Localization of overexpressed c-*myc* mRNA in polycystic kidneys of the *cpk* mouse

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Localization of overexpressed c-*myc* mRNA in polycystic kidneys of the *cpk* mouse. The C57BL/6J-*cpk* mouse has a form of autosomal-recessive polycystic kidney disease characterized by the rapid growth of large collecting duct cysts and the development of severe renal failure usually by three to four weeks of age. Previous studies had shown higher steady-state levels of proto-oncogene mRNA in these cystic kidneys. It is now shown using nuclear run-on transcription that the c-*fos* and c-*myc* proto-oncogenes are transcribed at higher rates in cystic kidneys, and thus that increased transcription, in part, may account for the increased mRNA levels. c-*myc* mRNA was detected by in situ hybridization in nephron anlagen and elongating tubules of normal and cystic kidneys during late fetal and early neonatal kidney development. Localization of c-*myc* expression in the normal kidney decreased with age over the three-week postnatal period. By contrast, c-*myc* mRNA was found in cysts as early as three days of age, with increased levels at two and three weeks. c-*myc* expression was also elevated in apparently normal, non-dividing proximal tubules in three-week-old cystic animals. On the basis of these findings, we suggest that c-*myc* expression is linked to the proliferation of cells engaged in the primary cystogenic process, and that expression of this gene in proximal tubule cells of severely azotemic animals reflects the compensatory response of residual tubular epithelial cells to progressive renal dysfunction.

Polycystic kidney disease (PKD) in humans can be inherited as an autosomal dominant [1] or autosomal recessive [2] trait, or can be provoked by environmental factors (acquired cystic disease) [3]. PKD is characterized by the growth of large epithelial-lined cysts derived from the nephrons and collecting ducts of affected kidneys [4]. Cysts are thought to initiate as small dilations in renal tubules, which then expand into fluid-filled cavities of relatively large size. In some cases, cyst formation appears to involve both increased cell proliferation and epithelial fluid secretion [5]. In addition, polycystic kidneys also display changes in tubular basement membrane [6, 7], tubular epithelial polarity [8], and growth factor expression [9]. Despite the growing list of morphological and biochemical abnormalities that have been found in polycystic kidneys, the pathogenesis of PKD which ultimately leads to kidney failure is not yet understood.

We are currently investigating the C57BL/6J-*cpk* mouse which manifests PKD in a manner similar to human autosomal-recessive polycystic kidney disease [10]. Autosomal-recessive PKD is characterized by the rapid development of renal cysts, primarily of collecting duct origin, and renal failure early in life [2]. Cystic disease in the affected *cpk/cpk* mouse begins with dilations of proximal tubules in fetal and newborn animals. The cortical and medullary collecting ducts start to enlarge shortly after birth, and by three weeks of age the affected animals have grossly distended abdomens due primarily to the expansion of these collecting duct cysts. The cystic mice die of kidney failure, usually during their fourth postnatal week.

Previous studies undertaken in our lab have shown marked overexpression of the c-*fos*, c-*myc*, and c-*Ki-ras* proto-oncogenes in *cpk* mouse kidneys [11–13]. The transient expression of c-*fos* and c-*myc* is usually associated with increased mitogenic activity, and when constitutively expressed or abnormally high is characteristic of unregulated cell growth [14]. In the developing kidney, c-*myc* expression occurs in uninduced nephrogenic mesenchyme and immature tubules, in association with the increased cell proliferation seen at that time [15]. Thus, the high levels of c-*fos* and c-*myc* mRNA in cystic kidneys may indicate that certain cells in these kidneys are in a state of persistent or unregulated mitogenic stimulation.

An examination of cell proliferation in the cystic kidneys of *cpk* mice has indicated that rates of cell division are somewhat elevated and that cell number increases about threefold over normal during the three-week postnatal period [10, 11]. While this increase in mitotic activity probably accounts for some of the elevated c-*fos* and c-*myc* expression, it has been noted that proto-oncogene expression increases out of proportion to the expression of histone H4, which is a marker of cell proliferation [10–13]. These results suggested to us that proto-oncogene expression may also occur in non-dividing cells and might be associated with other processes involved in the pathogenesis of polycystic kidney disease.

In order to understand the role of proto-oncogene overexpression in polycystic kidney disease, we have determined the level of regulation at which c-*fos* and c-*myc* expression is altered and in which kidney cells proto-oncogene expression is affected. The results suggest that two different processes may be responsible for elevated proto-oncogene expression in cystic kidneys; one occurring early in the disease that is associated

with increased cell proliferation and cystogenesis, and a second occurring late in the disease that is associated with the compensatory response to kidney failure.

Methods

Animals

C57BL/6J-*cpk* mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA), and have been maintained as a breeding colony at the University of Kansas Medical Center. Offspring of heterozygous breeders were examined at 16 gestational days, 3 postnatal days and at 1, 2, and 3 weeks of age.

Nuclear run-on transcription

Nuclei from the kidneys of three-week-old *cpk/cpk* polycystic mice ($N = 4$) and phenotypically normal littermates ($N = 5$) were isolated by the method of Lamars, Hanson and Meisner [16]. Run-on transcription analysis was carried out by modification of methods used by others [16, 17]. Briefly, 3×10^7 normal and cystic kidney cell nuclei were suspended in 200 μl of transcription buffer (25% glycerol, 2.5 mM $MgCl_2$, 0.05 mM EDTA, 75 mM Hepes pH 7.5, 100 mM KCl, 4 mM DTT, 0.5 mM CTP, 0.5 mM GTP, 1 mM ATP, 8.8 mM creatine phosphate, 0.04 mg/ml creatine kinase, 300 μCi of 800 Ci/mmol [α-^{32}P] UTP) and then incubated at room temperature for 15 minutes. The reactions were terminated by adding 260 μl of DNase I incubation buffer (30 mM Tris-HCl pH 7.4, 15 mM $CaCl_2$, 90 μg/ml yeast tRNA) and 50 μl of DNase I/proteinase K solution (0.5 mg/ml DNase I, 0.5 mg/ml proteinase K, 20 mM Tris-HCl pH 7.4, 10 mM $CaCl_2$) that had been preincubated 30 minutes at 37°C. Digestion of nuclei was carried out at 37°C for 30 minutes with frequent agitation. Seventy microliters of a solution containing 0.5 mM EDTA and 10% SDS were added and incubation continued for 15 minutes. Extraction was carried out with phenol/chloroform. The RNA was then precipitated by adding an equal volume of 20% TCA and incubating on ice. Unincorporated ^{32}P-UTP was removed from the pellet by multiple washings with 5% TCA. The RNA pellet was dissolved and then ethanol precipitated. The labeled RNA was redissolved in HENS/tRNA (20 mM Hepes pH 7.0, 5 mM EDTA, 50 mM NaCl, 0.1% SDS, 1 mg/ml yeast tRNA) and incorporation of radioactivity was measured by scintillation counting.

Nitrocellulose filters were slot-blotted with 1 μg each of linearized plasmids containing a c-*fos*, c-*myc*, hsp70 and preproEGF DNAs. After baking, the filters were prehybridized and hybridized in the manner used for Northern blots [18] with 1×10^7 cpm of transcribed RNA. Washing was carried out at 65°C for several hours in 5× SSC (SSC is 0.15 M NaCl, 0.015 M Na_3citrate, pH 7), 0.1% SDS, then for several more hours in 2× SSC, 0.1% SDS. The filters were autoradiographed and the relative intensity of the hybridization signal was determined by densitometry.

Cycloheximide treatment

Cycloheximide treatment was used in some animals to stabilize mRNAs to increase the sensitivity of in situ hybridization. Mice were injected intraperitoneally with 0.01 ml/g (body weight) of 5 mg/ml cycloheximide solution (dose = 50 μg/g body weight) made in 150 mM sodium bicarbonate, pH 8. Untreated controls were injected with the same volume of bicarbonate buffer.

In situ hybridization

The methods for in situ hybridization are described in Harding et al [19]. Briefly, mice were anesthetized with sodium pentobarbital and their kidneys were fixed by perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS) followed by immersion in the same fixative. Pieces of kidney were cryoprotected by soaking in 15% sucrose/PBS then embedded in ornithine carbamyl transferase (OCT) compound and snap-frozen in liquid N_2. Eight micrometer sections were mounted on poly-L-lysine coated slides and fixed an additional 15 minutes in 4% paraformaldehyde/PBS. After rinsing in PBS, slides were acetylated, washed in 2× SSC, and dehydrated in ethanol. Sections were incubated first in 5 mM $MgCl_2$/PBS and then in 0.25 M Tris, 0.1 M glycine, pH 7. A wash of 50% formamide, 2× SET (SET is 0.15 M NaCl, 2 mM EDTA, 0.03 M Tris, pH 8) was carried out at 37°C for 10 minutes. Hybridization solution, containing 2× SET, 10× Denhardt's solution, 250 mg/ml yeast tRNA, 50% formamide, 0.1 M DTT, 10% dextran sulfate, and approximately 0.05 μg/ml of ^{35}S-labeled cRNA probe was placed on the sections, then covered with a siliconized coverslip and immersed in paraffin oil. Hybridization took place overnight at 45°C. Excess paraffin oil was removed by washing in $CHCl_3$, and coverslips were removed by soaking in 4× SSC. Slides were washed in 50% formamide, 2× SET, 10 mM DTT for 15 minutes at 60°C, then incubated with RNase A (20 μg/ml RNase A, 3× SET, 100 μg/ml BSA) for 30 minutes at 37°C. After washing in 1× SSC at room temperature, the slides were washed a final time in 0.2× SSC, 0.1% mercaptoethanol at 50°C for 30 minutes, then dehydrated in ethanol. Autoradiography was performed with Kodak NTB-2 liquid emulsion. The sections were stained in hematoxylin and eosin following development.

DNA probes

The c-*myc*, c-*fos*, hsp70 and preproEGF subclones were supplied by Dr. G. Andrews. The c-*fos* clone consists of a 1.8 kb EcoRI/SstI fragment of a mouse clone from American Type Culture Collection subcloned into pSP64. This subclone spans the last three exons of the c-*fos* gene and was linearized with EcoRI prior to transcription. The c-*myc* clone consists of a 1.9 kb HindIII/SacI insert from a mouse c-*myc* clone obtained from the American Type Culture Collection. The insert was directionally cloned into pSP64 and when linearized with PvuII and transcribed, yields a 500-base cRNA probe containing exon 3 sequences. The c-*fos* and c-*myc* subclones do not contain exon 1 sequences, where termination of transcriptional elongation is thought to occur.

A mouse hsp70 clone was obtained from Dr. Richard Morimoto. The subclone consists of a 1.9 kb BamHI/EcoRI coding region fragment inserted into pSP64. This clone when linearized with XmnI yields a 990 base cRNA probe. The preproEGF clone has been previously described [9].

Results

Our previous studies showed approximately tenfold and 30-fold increases, respectively, in c-*fos* and c-*myc* steady state mRNA levels in cystic kidneys [11–13]. These elevated mRNA
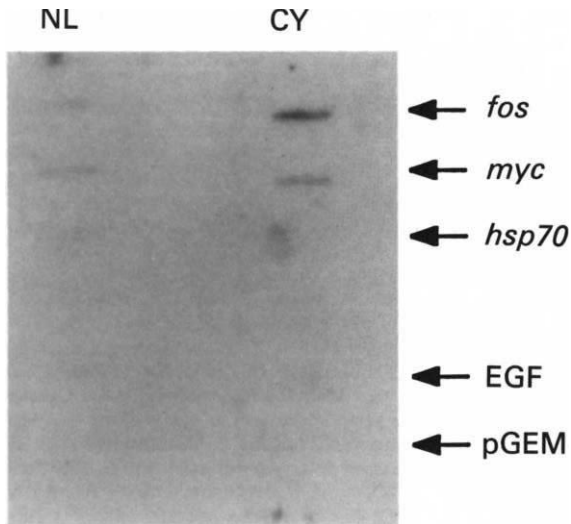
Fig. 1. *Nuclear run-on transcription analysis.* ^{32}P-labeled run-on transcripts synthesized in normal (NL) and cystic (CY) kidney cell nuclei from 3-week-old mice hybridized to cloned c-*fos*, c-*myc*, hsp70, preproEGF (EGF), and pGEM (Promega) plasmid vector DNAs slot-blotted and immobilized on nitrocellulose.

Fig. 2. *Induction of c-fos and c-myc mRNAs by cycloheximide treatment.* Northern blot analysis of c-*fos* and c-*myc* mRNAs carried out with total kidney RNA from 3-week-old normal and polycystic mice. **(A)** RNA isolated after a 1-hour treatment, from normal mice injected with carrier NaHCO$_3$ (lane 1) or cycloheximide (lane 2), from uninjected cystic mice (lane 3), or from cystic mice injected with carrier NaHCO$_3$ (lane 4) or cycloheximide (lane 5). **(B)** RNA isolated after a 3-hour treatment, from normal mice injected with carrier NaHCO$_3$ (lane 1) or cycloheximide (lane 2), or from cystic mice injected with carrier NaHCO$_3$ (lane 3) or cycloheximide (lane 4).

levels could be due either to increased gene transcription or to post-transcriptional mechanisms such as increased mRNA stability. To determine if increased rates of gene transcription are responsible for the increased mRNA levels, nuclear run-on transcription studies were carried out using nuclei isolated from three-week-old cystic and normal mouse kidneys (Fig. 1). This analysis showed that the c-*fos* and c-*myc* genes are transcribed, respectively, at 6.3-fold and 2.3-fold higher rates in cystic as compared to normal kidneys. In contrast, the hsp70 and preproEGF genes, which are transcribed at relatively low levels in normal kidneys, demonstrated no increase in cystic kidneys. This latter result is consistent with Northern blot evidence which showed that hsp70 mRNA is not significantly increased (Harding and Calvet, unpublished results) and that preproEGF mRNA levels are actually lower in cystic kidneys [9]. These results, therefore, show that some of the increased c-*fos* and c-*myc* mRNA may be accounted for by increased rates of gene transcription.

The protein synthesis inhibitor cycloheximide has been found to increase the amounts of c-*fos* and c-*myc* mRNA in cells expressing these genes [20–26]. Because of the increased transcriptional activity of these genes in cystic kidneys, we reasoned that cycloheximide might superinduce c-*fos* and c-*myc* mRNA. Previous studies by others indicated that c-*myc* expression can be superinduced in vivo in rat liver after partial hepatectomy [27]. Thus, we sought to amplify the difference in c-*fos* and c-*myc* mRNA levels seen between normal and cystic kidneys (Fig. 2A, lanes 1 and 4) by pretreating mice with cycloheximide. One hour after injection, the mice were sacrificed and total kidney RNA was isolated. Northern blot analysis demonstrated that cycloheximide treatment amplified the c-*fos* and c-*myc* mRNA steady-state levels in both normal and cystic kidneys (Fig. 2A, lanes 2 and 5), giving rise to much more c-*fos* and c-*myc* mRNA in the cystic kidneys than in the normals. This effect of cycloheximide treatment on c-*fos* and c-*myc*
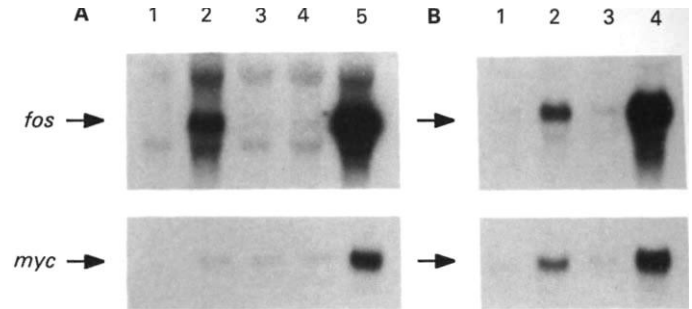
mRNA lasted several hours, since the amplified levels of these two mRNAs were also found three hours after injection (Fig. 2B). These results establish that cycloheximide can be used to augment the differential expression of c-*fos* and c-*myc* mRNAs in normal and cystic kidneys.

The kidney is a heterogeneous organ with a complex tubular architecture composed of many different cell types. Some tubular segments are profoundly altered in cystic kidneys while others appear essentially normal. While c-*myc* mRNA was found to be elevated in cystic kidneys at all stages examined, most of the increased expression was seen between two and three weeks, when the rate of cell proliferation is actually decreasing [10, 11, 13]. Therefore, an understanding of the role that the elevated c-*myc* expression may play in PKD depends upon a knowledge of which cell types account for this abnormal expression. To localize the increased c-*myc* expression in cystic kidneys, in situ hybridization was carried out using perfusion-fixed, frozen sections hybridized with an ^{35}S-labeled anti-sense RNA probe. As a control, sections of small intestine were hybridized alongside kidney sections. The intestinal epithelium is continually regenerating, with cell proliferation occurring at the base of the villi in the crypts of Lieberkuhn [28]. Therefore, it would be expected that most c-*myc* expression in the gut would be localized to the small population of proliferating cells in these crypts. Indeed, in situ hybridization with the c-*myc* probe demonstrated significant labeling of the crypts (Fig. 3A), indicating that the technique is quite specific. In some experiments, cycloheximide was used to increase the sensitivity of in situ hybridization. As shown in Figure 3B, cycloheximide pretreatment did not alter the pattern of c-*myc* expression in the gut, suggesting that it did not induce expression in cells not already expressing c-*myc*.

In both normal and cystic kidneys at 16 days in utero and three postnatal days of age, c-*myc* mRNA was localized primarily in the nephrogenic zone of the outer cortex (Fig. 4, A-D, arrows). While label could also be seen in cystic epithelium (Fig. 4D, arrowheads), most of the c-*myc* mRNA at these early time points appeared to be associated with normal developmental expression. At one week, labeling associated with the outer
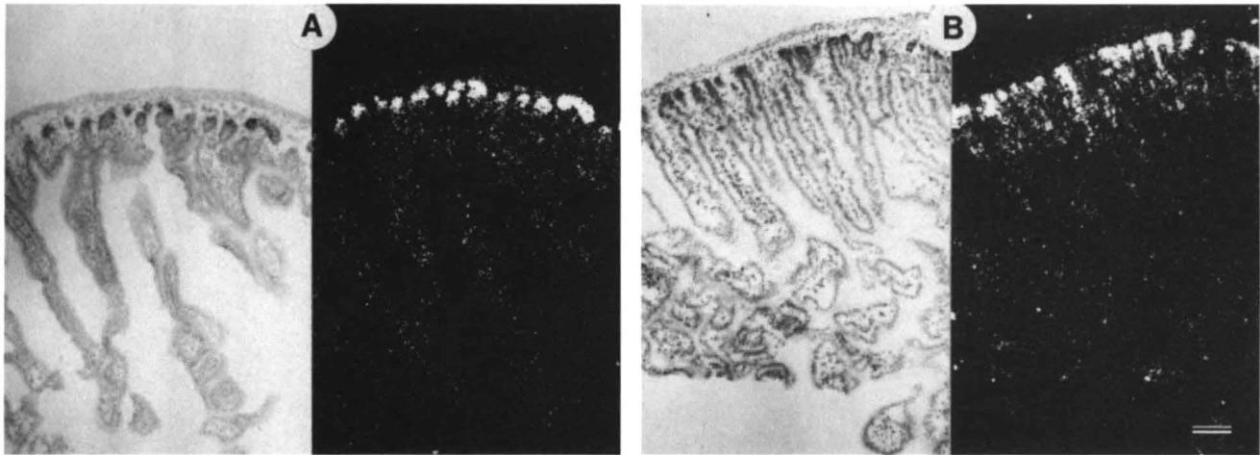
Fig. 3. *Localization of c-myc mRNA in intestinal crypts.* In situ hybridization bright and dark field photomicrographs of 3-week-old (**A**) untreated and (**B**) 3-hour cycloheximide-treated mice. Bar = 100 μm.
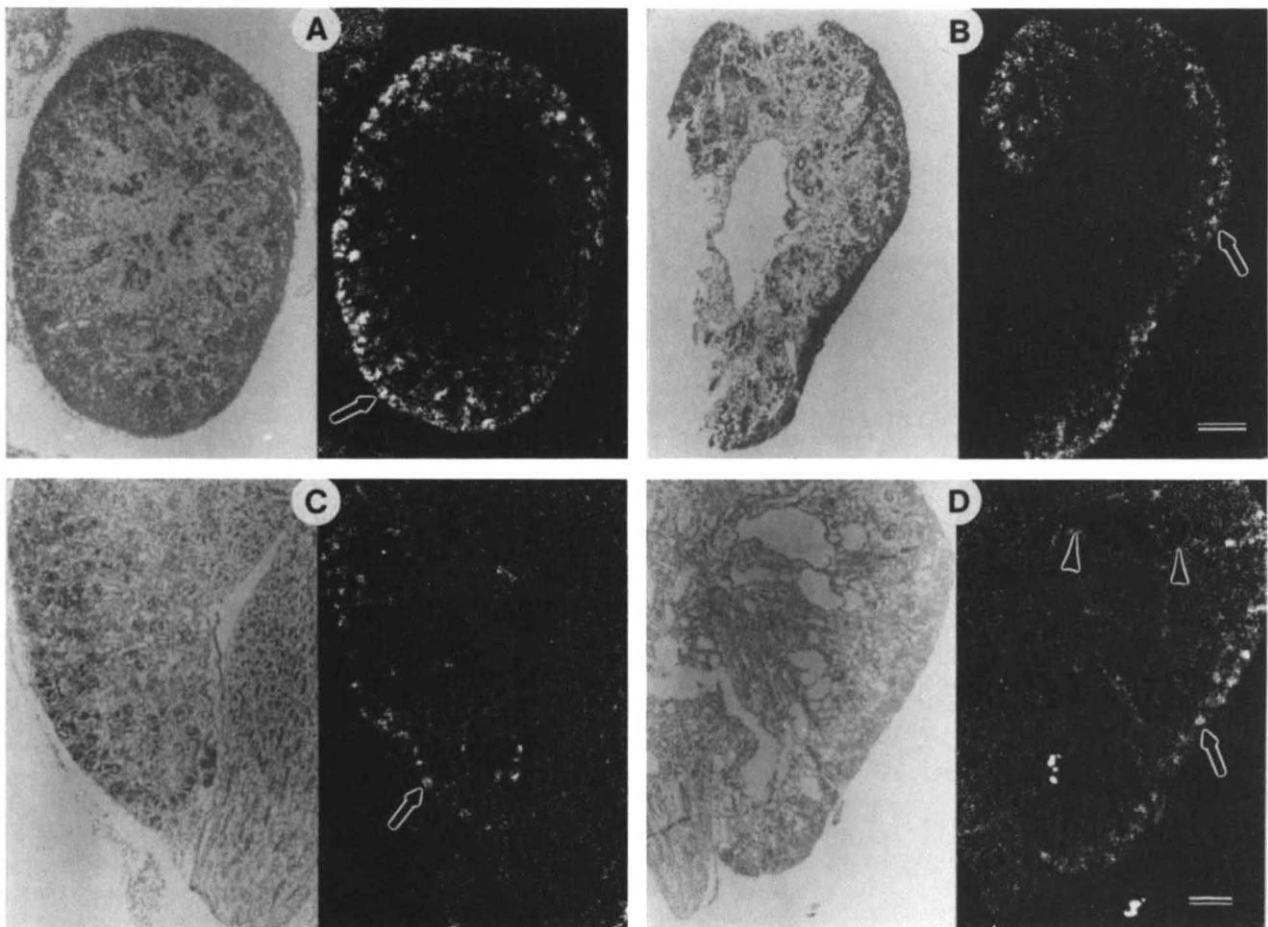

Fig. 4. *Localization of c-myc mRNA in nephrogenic kidneys.* In situ hybridization bright and dark field photomicrographs of nephrogenic vesicles (arrows) in (**A**) normal and (**B**) cystic kidneys at 16 gestational days, and in (**C**) normal and (**D**) cystic kidneys at 3 postnatal days. Labeled cysts (arrowheads) can be seen at 3 days. Bar = 200 μm.

cortex was seen to decline as the number of newly developing nephrons decreased, but was apparent in the medullary rays (Fig. 5A, B, small arrows). In the one-week cystic kidney, more

probe appeared to hybridize to the outer medulla in association with newly developing collecting duct cysts (Fig. 5B, arrowheads), with less labeling occurring near the tip of the papilla
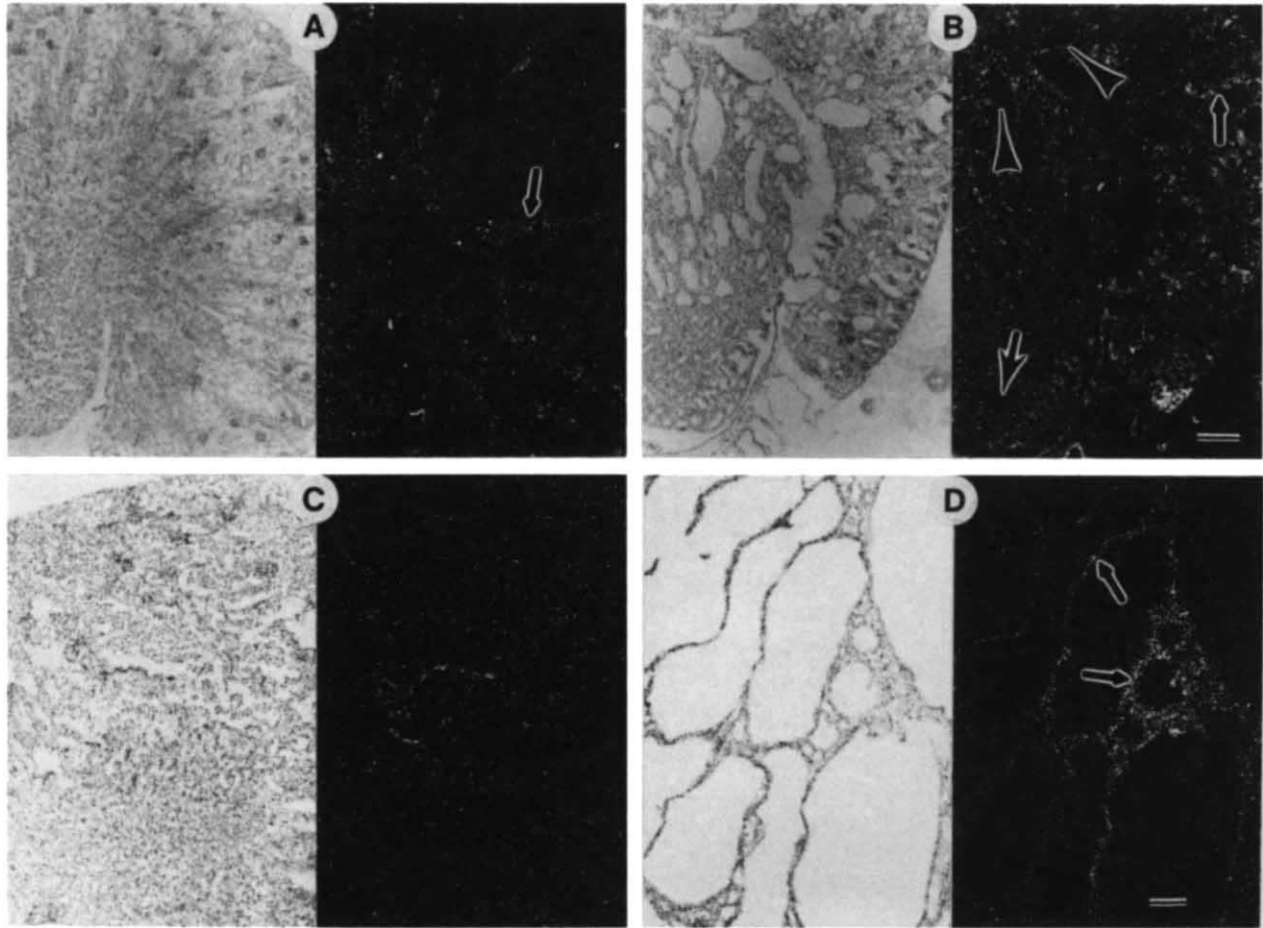
Fig. 5. *Localization of c-myc mRNA in maturing normal and cystic kidneys.* In situ hybridization bright and dark field photomicrographs of 1-week (**A**) normal and (**B**) cystic kidneys showing medullary rays (small arrows), labeled cysts (arrowheads), and the tip of the renal papilla (large arrow), and 2-week (**C**) normal and (**D**) cystic kidneys showing collecting duct cystic epithelium (arrows). (A,B) Bar = 200 μm; (C,D) Bar = 100 μm.

(Fig. 5B, large arrow). At two weeks of age, the normal kidney contained much less c-*myc* mRNA (Fig. 5C). However, the cystic kidney had generally higher levels of c-*myc* expression, in particular in the epithelial lining of collecting duct cysts (Fig. 5D, arrows). It is important to note that specific localization of c-*myc* mRNA to tubule segments comprising normal-appearing nephrons was not seen in two-week cystic kidneys. At three weeks, the difference in c-*myc* expression between normal and cystic kidneys was quite pronounced. Very little c-*myc* mRNA was detected in the normal kidney (Fig. 6A), and high levels of c-*myc* mRNA were detected in the cystic kidney (Fig. 6B-D). Labeling of the cystic epithelium was somewhat heterogeneous, with some cysts showing more hybridization than others (Fig. 6C, arrows), and with some regions within single cysts having more hybridization than other regions. Surprisingly, in addition to the cystic epithelium, a large amount of the c-*myc* probe hybridized to normal-appearing proximal tubules in the cystic kidney (Fig. 6B, D, arrows). Such a large concentration of c-*myc* mRNA in the proximal tubules was unexpected, since there were no indications of increased rates of cell proliferation in these proximal tubules in the same tissue sections used in this study or in previous studies that specifically addressed this

issue in the three-week *cpk* mouse kidney [10]. Thus, both the cyst wall epithelium *and* proximal tubules seem to contribute to the overexpression of c-*myc* mRNA detected in whole kidney RNA by Northern blot hybridization.

To increase the sensitivity of in situ hybridization, three-week-old mice were pretreated with cycloheximide. As noted above, cycloheximide did not alter the pattern of c-*myc* hybridization in the intestines of normal mice (Fig. 3B). In normal kidneys, cycloheximide treatment allowed the detection of c-*myc* mRNA in proximal tubules (Fig. 6E, arrow); however, no labeling was observed in collecting ducts anywhere in the kidney, this being particularly evident in the medulla (Fig. 6E, bottom). The lack of c-*myc* expression in normal collecting ducts even after cycloheximide enhancement is significant because it demonstrates that any c-*myc* expression detected in these cells in cystic kidneys is clearly abnormal. In cystic kidneys, the level of c-*myc* mRNA in the cyst wall epithelium (small arrow) and proximal tubules (large arrow) was greatly amplified by cycloheximide treatment (Fig. 6F). The increased levels of c-*myc* mRNA made it possible to detect some level of expression in almost all of the collecting duct cysts, confirming
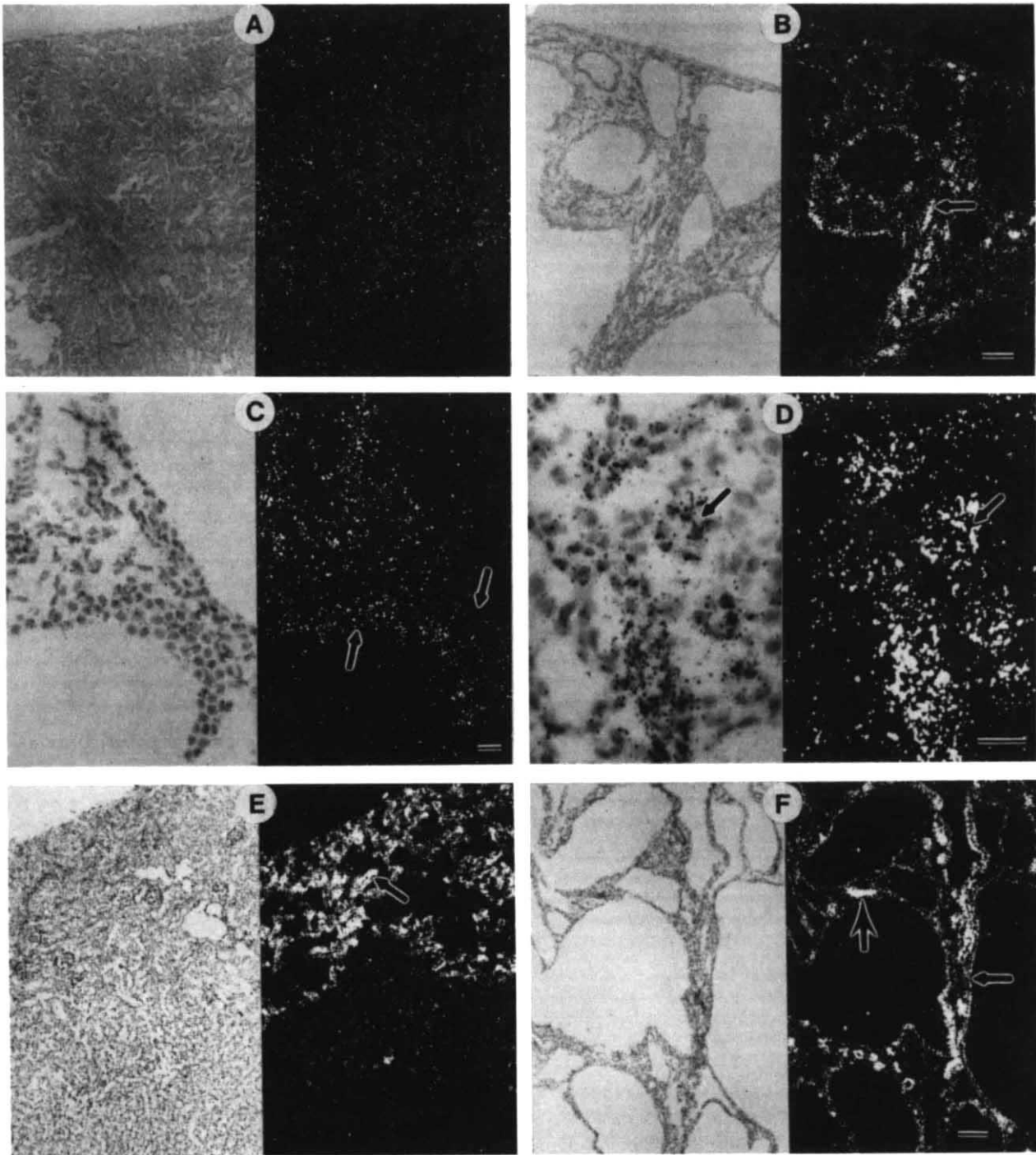
Fig. 6. *Localization of c-myc mRNA in 3-week-old normal and cystic kidneys.* In situ hybridization bright and dark field photomicrographs showing **(A)** normal and **(B)** cystic kidneys under low power magnification demonstrating proximal tubule labeling (arrow), **(C,D)** cystic kidneys under higher magnification demonstrating **(C)** the variability of cyst labeling (arrows) and **(D)** proximal tubule labeling (arrows), and **(E)** normal and **(F)** cystic kidneys 3 hours after cycloheximide treatment showing **(E)** a normal kidney with labeled proximal tubules (arrow) throughout the cortex, and **(F)** a cystic kidney with labeled cysts (small arrow) and proximal tubules (large arrow). (A,B,E,F) Bar = 100 μm; (C,D) Bar = 40 μm.

that the majority of cells lining cysts abnormally express c-*myc* mRNA.

Discussion

Previous studies had shown that several proto-oncogene mRNAs are markedly elevated in the cystic kidneys of *cpk* mice

[11–13]. We have now found that the c-*fos* and c-*myc* genes are transcribed at higher rates in cystic compared to normal kidneys. This difference in transcription rates may account, in part, for the elevation of c-*fos* and c-*myc* mRNAs in cystic kidneys. Transcription rates of the c-*fos* and c-*myc* genes appear to be selectively elevated, since the transcription of two

other genes was not increased. This would suggest that the *cpk* mutation directly or indirectly affects signal transduction mechanisms responsible for the transcriptional regulation of the c-*fos* and c-*myc* genes.

The rates of transcription of the c-*fos* and c-*myc* genes were found to be elevated in cystic kidneys approximately 6.3-fold and 2.3-fold, respectively. It should be noted that the steady state c-*fos* and c-*myc* mRNA levels were found previously to be elevated about tenfold and up to 30-fold, respectively [11–13]. For c-*myc*, therefore, it appears that there is only a relatively modest increase in transcription rate compared to the large increase in mRNA amount in cystic kidneys. This would suggest that there is also a significant post-transcriptional component to the regulation of c-*myc* gene expression in cystic kidneys. Indeed, it has been reported by Asselin and Marcu [29] that post-transcriptional mechanisms are largely responsible for the marked induction of c-*myc* mRNA levels in regenerating mouse kidneys following folic acid-induced injury [18], and in contrast that c-*fos* is regulated primarily at the transcriptional level in these kidneys [29].

We have shown that the elevation in c-*fos* and c-*myc* mRNAs in *cpk* mouse kidneys can be greatly amplified by treatment with the protein synthesis inhibitor cycloheximide. Although cycloheximide treatment also raised the level of these mRNAs in the kidneys of phenotypically normal littermates, this increase was not nearly as dramatic as that seen in cystic kidneys. Cycloheximide is known to cause superinduction of a number of proto-oncogene and growth factor mRNAs due both to increased gene transcription and to increased mRNA half-life [20–26, 30–32]. It is important to note, however, that cycloheximide by itself probably does not induce increases in c-*fos* and c-*myc* mRNA levels in quiescent cells not previously expressing these two genes [21, 22, 25]. Based on the cycloheximide results, therefore, it would seem that the c-*fos* and c-*myc* genes are probably under a state of increased or continuous transcriptional stimulation in the cystic kidney, in agreement with the nuclear run-on results.

Previous studies had indicated that abnormal cell proliferation occurs in the cystic kidneys of *cpk* mice [11, 13]. It had also been shown that c-*myc* mRNA levels are elevated in cystic kidneys even at early stages of cyst development. Therefore, the elevation in c-*myc* expression may be a reflection of this abnormal cell proliferation, and the localization of c-*myc* overexpression in the cyst wall epithelium can be attributed to the increased cell proliferation necessary for cyst formation. Since some cysts had much higher levels of c-*myc* mRNA than others, it is possible that epithelial proliferation rates vary between cysts. Cysts are heterogeneous in size, which may be the result of different growth rates or of variability in the onset of the initiation and/or termination of cyst growth. This variation in cyst growth could be linked to epigenetic factors.

Recent evidence obtained from studies using transgenic mice which express the SV40 large T antigen [33, 34] or c-*myc* [35, 36] genes suggests that proto-oncogene overexpression may initiate cyst formation. Transgenic mice expressing a c-*myc* gene driven by an SV40 enhancer developed polycystic kidneys, with a predominance of cysts arising from collecting tubules [35, 36]. In these studies, in situ hybridization revealed that high levels of c-*myc* mRNA were localized in the cyst wall epithelium. Similar experiments with the SV40 enhancer and

large T antigen gene resulted in transgenic animals that developed tubular cysts [36]. The SV40 enhancer may play a role in the temporal and tissue specific expression of these transgenes and could be critical for development of renal cysts in these transgenic animals [37–39]. These transgenic studies support the idea that the elevated c-*myc* expression observed in the *cpk* mouse kidney may be directly involved in the pathogenesis of PKD. In the *cpk* mouse, however, it is likely that the primary defect is in a gene that, when abnormally expressed, leads to the misregulation of a number of genes including c-*myc*.

Cell proliferation and proto-oncogene expression are frequently associated with early development. c-*myc* expression has been found in several tissue types in developing mouse [40, 41] and human [42] embryos. In the kidney, c-*myc* expression is highest in the early fetus (fetal day 12) and decreases during tubule differentiation from fetal day 12 through newborn [15]. We have also seen a decline in c-*myc* expression in the developing kidney, which we have examined from fetal day 16 through three weeks of postnatal development [13] (Figs. 4–6). Of particular significance is the observation that very little, if any, c-*myc* expression can be detected in collecting ducts at any developmental stage [15] (Figs. 4–6).

The increased cell proliferation and c-*myc* expression in cystic kidneys may be an indication that cyst wall cells are not fully differentiated. Constitutive proto-oncogene expression is frequently associated with a less-differentiated state [43, 44]. In particular, expression of the c-*myc* gene has been shown to block differentiation of mouse erythroleukemia cells [45], F9 teratocarcinoma cells [46], pre-B-cells [47], and rat myoblasts [48]. Furthermore, expression of the retroviral v-*myc* oncogene in avian sympathetic neurons maintains the cells in an immature, proliferating state [49]. It is possible, therefore, that the *cpk* mutation working through c-*myc* and perhaps other genes interferes with normal kidney development and differentiation. Indeed, other lines of evidence are consistent with the idea that cystic kidneys have a developmental abnormality. The preproEGF gene, which is normally expressed at high levels in distal tubules starting at one week and increasing through three weeks of age, has been found to be significantly underexpressed in cystic kidneys [9]. This lack of EGF expression may be a result of abnormal distal tubule development. We have also discovered that the sulfated glycoprotein-2 (SGP-2) gene is persistently expressed in collecting duct cysts, where in normal kidneys it is developmentally down-regulated, suggesting that cyst epithelial cells are not terminally differentiated [19]. Furthermore, the polarity of cyst epithelial cells has been found to be reversed with respect to Na-K ATPase, a characteristic of immature, relatively undifferentiated collecting duct epithelium [8]. Thus, it appears that cyst epithelial cells, as a consequence of the abnormal gene expression associated with the *cpk* mutation, do not differentiate normally.

Our finding that c-*myc* mRNA is expressed at elevated levels in proximal tubules of three-week cystic kidneys was surprising since previous work revealed no evidence for proliferation of these cells [10]. The c-*fos* and c-*myc* proto-oncogene products are thought to play a role in the transition from G_0 through the G_1 phase of the cell cycle. However, there is also evidence that c-*myc* can be disassociated from cell division. It has been shown, for example, that c-*myc* expression is high in Xenopus oocytes, which are not dividing, and that it does not increase

during the subsequent cleavage stage in which there is rapid cell division [50, 51]. Thus, it was found in those studies that there was no correlation between the onset of DNA synthesis and c-*myc* expression [51]. Furthermore, during development of the human embryo, some rapidly growing tissues express c-*myc* mRNA while others express very little [42]. These observations, and our own, emphasize that caution must be used in the interpretation of c-*myc* expression as a simple marker for mitotic activity.

As noted earlier, the first detectable changes in the *cpk* mouse kidney are in the proximal tubules. However, these changes are not a prominent feature of the latter stages of the disease process, and at three weeks of age morphologically normal proximal tubules are present, although in reduced number. At three weeks, the elevated c-*myc* expression in proximal tubules may represent a secondary effect of kidney failure. Acute renal injury is known to elicit a compensatory response accompanied by cell proliferation [18, 52–54]. A related process may occur in cystic kidneys, where a loss of normal renal function might lead to an attempt to increase proximal tubule mass. However, the absence of cell division in these proximal tubules would indicate that this compensatory, regenerative process cannot progress to the proliferative stage, and the elevated c-*myc* expression would suggest that these cells are blocked in G_1, caused perhaps by their inability to produce enough G_1-specific cell cycle proteins to proceed into S phase.

In conclusion, our results establish that the elevated c-*myc* expression in the cystic kidneys of *cpk* mice is associated with the cyst wall epithelium and thus that it is probably associated with the increased rate of proliferation of this cell population. While c-*myc* mRNA levels are elevated in cystic kidneys during the first two postnatal weeks, this increase is not dramatic until three weeks of age. This may be an indication that polycystic kidney disease progresses in two stages in the *cpk* mouse [13]. In the first stage, the elevated (relatively low, but above-threshold) c-*myc* expression may lock tubule cells in a less-differentiated state, leading to the initiation of cyst formation and the expansion of this population of cells, resulting in cyst growth. In the second stage, the high c-*myc* expression in non-proliferating proximal tubules may be a consequence of secondary abnormalities resulting from formation of the very large collecting duct cysts and the associated renal failure.

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