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Elevated c-*myc* protooncogene expression in autosomal recessive polycystic kidney disease

(*cpk* mouse/compensatory hypertrophy/uninephrectomy/folic acid/acute renal failure)

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ABSTRACT **The polycystic kidney diseases (PKDs) are a group of disorders characterized by the growth of epithelial cysts from the nephrons and collecting ducts of kidney tubules. The diseases can be inherited or can be provoked by environmental factors. To investigate the molecular basis of the abnormal cell growth associated with PKD, c-*myc* protooncogene expression was studied in a mouse model for autosomal recessive PKD. Homozygous recessive C57BL/6J (*cpk/cpk*) mice develop massively enlarged cystic kidneys and die from renal failure shortly after 3 weeks of age. Quantitative dot blot and RNA blot hybridization experiments in which whole kidney poly(A)$^+$ RNA was hybridized with a c-myc RNA probe showed a 2- to 6-fold increase in c-myc mRNA at 2 weeks, and a 25- to 30- fold increase in c-myc mRNA at 3 weeks of age in polycystic mice, as compared to normal littermates. c-*myc* expression was also examined under two conditions in which kidney cell growth was experimentally induced in normal adult mice: compensatory renal hypertrophy and tubule regeneration following folic acid-induced renal cell injury. While compensatory hypertrophy resulted in only a small (<3-fold) increase in c-myc, folic acid treatment gave rise after 24 hr to a 12-fold increase in c-myc mRNA. The induction of c-*myc* by folic acid is consistent with increased cellular proliferation in regenerating tubules. In contrast, polycystic kidneys show only a minimal increase in cellular proliferation over that seen in normal kidneys, while c-*myc* levels were found to be markedly elevated. Thus, the level of c-*myc* expression in cystic kidneys appears to be out of proportion to the rate of cell division, suggesting that elevated and potentially abnormal c-*myc* expression may be involved in the pathogenesis of PKD.**

Elevated protooncogene expression has been demonstrated in embryonic and neonatal tissues (1), in cultured cells stimulated with growth factors (2–5), in regenerating liver after partial hepatectomy or toxic injury (6, 7), and in a number of malignant tumors (8, 9). All of these studies have suggested that protooncogenes are involved in the regulation of cellular proliferation. In particular, expression of the c-*myc* gene has been associated with numerous types of normal and abnormal cell growth (10). Expression of the c-*myc* protooncogene occurs transiently in growth-stimulated cells and appears to be an early event that is temporally correlated with the transition from G_0 to G_1 (11). In cases in which the gene is abnormally expressed there may be high levels or constitutive expression of c-myc mRNA (5, 8, 9). While the function of c-*myc* is not known, there is evidence to suggest that it encodes a nuclear protein that may be involved in DNA synthesis (12).

The polycystic kidney diseases (PKDs) are a group of disorders characterized by massive kidney enlargement resulting from the formation of countless epithelial-lined cysts that develop from the nephrons and collecting ducts of renal tubules (13). The disease can be inherited as an autosomal dominant (AD) PKD or as an autosomal recessive (AR) PKD trait (14, 15), or it can be provoked by environmental factors (acquired PKD) (16, 17). Several observations have suggested that potentially abnormal cell growth may be involved in cyst formation (18, 19). There is an increased surface area of cysts and thus increased numbers of cells compared to normal tubules, although in some forms of PKD, cyst growth occurs over a long period of time (20). The presence of undifferentiated cells and micropolyps has also been observed (18, 20), and there is an increased incidence of tumors in certain forms of PKD (21–23). While markedly elevated levels of cellular proliferation are not necessarily required to explain these findings, they do suggest that abnormal regulation of cell growth control may occur in PKDs.

To learn more about the molecular basis of these diseases, we have investigated c-*myc* gene expression in a mouse model of ARPKD. We have also determined the levels of c-*myc* expression under two other conditions typified by increased kidney cell growth: tubule cell regeneration after folic acid-induced acute renal injury and compensatory hypertrophy after contralateral uninephrectomy. While c-*myc* expression was observed in each of these models of renal cell growth, extremely high levels of c-myc mRNA were found in cystic kidneys, suggesting that abnormal c-*myc* expression may play a role in the pathogenesis of PKD.§

METHODS

Animals. C57BL/6J (*cpk/cpk*) mice, which have a form of ARPKD (24), were obtained from The Jackson Laboratory and maintained as a breeding colony in our facilities. Homozygous recessive mice develop massively enlarged cystic kidneys, become azotemic, and die soon after 3 weeks of age. Heterozygotes and homozygous dominant mice are phenotypically normal. Cystic mice were sacrificed at the age of 2 weeks and 3 weeks, and kidneys were processed for RNA isolation. Noncystic littermates served as controls. Retroorbital venous blood was obtained for blood urea nitrogen determinations.

Acute renal injury was induced in normal adult CF-1 mice (25–30 g) by intraperitoneal injection of 250 or 300 mg of folic acid per kg of body weight in 150 mM $NaHCO_3$. Controls received only $NaHCO_3$. Mice were sacrificed after 24 hr and kidneys were processed for RNA isolation.

To study compensatory hypertrophy, normal adult CF-1 mice were subjected to left uninephrectomy. The left kidneys served as controls. Mice were sacrificed after 3, 6, 12, or 24

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Abbreviations: PKD, polycystic kidney disease; ADPKD, autosomal dominant PKD; ARPKD, autosomal recessive PKD.

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hr and right kidneys were removed. Left and right kidneys were processed separately for RNA isolation.

RNA Isolation and Hybridization. Whole kidney RNA was isolated using the technique of Chirgwin *et al.* (25). Samples were enriched for poly(A)$^+$ RNA by one cycle of oligo(dT)-cellulose chromatography. Yields of poly(A)$^+$ RNA were 7–11% of total RNA. Poly(A)$^+$ RNA samples were denatured in 2.2 M formaldehyde/50% formamide and electrophoresed in 2.2 M formaldehyde/1.5% agarose gels. RNA was transferred to nitrocellulose filters according to Thomas (26). Alternatively, poly(A)$^+$ RNA was denatured in CH_3HgOH and dotted directly onto nitrocellulose filters. Filters were baked at 60°C–70°C overnight.

Filters were prehybridized at 66°C in 3× SET (20× SET is 3 M NaCl/0.04 M EDTA/0.6 M Tris·HCl, pH 8)/0.1% $NaDodSO_4$/10× Denhardt's solution (10× Denhardt's solution is 0.2% Ficoll/0.2% polyvinylpyrrolidone/0.2% bovine serum albumin) for 2 hr, and then in 3× SET/0.1% $NaDodSO_4$/10× Denhardt's solution/250 μg of tRNA per ml for 2 hr. Hybridizations were performed overnight at 66°C in 3× SET/0.1% $NaDodSO_4$/20 mM sodium phosphate buffer, pH 7.8/10× Denhardt's solution/250 μg of tRNA per ml/10% dextran sulfate, with 10^6 cpm of probe per ml of hybridization solution. c-*myc* filters were washed at 66°C in 1× SSC/0.1% $NaDodSO_4$ for 1 hr and in two changes of 0.3× SSC/0.1% $NaDodSO_4$ for 1 hr each (20× SSC is 3 M NaCl/0.3 M trisodium citrate). Histone H4 filters received a final wash in 0.1× SSC/0.1% $NaDodSO_4$ rather than 0.3× SSC/0.1% $NaDodSO_4$. Filters were then exposed to Kodak XAR film. Autoradiographs were quantitated by densitometry.

Plasmid DNA, pSVc-myc1, containing mouse c-*myc* exons 2 and 3 was obtained from American Type Culture Collection (27). A *Hin*dIII/*Sac* I fragment of this mouse DNA was subcloned into pSP64 (Promega Biotec, Madison, WI). This plasmid was linearized with *Pvu* II prior to transcription, and radiolabeled [^{32}P]RNA probes specific for exon 3 were generated using SP6 RNA polymerase. Plasmid DNA pHu4A (28) containing human histone H4 sequences was provided by D. Beer (University of Kansas Medical Center). A *Hin*dIII/*Sac* I fragment of this DNA was subcloned into pGEM3blue (Promega Biotec). This plasmid was linearized with *Sac* I prior to transcription with SP6 polymerase for generation of radiolabeled probes. Specific activities were calculated to be approximately 1–2 × 10^9 cpm/μg.

RESULTS

Elevated expression of the protooncogene c-*myc* is often associated with cell proliferation. To determine whether this is also the case in the kidney and to set a standard of comparison for c-*myc* expression under conditions in which kidney cells have been stimulated to proliferate, mice were given a toxic dose of folic acid, and 24 hr later kidney RNA was isolated and analyzed. Folic acid is one of the most potent stimuli for cellular proliferation in the kidney. The administration of a single large dose of folic acid is known to be followed within ≈24 hr by the onset of DNA synthesis, probably as a result of tubule cell regeneration following folic acid-induced injury (29–32). Fig. 1 shows hybridization analyses for whole kidney RNA samples from control and folic acid-treated mice. Dots in columns 1 and 2 represent total kidney RNA, and those in columns 4 and 5 represent poly(A)$^+$ RNA. As a positive control, HeLa cell cytoplasmic RNA, which is known to be high in c-myc (33), is shown by the dots in column 3. The specificity of hybridization to c-myc mRNA was confirmed by RNA blot hybridization (for example, see Fig. 4). Densitometry of dot blots and RNA blots showed an ≈12-fold enhancement of steady-state c-myc mRNA levels induced by folic acid. Thus, c-*myc* gene expression can be significantly increased in the adult kidney

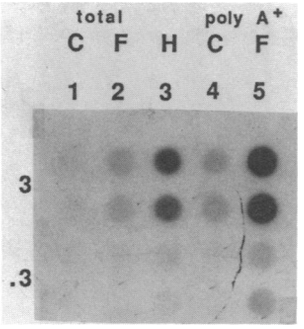

FIG. 1. Enhanced expression of c-*myc* in regenerating kidneys after folic acid-induced renal cell injury. Acute renal injury was induced in normal adult mice with a single injection of folic acid (F). Controls (C) received only $NaHCO_3$. Kidneys were removed after 24 hr and whole kidney total RNA or poly(A)$^+$ RNA was applied to nitrocellulose in duplicate 3- or 0.3-μg dots. HeLa cell cytoplasmic RNA (H) was included as a positive control. The filter was hybridized with a [^{32}P]cRNA probe specific for exon 3 of the c-*myc* gene.

(compare columns 4 and 5) following folic acid-induced renal cell injury.

Kidney cell growth can also be stimulated by unilateral nephrectomy (34). Cell growth as a result of unilateral nephrectomy differs from that induced by folic acid, however, since the major response of the contralateral kidney is to undergo hypertrophy rather than hyperplasia. The RNA blot hybridization in Fig. 2 shows c-myc mRNA in controls (0 hr) and in kidneys undergoing compensatory hypertrophy (3–24 hr). Lanes 2–5 represent poly(A)$^+$ RNA samples from the contralateral kidneys after 3, 6, 12, and 24 hr, respectively. There appears to be a low but reproducible level of c-myc mRNA in the normal adult kidney. Uninephrectomy causes a slight decrease in c-myc mRNA levels 3 and 6 hr later (lanes 2 and 3) followed by a modest increase (<3-fold) at 12 and 24 hr. Other experiments (data not shown) indicate that c-myc levels return to baseline by 96 hr after uninephrectomy. The increase in c-myc mRNA caused by uninephrectomy is modest compared to that induced by folic acid, and its significance is unclear.

To determine whether elevated levels of c-myc mRNA are associated with PKD, poly(A)$^+$ RNA was prepared from the

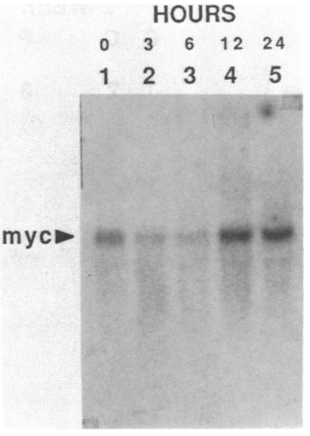

FIG. 2. Expression of c-myc mRNA during compensatory renal hypertrophy. Normal adult mice were subjected to left uninephrectomy. Left kidneys served as 0-hr controls (lane 1). Right kidneys were removed at 3, 6, 12, and 24 hr (lanes 2–5). Samples (10 μg) of poly(A)$^+$ RNA were electrophoresed, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled c-myc RNA probe.

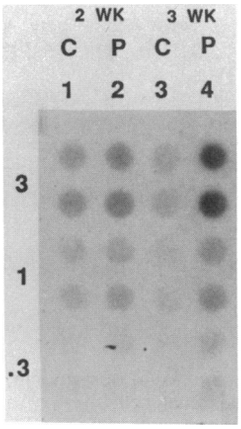

FIG. 3. Elevated expression of c-*myc* in ARPDK. Duplicate 3-, 1-, and 0.3-μg samples of poly(A)$^+$ RNA from 2-week-old (2 WK) and 3-week-old (3 WK) control (C) and polycystic (P) mice were applied to nitrocellulose and hybridized as described in Fig. 1.

kidneys of C57BL/6J (*cpk/cpk*) mice, which have ARPKD. By 2 weeks after birth, the abdomens of cystic mice are distended from the markedly enlarged polycystic kidneys, yet blood urea nitrogen levels (mg/dl) are only modestly elevated (mean ± SD: controls, 26.1 ± 5.6; polycystic, 37.1 ± 9.5). At 3 weeks of age, these kidneys weigh ≈10 times more than those of normal littermates (V. H. Gattone, personal communication), and blood urea nitrogen levels are markedly increased (controls, 30.0 ± 5.9; polycystic, 100.9 ± 30.9). Shortly after 3 weeks of age, cystic mice die from renal failure. Fig. 3 shows dot blot hybridizations for control and polycystic kidneys from 2- and 3-week-old mice. Two-week-old noncystic mice (column 1) exhibit a low level of c-*myc* expression, and this decreases by 3 weeks of age (column 3). In contrast, polycystic mice show increased c-*myc* at 2 weeks (column 2) and a further increase at 3 weeks of age (column 4).

Fig. 4*A* shows RNA blot hybridizations for 2- and 3-week-old control and polycystic mice from separate litters, showing the degree of reproducibility in these experiments. From the RNA blots in Fig. 4*B*, it is clear that c-*myc* expression declines in the kidneys of normal mice (lanes 1–3) with increasing age (2 weeks, 3 weeks, and adult). In contrast, polycystic kidneys show striking elevations in c-*myc* levels (lanes 4 and 5). Densitometry of several RNA blots has indicated that there is a 2- to 6-fold increase in c-*myc* expression at 2 weeks, and a 25- to 30-fold increase in c-*myc* expression at 3 weeks of age in polycystic mice. As mentioned previously, folic acid treatment markedly stimulates renal cell proliferation and at 24 hr results in a 12-fold increase in c-*myc* expression in the adult mouse kidney.

To assess the degree of cellular proliferation in polycystic kidneys at 2 weeks and 3 weeks of age, total RNA was hybridized with a histone H4 probe. The RNA blot in Fig. 5 shows histone H4 mRNA levels in control and polycystic kidneys from 2-week-old (lanes 1 and 2) and 3-week-old (lanes 3 and 4) mice. These results show only minimal increases in histone H4 mRNA levels in polycystic kidneys and thus suggest that the rates of cellular proliferation are very similar, as judged by this technique, despite the striking increases in c-myc mRNA observed at the same ages.

DISCUSSION

We have demonstrated expression of c-myc mRNA in neonatal and adult mouse kidneys and have also shown decreasing c-*myc* expression with age. This confirms previous observations (35) and suggests that c-*myc* may have an important role during early kidney growth and that there may be a role for c-*myc* in the adult kidney as well. We have also examined c-myc mRNA levels under three conditions in which kidney cell growth has been induced: after folic acid treatment, during compensatory renal hypertrophy, and in ARPKD.

Folic acid is known to be a potent stimulus of cell proliferation in the kidney. Studies in rats have shown increases in [^{3}H]thymidine incorporation of 4- to 16-fold in renal cortex and 20- to 50-fold in the outer medulla (29–31). Studies in mice have shown similar increases in renal cell proliferation induced by folic acid (32). In both cases, these increases are paralleled by corresponding increases in mitotic

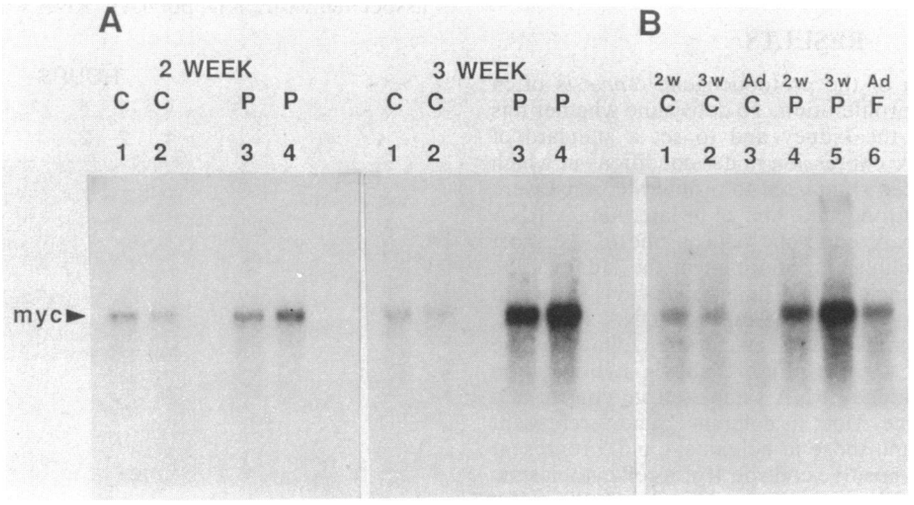

FIG. 4. Elevated expression of c-myc mRNA in ARPKD. (*A*) Samples (5 μg) of poly(A)$^+$ RNA from 2- and 3-week-old control (C) and polycystic (P) mice. Samples in lanes 1 and 3 in each set and in lanes 2 and 4 in each set represent normal and cystic littermates, respectively (four different litters are represented). (*B*) Samples (10 μg) of poly(A)$^+$ RNA from 2- and 3-week-old noncystic control (C) *cpk* mice (2w and 3w, lanes 1 and 2), from normal control (C) adult CF-1 mice (Ad, lane 3), from 2- and 3-week-old polycystic (P) mice (2w and 3w, lanes 4 and 5), and from folic acid-treated (F) adult CF-1 mice (Ad, lane 6). RNAs were electrophoresed and hybridized as described in Fig. 2. Hybridization of these samples on the same blot has permitted a more direct quantitative comparison of the levels of c-myc mRNA in each preparation.

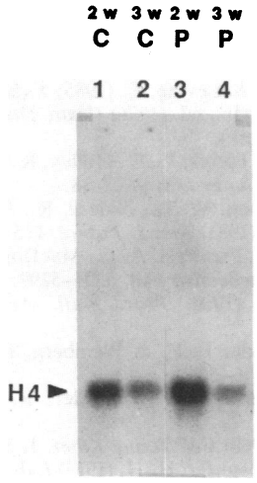

FIG. 5. Expression of histone H4 mRNA in ARPKD. Samples (5 μg) of total RNA from 2- (2w) and 3-week-old (3w) control (C) and polycystic (P) mice. Samples in lanes 1 and 2 and in lanes 3 and 4 represent normal and cystic littermates, respectively. RNAs were electrophoresed and hybridized as described in Fig. 2. In this experiment (and in others), there was a <2-fold difference in histone H4 mRNA levels between normal and cystic mice at ages 2 and 3 weeks.

indices. The data presented here (Figs. 1 and 4) demonstrate significantly elevated levels of c-myc mRNA in kidneys of folic acid-treated mice. This clearly demonstrates increased renal protooncogene expression in a nonmalignant pathologic condition in otherwise normal adult animals. The elevated c-myc levels most likely reflect increased cellular proliferation that accompanies the repair process induced by folic acid injury, a situation analogous to liver regeneration after toxic insult (6). It is reasonable to suppose that renal cell proliferation involves some of the same molecular mechanisms observed in other proliferating cell types and in tumorigenesis.

Baserga *et al.* (32) have noted that folic acid is more effective in stimulating cell proliferation when dissolved in 300 mM Na_2CO_3 rather than in 300 mM $NaHCO_3$. In fact, we have observed induction of c-myc by 300 mM Na_2CO_3 alone (data not shown). Since this concentration of Na_2CO_3 is both hypertonic and alkaline, we do not know whether this induction of c-myc mRNA represents a proliferative response induced by the high salt or perhaps a more general stress response in the animal (or both). In the experiments reported in Figs. 1 and 4, we used 150 mM $NaHCO_3$, which alone does not induce c-myc (see controls).

The increase in c-myc mRNA observed after uninephrectomy is quite modest (Fig. 2) and is consistent with observations made by other investigators (36, 37). It is unclear whether this is related to a small amount of hyperplasia or whether this is a manifestation of hypertrophy. Fine (34) has recently suggested that the stimulus for hypertrophy and hyperplasia may be the same. If this is the case, it is conceivable that some of the early molecular mechanisms may be similar in cells stimulated to undergo either type of growth.

The extent of c-*myc* expression in the kidneys of autosomal recessive polycystic mice (Figs. 3 and 4) is surprising in spite of the significant kidney enlargement observed at 2 and 3 weeks of age. In actuality, this kidney enlargement is due primarily to the formation of numerous fluid-filled cysts rather than to significantly increased cellular proliferation. Histone H4 gene expression, which is tightly coupled to DNA synthesis (38), was used as a measure of the relative rates of cell division in the kidneys of 2- and 3-week-old animals. At 2 and 3 weeks of age, there was little difference in histone H4 mRNA levels between control and polycystic kidneys (Fig. 5; unpublished data). These results are in agreement with measurements of mitotic indices at ages newborn to 3 weeks, which indicated that the rate of cellular proliferation is only marginally increased (<2-fold) in cystic compared to noncystic mice (V. H. Gattone, personal communication). It should be noted that histone H4 mRNA levels in cystic kidneys decreased between 2 and 3 weeks of age, while c-myc mRNA levels increased. Thus, the level of c-*myc* expression in cystic kidneys appears to be out of proportion to the degree of cellular proliferation, and this suggests a distinct abnormality in the regulation of c-*myc* gene expression. This indicates that elevated c-*myc* expression is not simply a marker for proliferation in ARPKD and suggests that abnormal c-*myc* expression may be involved in the pathogenesis of PKD.

While the specific cell types expressing c-*myc* cannot be defined using whole kidney RNA, the lack of a significant inflammatory response as determined by histological inspection (V. H. Gattone, personal communication) would rule out inflammatory cell infiltration as a source of the c-myc mRNA. Additional studies will be required to determine whether the abnormal c-*myc* expression is localized to cysts or is a more general response of the kidney. Blood urea nitrogen measurements have indicated that polycystic mice are mildly azotemic by 2 weeks and severely azotemic by 3 weeks of age. Thus, the increased c-*myc* expression could be due to some aspect of the azotemic state, which could increase c-myc mRNA levels by a variety of mechanisms. There is little or no increase in c-myc mRNA in the livers of 2- or 3-week-old polycystic mice (data not shown), suggesting that azotemia is not producing a generalized elevation of c-myc mRNA in all organs of the body.

c-*myc* may be only one of several genes that is abnormally expressed in polycystic kidneys. The *cpk* mice used as a model for PKD inherit the disease as an autosomal recessive trait. It is difficult to envision a recessive disease that is caused by the overexpression of a gene, as observed for c-*myc* in these studies. It is more likely that the ARPKD defect results in a deficient or abnormally functioning protein and that the expression of this primary defect triggers a series of other events, which may include the overexpression of c-*myc*. However, while c-*myc* is not necessarily the primary cause of the disease, its overexpression may contribute to the abnormal cell growth associated with cyst formation. It is also possible that other protooncogenes are abnormally expressed and that these, too, contribute to the abnormal phenotype.

The more common form of polycystic kidney disease in humans is dominantly inherited (ADPKD) and appears to be caused by a single defective gene (39). There is also a rare infantile PKD in humans, which is recessive (14, 15). In addition, PKD can be induced by environmental factors (acquired PKD) (21). The various types of PKD show different characteristics with respect to their rates of cyst growth and to cyst localization, both within the kidney and in other organs (14, 15, 22). ADPKD, for example, gives rise not only to cysts in the kidney but also to cysts in the liver and pancreas, to intestinal diverticula, and to cerebral aneurysms (14, 15, 39, 40). The heterogeneity manifested in the different types of PKD would suggest that more than one gene is involved as their primary cause. It may also be possible that protooncogenes are differentially expressed in the various forms of PKD. Thus, an understanding of protooncogene expression in the PKDs may provide useful markers for diagnosis and, in addition, should lead to a better understanding of the pathogenesis of the various disease states.

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