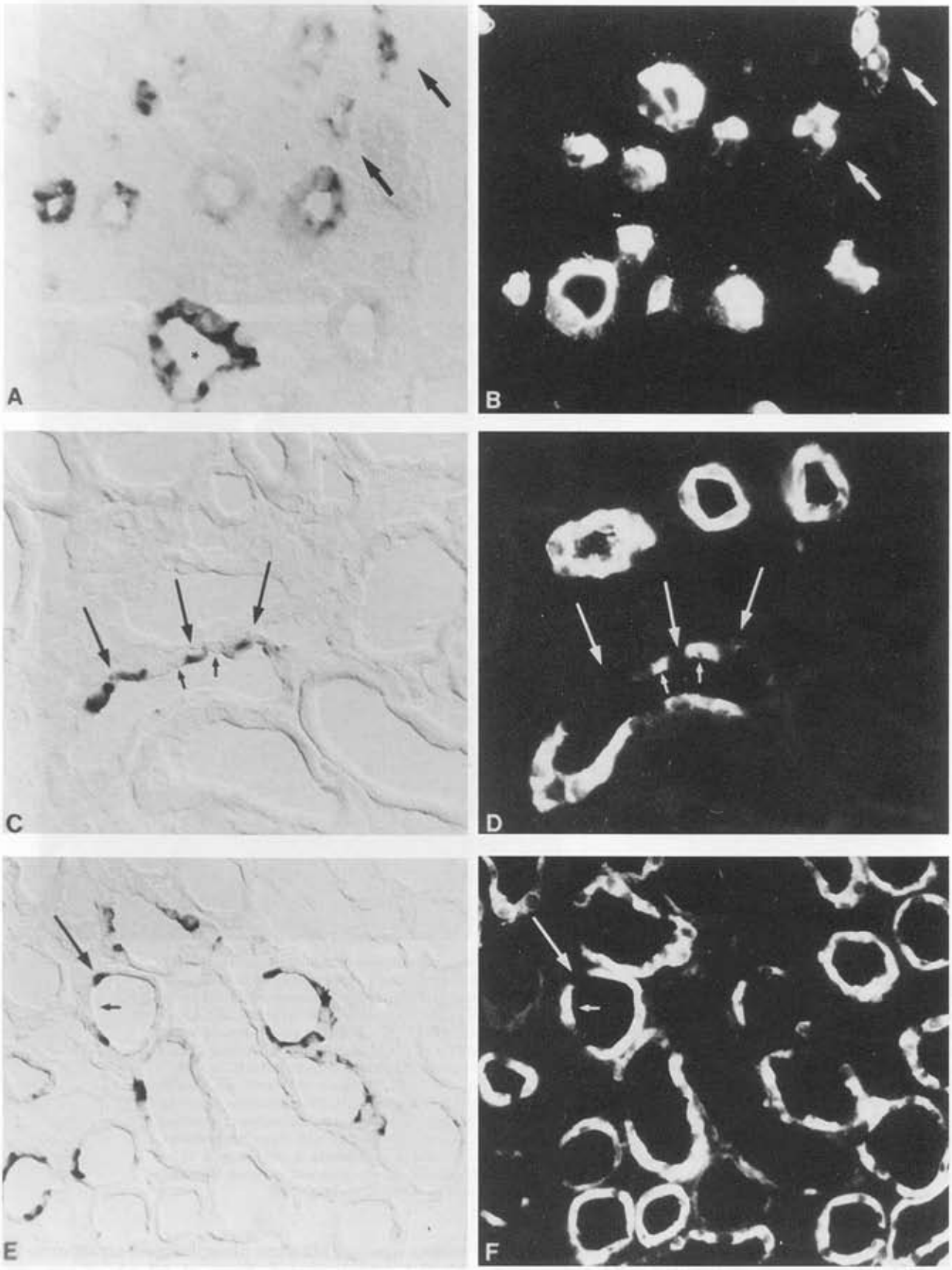
Conflicting results concerning the cell polarity of cyst-lining cells were published in other animal models of PKD as well as human ADPKD. One of the earliest reports in support of a change in cell polarity was obtained in several kidney specimens from human ADPKD patients. It could be shown that both in kidney sections and in tissue culture Na^+-K^+-ATPase was missorted to the apical membrane and that the abnormal distribution was accompanied by a ouabain-inhibitable Na^+ transport from basolateral to apical. This missorting event, however, was restricted to Na^+-K^+-ATPase and was not found in the case of other polarity markers (39). The mislocation of Na^+-K^+-ATPase in human ADPKD was confirmed in a subpopulation of cyst wall cells by some investigators (6), whereas others demonstrated the regular basolateral sorting of Na^+-K^+-ATPase in human ADPKD (15).

In the *cpk/cpk* mouse model of autosomal recessive polycystic kidney disease (ARPKD), an apical location of Na^+-K^+-ATPase was reported in cysts originating from the collecting duct but not in cysts derived from the proximal tubule. Again, no other basolateral or apical markers were missorted (1). A recent immunohistochemical study of advanced stages of PKD in *cpk/cpk* and *pcy/pcy* mice, glucocorticoid-induced PKD in mice and diphenylthiazole-induced PKD in rats demonstrated an increase of apical Na^+-K^+-ATPase staining in cysts (25), whereas, in early stages of diphenythiazole-induced PKD, other investigators found no missorting of Na^+-K^+-ATPase (7).

The discrepancies in some of these studies may be related to the heterogeneity of the models and samples. Our results in the Han:SPRD (cy/+) rat clearly show the maintenance of cell polarity and therefore do not support the hypothesis that a change in cell polarity represents a pathogenetic factor of cystogenesis in this model of ADPKD.

Differentiation and cyst formation. Our characterization of cyst wall epithelia at postnatal *days 21* and *38* demonstrated that the cells were mainly in a differentiated state at the beginning of cyst formation. At 21 days of age, the majority of proximal cysts still showed a normal epithelium with strong alkaline phosphatase activity, whereas, at 38 days of age, an increased loss of alkaline phosphatase was observed in cyst wall cells. The loss of markers normally expressed by fully differentiated proximal tubules was accompanied by the expression of clusterin mRNA, which appears transiently during very early stages of regular nephron formation (13, 18). Although, in the rat, differentiation markers such as alkaline phosphatase, aquaporin-1, and NaSi-1 cotransporter appear already in the first postnatal week of normal renal development (Refs 2, 8; unpublished observations), we cannot exclude the pos-

Fig. 10. *A* and *B*: combined in situ hybridization for clusterin mRNA (*A*) and immunohistochemical staining with an anti-aquaporin-1 antibody (*B*). Section through inner stripe of a kidney from a 38-day-old Han:SPRD (cy/+) rat demonstrates clusterin-positive descending thin limbs, which can easily be recognized by staining with an antibody against aquaporin-1 (arrows in *A* and *B*). *A*: * dilated thick ascending limb profile. *C–F*: sections through cortex and inner stripe of a kidney from a 38-day-old Han:SPRD (cy/+) rat subjected to combined in situ hybridization for clusterin mRNA (*C* and *E*) and immunohistochemistry with an anti-Tamm-Horsfall protein (THP) antibody, a marker for the thick ascending limb (*D* and *F*). Only one of four THP-positive profiles (*D*) in cortex expresses clusterin (*C*). In inner stripe, many more THP-positive profiles can be detected (*F*), and correspondingly more thick ascending limbs which express clusterin mRNA (*E*). Of note, profiles of thick ascending limbs apparently are distended. *C–F*: large arrows point to clusterin-positive, THP-negative cells; small arrows to clusterin-negative, THP-positive cells. Magnifications: *A* and *B*, ×391; *C* and *D*, ×384; *E* and *F*, ×246.


A
B
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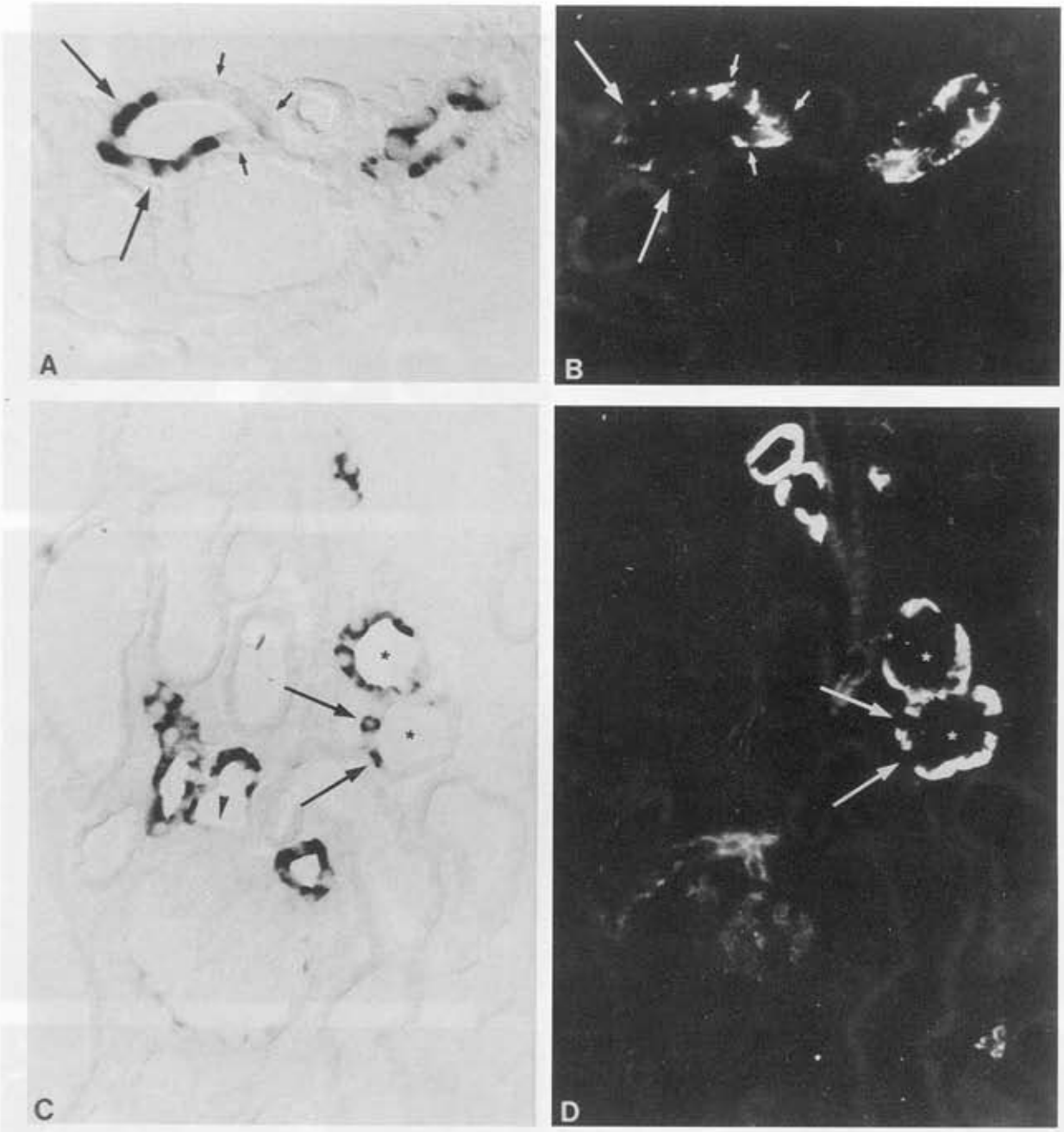

Fig. 11. *A* and *B*: combined in situ hybridization for clusterin mRNA (*A*) and immunohistochemical staining with an antibody against the α_1-subunit of Na^+-K^+-ATPase (*B*) on a kidney section through outer cortex of a 38-day-old Han:SPRD (*cy*/+) rat. Na^+-K^+-ATPase is strongly expressed in a basolateral fashion in distal convoluted tubule (*B*). Clusterin mRNA-positive cells (*A*) show a weaker but still clearly visible expression of Na^+-K^+-ATPase (*B*). Large arrows mark clusterin mRNA-positive cells with a low expression level of Na^+-K^+-ATPase; small arrows mark clusterin mRNA-negative cells with a high expression level of Na^+-K^+-ATPase. *C* and *D*: staining with an antibody against the Na^+/Ca^{2+} exchanger (*D*) shows that clusterin mRNA (*C*) is expressed in connecting tubules (*C* and *D*, *) in kidneys from 38-day-old Han:SPRD (*cy*/+) rats. Most cells in a given profile that express clusterin mRNA also stain positive for the Na^+/Ca^{2+} exchanger and therefore represent connecting tubule cells. Cells not recognized by the antibody against the Na^+/Ca^{2+} exchanger probably are intercalated cells but may also represent connecting tubule cells that have lost the Na^+/Ca^{2+} exchanger (arrows). Tubule with clusterin-positive and -negative portions, which lies close to the extraglomerular mesangium of a glomerulus, probably represents a profile of a thick ascending limb including the macula densa (*C*, arrowhead). Although macula densa does not express clusterin mRNA, the adjacent cells of thick ascending limb do. Magnifications: *A* and *B*, ×384; *C* and *D*, ×246.

sibility that the less differentiated cells in the cysts grow out from remnants of tubular cells arrested in development. In our study, however, noncystic proximal tubules were always stained homogeneously with antibodies against alkaline phosphatase, aquaporin-1, and NaSi-1 cotransporter. Even at 60 wk of age, we observed the continued formation of many cortical cysts with the regular expression of alkaline phosphatase,

NaSi-1 cotransporter, and Na^+-K^+-ATPase. The increase in the number of cysts at ~21 days after birth, when nephron development is virtually complete, would also argue against the hypothesis that the cysts originate from undifferentiated tissue. Our data are in agreement with a recent analysis of aquaporin-1 expression in proximal cysts from human ADPKD kidneys, where no profound differences between control and polycystic tissue could be demonstrated in the early stages of the disease (11).

The changes described in our study occured at a very advanced stage of nephron development and therefore would speak against an early differentiation defect as a cause of cyst formation, although we cannot rule out a late defect in differentiation. Cyst formation therefore is unlikely to be caused by an arrest in an early immature state but rather by the loss of maturity.

Pattern of clusterin expression suggests that whole nephrons are affected by PKD. Another important finding in our study was the expression of clusterin not only in cysts but also in virtually all other nephron segments including parietal cells of Bowman's capsule, although we have no evidence for clusterin expression in the ascending thin limb (for a summary of those data, see Table 1). Only a portion of all nephrons, however, expressed clusterin mRNA. The high clusterin mRNA levels in Han:SPRD (*cy*/+) rat kidneys previously detected by Northern blot analysis (10) are therefore due to the expression of clusterin mRNA in cyst wall cells and noncystic nephron segments. The topographical arrangement of clusterin mRNA-positive tubules in polycystic kidneys at 38 days of age leads us to speculate that the clusterin-positive distal segments may belong to the same nephrons affected by cyst formation in the proximal tubule, although no further evidence for this hypothesis was obtained. In the early stages of cyst formation, only few tubules expressed clusterin mRNA. This finding was particularly striking when kidneys from 21- and 38-day-old Han:SPRD (*cy*/+) rats were compared with each other.

When roughly estimating the amount of clusterin mRNA expression by the time-dependent appearance of the hybridization signal during color development, a specific and strong signal was obtained very early in distal tubular profiles before the appearance of the signal in cyst-lining epithelia of the proximal tubule. After color development was terminated, the most intense signals were seen in tubular profiles of the thick ascending limb, distal convoluted tubule, and connecting tubule. The highest levels of clusterin mRNA therefore is not expressed by cyst wall epithelia of the proximal tubules. Even in the 60-wk-old (*cy*/+)animal, very intense hybridization signals for clusterin mRNA were predominantly detected in noncystic distal nephron portions.

Double labeling experiments performed with the clusterin probe and an anti-Na^+-K^+-ATPase antibody to identify distal convoluted tubule cells showed that clusterin and Na^+-K^+-ATPase were expressed in the same cells. Likewise, connecting tubule cells still coexpressed clusterin and the basolateral Na^+/Ca^{2+} exchanger. It was not possible, however, to find cells in thick ascending limbs that expressed both clusterin and THP, despite the presence of many clusterin mRNA-positive thick ascending limbs. This expression pattern of clusterin in the thick ascending limb was similar to the situation in the cysts, where clusterin-positive cells did not express alkaline phosphatase, aquaporin-1, and NaSi-1 cotransporter. Therefore, in clusterin-positive cells, the expression of at least some proteins such as alkaline phosphatase, aquaporin-1, NaSi-1 cotransporter, and THP seems to be shut off. Our findings extend previous reports on clusterin expression in human PKD (12) and polycystic kidneys from *cpk/cpk* mice (18). Whereas there clusterin expression seems to have been more prominent in epithelia of cysts, we find the highest levels of clusterin mRNA in noncystic nephron portions. The different distribution may reflect the genetic and morphological variability in human PKD, *cpk/cpk* mice, and Han:SPRD (*cy*/+) rats.

We want to point out the fact that we could not obtain any evidence for clusterin mRNA expression in collecting ducts of Han:SPRD (*cy*/+) rats, which may indicate that collecting ducts are not affected by PKD in this rat model of ADPKD. Such a hypothesis would be supported by the fact that only in homozygous (*cy/cy*) animals, where both alleles of the PKD gene are mutated, also collecting ducts are cystically transformed early in the course of the disease (9, 34). This different susceptibility to PKD may reflect the developmental origins of collecting ducts and nephrons. Whereas the collecting ducts develop from the invading ureter bud, the nephrons originate from the metanephrogenic mesenchyme. This interpretation is strengthened by the fact that in human ADPKD a mutation in the second allele of *PKD1* was found in cystically transformed nephrons. The mutation in the second allele is thought to be a somatic mutation, which would

Table 1. *Expression of clusterin mRNA in kidneys of Han:SPRD (cy/+) rats and coexpression of clusterin mRNA with various marker proteins in the same cells*

Nephron Segments and Collecting Duct	Marker	Clusterin mRNA	Coexpression
Podocytes	−*	−	NA
Parietal epithelial cells	−*	+	NA
Proximal tubule	Alkaline phosphatase, aquaporin-1, NaSi-1 cotransporter	Normal profiles, − cysts, +	Normal profiles, NA cysts, no
DTL	Aquaporin-1	+	Yes
ATL	−	ND	NA
TAL	Tamm-Horsfall protein	+	No
DCT	Na^+-K^+-ATPase	+	Yes
CNT	Na^+-Ca^{2+} exchanger	+	Yes
Collecting duct	Aquaporin-2	−	NA

DTL, descending thin limb; ATL, ascending thin limb; TAL, thick ascending limb; DCT, distal convoluted tubule; CNT, connecting tubule; NA, not applicable; ND, not determined. *Identified by morphological means.

explain why only a certain portion of the nephrons are affected by the disease (3, 27).

Our study shows that dedifferentiation in the course of cyst formation in the Han:SPRD (*cy*/+) rat appears late when renal development has progressed far. Cyst-lining cells did not undergo a change in cell polarity. Based on the observation that clusterin is a very efficient mediator of cell-cell adhesion, it can be speculated that clusterin serves to establish and maintain epithelial integrity, similar to the situation during renal development when new epithelia have to be formed. We believe that clusterin is protective rather than damaging to cells and an indicator rather than a mediator of cell injury. The distribution of clusterin mRNA leads us to speculate that polycystic disease affects the entire nephron in the Han:SPRD (*cy*/+) rat model of ADPKD, although cystic changes occur in the proximal tubule.

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